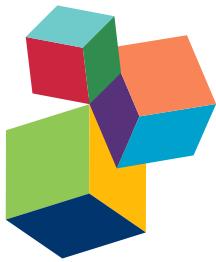


# ACTH ACTION IN THE ADRENAL CORTEX: FROM MOLECULAR BIOLOGY TO PATHOPHYSIOLOGY

EDITED BY: Nicole Gallo-Payet, Antoine Martinez and André Lacroix

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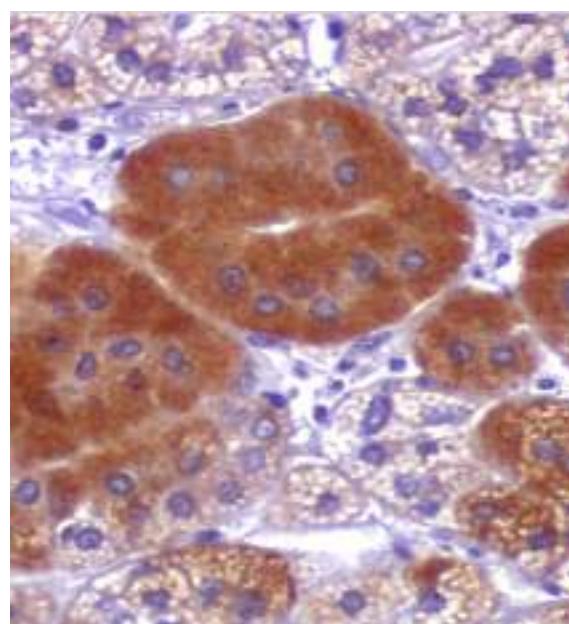
# ACTH ACTION IN THE ADRENAL CORTEX: FROM MOLECULAR BIOLOGY TO PATHOPHYSIOLOGY

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Immunohistochemical detection of corticotropin in the adrenal glands of a patient with primary macronodular adrenal hyperplasia. Corticotropin positive cells (brown) were found in isolated cells or in cell clusters in the adrenal subcapsular region and in the hyperplastic macronodules. Image was given by the authors, Estelle Louiset and Hervé Lefebvre. Similar images could be seen in Estelle Louiset, Céline Duparc, Jacques Young, Sylvie Renouf, Milène Tetsi Nomigni, Isabelle Boutelet, Rossella Libé, Zakariae Bram, Lionel Groussin, Philippe Caron, Antoine Tabarin, Fabienne Grunenberger, Sophie Christin-Maitre, Xavier Bertagna, Jean-Marc Kuhn, Youssef Anouar, Jérôme Bertherat, and Hervé Lefebvre. N. Engl J Med 369:2115-2125 (2013).

By stimulating adrenal gland and corticosteroid synthesis, the adrenocorticotrophic hormone (ACTH) plays a central role in response to stress. In this Research Topic, a particular attention has been given to the recent developments on adrenocortical zonation; the growth-promoting activities of ACTH; the various steps involved in acute and chronic regulation of steroid secretion by ACTH, including the effect of ACTH on circadian rhythms of glucocorticoid secretion. The Research Topic also reviews progress and challenges surrounding the properties of ACTH binding to the MC2 receptor (MC2R), including the importance of melanocortin-2 receptor accessory protein (MRAP) in MC2R expression and function, the various intracellular signaling cascades, which involve not only protein kinase A, the key mediator of ACTH action, but also phosphatases, phosphodiesterases, ion channels and the cytoskeleton. The importance of the proteins involved in the cell detoxification is also considered, in particular the effect that ACTH has on protection against reactive oxygen species generated during steroidogenesis. The impact of the cellular microenvironment, including local production of ACTH is discussed, both as an important factor in the maintenance of homeostasis, but also in pathological situations, such as severe inflammation. Finally, the Research Topic reviews the role that the pituitary-adrenal axis may have in the development of metabolic disorders. In addition to mutations or alterations of expression of genes encoding components of the steroidogenesis and signaling pathways, chronic stress and sleep disturbance are both associated with hyperactivity of the adrenal gland. A resulting effect is increased glucocorticoid secretion inducing food intake and weight gain, which, in turn, leads to insulin and leptin resistance. These aspects are described in detail in this Research Topic by key investigators in the field.

Many of the aspects addressed in this Research Topic still represent a stimulus for future studies, their outcome aimed at providing evidence of the central position occupied by the adrenal cortex in many metabolic functions when its homeostasis is disrupted. An in-depth investigation of the mechanisms underlying these pathways will be invaluable in developing new therapeutic tools and strategies.

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# Editorial: ACTH Action in the Adrenal Cortex: From Molecular Biology to Pathophysiology

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**Keywords:** adrenal cortex, ACTH, cortisol, aldosterone, signaling, MC2R, adrenal tumors, Cushing's syndrome

## Editorial on the Research Topic

### ACTH Action in the Adrenal Cortex: From Molecular Biology to Pathophysiology

With this Research Topic, we want to celebrate 80 years of research on the role of adrenocorticotrophic hormone (ACTH) on the adrenal cortex, from the pioneering work by Selye, who introduced the concept of “general adaptation syndrome” in a short Letter to Nature (1). Selye described the general adaptation syndrome, later renamed stress, as a response of the body to demands placed upon it. The system whereby the body copes with stress, the hypothalamo-pituitary-adrenal (HPA) axis, was also first described by Szabo et al. (2). From a historical standpoint, Selye actively avoided using the term “stress” until 1946. It was Walter Cannon who actually developed the term stress in his work relating to the fight-or-flight response in 1914 (2, 3). Because it was clear that most people viewed stress as some unpleasant threat, Selye had to create a new word, “stressor,” in order to distinguish between stimulus and response (3). It is also important to remember that Selye was the first to describe “corticoids” and to propose that glucocorticoids and mineralocorticoids (also named by Selye) regulated not only carbohydrate and electrolyte metabolism, respectively, but also exerted anti- or pro-inflammatory effects (2, 4, 5).

By stimulating adrenal corticosteroids synthesis, the ACTH, first isolated in 1943 (6) and then synthesized in 1960s and 1970s (see the review by Ghaddhab et al.), plays a central role in homeostasis and stress response and thus is a key component of the HPA axis. This Research Topic is a compendium of reviews and original contributions, which summarize classical views, novel findings, and challenges on the various mechanisms involved in ACTH action, from the binding of ACTH to its receptor to steroid secretion. The Research Topic also reviews the pathological consequences of the disruption of specific components of these pathways.

The adult mammalian adrenal cortex is composed of three concentric layers, each having specific functional and morphological properties. ACTH is the main stimulus of the zona fasciculata and zona reticularis, stimulating glucocorticoids secretion, while angiotensin II and potassium are the main stimuli of aldosterone secretion by the zona glomerulosa. Glucocorticoids (cortisol, corticosterone, cortisone) are implicated in a broad range of metabolic functions, including anti-inflammatory responses, stress response, and behavior. However, chronically elevated glucocorticoid levels alter body protein synthesis, fat distribution, increase visceral adiposity, and are responsible for metabolic abnormalities, such as hypertension, type 2 diabetes, or sleep disturbance. On the other hand, aldosterone stimulates sodium reabsorption, hence maintaining blood volume and pressure in sodium-depleted conditions. Excessive aldosterone secretion not only leads to hypertension and

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electrolyte imbalance but is also associated with much increased risks of cardiometabolic complications as compared to similar levels of essential hypertension. As reviewed by Funder and El Ghorayeb et al., there is recent evidence that aldosterone secretion is highly sensitive to very low doses of ACTH and that alteration in melanocortin 2 receptor (MC2R) expression is observed in adrenal tissues of patients with primary aldosteronism. Together, these results indicate that ACTH may be an important mediator of inappropriate hypersecretion of aldosterone in this very prevalent disease (approximately 10% of humans with hypertension).

From the recent findings on a number of proteins involved in functional activity of the cortex, Vinson discusses the significance of the standard model of morphological zonation vs. functional zonation. Lerario et al. detail how adrenocortical cells are homeostatically derived from a pool of progenitors localized in the subcapsular region of the zona glomerulosa. These cells undergo a continuous process of centripetal migration. In their review, these authors summarize established and emerging concepts that regulate the biology of the progenitor cell niche. An emphasis is placed on the interactions between extracellular matrix (ECM) components and their cell surface receptors, and with the ECM-embedded signaling molecules, including ligands of the sonic hedgehog and Wnt signaling pathways. Lotfi and de Mendonca review the cascade of events involved in the regulation of proliferation and growth by ACTH and other POMC-derived peptides. Key findings regarding signaling pathways and modulation of genes and proteins required for the regulation of murine adrenal growth are summarized. It is also well known that there are sex differences in adrenal cortex structure and function. In the rat, they are manifested as larger adrenal cortex and higher corticosterone secretion in females compared with males. Jopek et al. have investigated the transcriptome profile of rat adrenal after gonadectomy and testosterone or estradiol replacement. As developed in his article, results were unexpected and the physiological relevance of these findings are discussed.

Dumbell et al. review the importance of circadian clocks and their interactions with the immune system. The review highlights the importance of timing in treatment of many chronic disorders with a chronic inflammatory background. In particular, as documented by Engeland et al., the effect of stress differs depending on the time of day when acute stress is administered. How stress affects circadian rhythms of glucocorticoids secretion and their impact on health is reviewed by Nicolaides et al. Accumulating evidence suggests that dysfunction of the former may result in dysregulation of the latter, and *vice versa*. The authors review the functional components of the two systems and discuss their multilevel interactions during excessive or prolonged activity of the HPA axis. In particular, as discussed by Moeller et al., disruption of circadian timing, such as after inter-time zone travel, shift work, and mistimed eating, can have consequences for cardiovascular, metabolic, and mental health and, crucially, immune function.

Adrenocorticotrophic hormone exerts its role through binding to the G protein-coupled receptor, MC2R, which activates the adenylyl cyclase cascade leading to cAMP production and subsequent activation of cAMP-dependent protein kinase A (PKA). PKA is the main kinase responsible for the phosphorylation of specific transcription factors, which in turn regulate free

cholesterol availability and activate the expression of steroidogenic enzymes. Six groups detail the current knowledge surrounding the mechanisms of ACTH binding to its receptor. The MC2R is unusual in that it is dependent on a small accessory protein, melanocortin receptor accessory protein (MRAP), which is essential for both trafficking of MC2R to the plasma membrane and for ACTH binding and activation of MC2R (reviewed by Clark et al., Ghaddhab et al., and Fridmanis et al.). MRAP is a single chain protein with one membrane-spanning domain. Thanks to an elegant tool, Maben et al. present new evidence on the dual topology of MRAP. The publication supports previous findings of an antiparallel homodimer structure for MRAP and indicates that partners of the MRAP dimer maintain a fixed orientation during trafficking and integration in the plasma membrane. In the human genome, there are two *MRAP* paralog *MRAP* genes. Some features of this gene family are unique. Dores addresses new hypotheses on the evolution of MCRs and MRAPs, highlighting important considerations on the origin of these key proteins in stress axis in vertebrates. One hypothesis is that differences in affinity between MCR-MRAP interactions may affect the trafficking of certain receptors to the cell membrane or allow activation of the receptor by its ligand. In their review, Maben et al. and Fridmanis et al. detailed how specific mutations in the region of the N-terminal tail of MRAP1 and MRAP2 are essential for promoting only the trafficking of receptors to the plasma membrane (MRAP2) or essential for ACTHR/MC2R ligand recognition and functionally (MRAP1).

Even if the structure of ACTH has been known for many years, the exact mechanism of activation of MC2R by ACTH is still unknown. ACTH is the only known naturally occurring ligand for this receptor. The lack of redundancy and high degree of ligand specificity suggests that antagonism for this receptor could provide a useful therapeutic tool for some difficult-to-treat diseases such as congenital adrenal hyperplasia and primary bilateral macronodular adrenal hyperplasia (BMAH), as well as in Cushing's disease or stress-related pathologies. In their reviews, Clark et al. and Ghaddhab et al. discuss the scientific and clinical rationale for the development of an ACTH receptor antagonist. However, as reviewed by the two groups, the design of a specific peptide acting as an MC2R antagonist remains a challenge due to the difficulty of designing a tridimensional peptide specific for only one of five MCR receptors. Indeed, the first 13 residues of ACTH are active on all the other melanocortin receptors and thus it seems that this tetrabasic region acts as a key to unlock the MC2R-MRAP complex. In addition, all melanocortin receptors share the same amino acid sequences H6F7R8W9 (the "message sequence"), which is important for melanocortin receptor stimulation and which bind to all MCR receptors, while a.a. 15–19 (KKRRP sequence) (the "address sequence"), is essential for the binding of ACTH to MC2R. If they are not selective, these peptides could be source of severe secondary effects due to numerous roles of five MCRs. Moreover, a small, non-peptide molecule that can be delivered orally would be more desirable. Interestingly, in neonates, Nensey et al. detailed that stress-induced increases in corticosterone demonstrate a unique shift from ACTH independence at postnatal day 2 (PD2) to ACTH dependence at day 8 (PD8). If the corticosterone response to

stress in PD2 pups can be blocked by antagonizing the MC2R, it would suggest that a bioactive form of ACTH (not measured by immunoassay) is working through the MC2R in PD2 pups. Nensey et al. describe the effect of two novel MC2R antagonists, GPS1573 and GPS1574, on the corticosterone response to ACTH in these two neonatal stages of development.

Several reviews and original contributions develop recent advances on the mechanisms by which ACTH stimulates steroidogenesis. The acute response of ACTH is initiated by the mobilization of cholesterol from lipid stores (Shen et al.) and its delivery to the inner mitochondrial membrane (Lee et al. and Midzak and Papadopoulos), a process that is mediated by the steroidogenic acute regulatory protein (StAR) (Clark), while the chronic response results in the increased coordinated transcription of genes encoding steroidogenic enzymes (Ruggiero and Lalli). One of the initial events of ACTH action is the regulation of expression and function of scavenger receptor class B type I (SR-B1). Shen et al. review how both acute and chronic ACTH treatments can modulate transcription, posttranscriptional stability, phosphorylation, and dimerization status of SR-B1, as well as the interaction of SR-B1 with other protein partners, all of these being important for SR-B1 to mediate lipoprotein-derived cholesterol esters uptake and the supply of cholesterol to mitochondria. Once cAMP is stimulated, activation of the cAMP-dependent PKA results in an acute increase in expression and activity of StAR. As reviewed by Clark, StAR plays an essential role in steroidogenesis in coordinating ACTH-dependent movement of cholesterol across mitochondrial membranes. The model for StAR translation and phosphorylation at the outer mitochondrial membrane, the site for StAR function, is presented. A model for the spatiotemporal regulation of StAR by cAMP is presented by Lee et al. The C-terminal cholesterol binding domain of StAR initiates mitochondrial inter-membrane contacts to rapidly direct cholesterol to the cholesterol side chain cleavage enzyme (Cyp11a1) in the inner mitochondrial membrane. The conserved StAR N-terminal regulatory domain (NTRD) controls the transit time that determines extra-mitochondrial StAR effects on cholesterol homeostasis. On the other hand, Midzak and Papadopoulos highlight the functional importance of the assembly of large multimeric protein complexes in mitochondrial cholesterol transport, steroidogenesis, and mitochondria-endoplasmic reticulum contact. The complex includes more than 10 proteins crossing the outer and inner mitochondrial membranes. The importance of StAR in steroidogenesis is illustrated by two cases of lipid congenital adrenal hyperplasia (LCAH) due to homozygous *StAR/STARD1* mutations. Khoury et al. review the impact of these mutations on steroidogenesis and fertility in LCAH patients. Their publication examines the correlation between the molecular structure of STARD1 with its function in all steroidogenic tissues. Ruggiero and Lalli review the impact of ACTH signaling on transcriptional regulation of steroidogenic genes. They provide a general view of the transcriptional control exerted by the ACTH/cAMP system on the expression of genes encoding steroidogenic enzymes in the adrenal cortex. A special emphasis is put on the transcription factors required to mediate ACTH-dependent transcription of steroidogenic genes. On the other hand, the regulation of mRNA stability has emerged as a

critical control step in dynamic gene expression. This process occurs in response to modifications of the cellular environment, including hormonal variations. In the adrenal cortex, Desroches-Castan et al. explain how ACTH or cAMP elicit changes in the expression, phosphorylation, and localization status of major mRNA-destabilizing protein.

As reviewed by Paz et al., steroidogenesis depends not only on PKA-mediated Ser/Thr phosphorylation but also on the activity of protein tyrosine phosphatases, which have been implicated in StAR expression and steroidogenesis. Furthermore, arachidonic acid and its metabolites play a key role in the hormonal control of steroidogenesis by regulating both the expression and function of StAR. The role of exchange protein activated by cAMP (EPAC) and the actin cytoskeleton in cAMP-mediated actions of ACTH is updated by Lewis et al. In adrenocortical cells in culture, cAMP induces characteristic changes in cell shape and a concomitant reorganization of actin microfilaments. This cytoskeletal reorganization allows the correct positioning of lipid droplets, endoplasmic reticulum, and mitochondria where cholesterol and its metabolites are transported and metabolized. However, the effects of EPAC on F-actin remodeling are not correlated to steroidogenesis and may, therefore, contribute to other aspects of adrenal physiology. Their findings indicate that EPAC2B has a role in cell motility, suggesting rather an implication in adrenal pathophysiology, such as adrenal cancer cell invasion. Finally, in addition to the cAMP–PKA pathway, multiple interactions exist between various signaling cascades. Spat et al. summarize the positive cross talk interactions between various signaling pathways, including calcium and cAMP, on gene expression, mitochondrial function, and thus on steroid secretion. In particular, the contribution of the various calcium channels expressed in adrenocortical cells to steroid production is reviewed. Rossier details the involvement of T-type and L-type calcium channels in the regulation of intracellular calcium concentration and aldosterone secretion in zona glomerulosa cells. In parallel with steroidogenesis, Pastel et al. review the importance of aldo-keto reductases 1B (AKR1B) in adrenal cortex physiology. Adrenal activities generate large amounts of harmful aldehydes from lipid peroxidation and steroidogenesis, which can both be reduced by AKR1B proteins. In addition, chronic effects of ACTH result in a coordinated regulation of genes encoding the steroidogenic enzymes and some AKR1B isoforms. The review describes the molecular mechanisms accounting for the adrenal-specific expression of some AKR1B genes. Using data from recent mouse genetic models, the authors establish a connection between the enzymatic properties of these AKR1B proteins and the regulation of adrenal functions.

The functional activity of the adrenal cortex is the result of an integration of not only cell-specific regulatory processes but also of the paracrine interactions between adjacent cells, as well as paracrine factors, such as ACTH, locally produced in the medulla. How ACTH interacts with the adrenal microenvironment to modulate corticosteroid secretion was reviewed by Bell and Murray who described the fundamental concepts and implication of gap junctions in steroidogenesis, cell proliferation and cancer. For instance, cells of the zona glomerulosa, which have fewer gap junctions than the two other cortical zones, may be less

dependent on cell–cell communication for normal function than the cells of the zona fasciculata or the zona reticularis. In their review, Kanczkowski et al. discuss the importance of the adrenal gland microenvironment in the regulation of stress-induced hormone secretion. Among these, adrenocortical–chromaffin cell interactions, the immune adrenal cross talk and the adrenal vascular system play major roles. Lefebvre et al. highlight the importance of ACTH in the interactive and paracrine regulation of adrenal steroid secretion in physiological and pathophysiological conditions. Indeed, ACTH has been found to be abnormally synthesized in bilateral macronodular adrenal hyperplasia (BMAH) responsible for hypercortisolism. In these nodules, ACTH is detected in a subpopulation of adrenocortical cells that express gonadal markers.

Finally, the Research Topic reviews the pathological consequences of a defect in ACTH signaling. Chronic ACTH excess leads to chronic cortisol excess. In the review by Bertagna, all the pathological situations involving an increase in ACTH are reviewed. Some of these include Cushing's syndrome, excess adrenal androgens, chronic adrenal mineralocorticoid excess, and low aldosterone levels. Indeed, in human, chronic ACTH treatment may induce a sustained increase in cortisol and 11 deoxycorticosterone (two products for the zona fasciculata), in parallel with the low aldosterone levels, eventually creating a state of chronic mineralocorticoid excess. Prolonged *in vivo* stimulation with ACTH, through the activation of a local and complex network of autocrine growth factors, including non-ACTH POMC-peptides, may also affect cell proliferation and hyperplasia, thus participating in the amplified response of the chronically stimulated gland, and the weight of each gland can be greatly increased.

As reviewed by Funder, aldosterone may be acutely stimulated by ACTH that could thus play a role in driving inappropriate aldosterone secretion, as detailed in some examples. El Ghorayeb et al. review the role of ACTH and other hormones in the regulation of aldosterone production in primary aldosteronism, focusing on the aberrant expression of MC2R compared to other GPCRs and their ligands. Alterations in the cAMP-dependent signaling pathway have been implicated in the majority of benign adrenocortical tumors (ACTs) causing Cushing's syndrome. Villares Fragoso et al. revisit how *GNAS* (*gsp*) mutations were associated with adrenal cortical tumors and hyperplasia. Although somatic activating mutations in *GNAS* are a rare molecular event in bilateral macronodular hyperplasia, these mutations have recently been identified in approximately 10% of cortisol-secreting unilateral adenomas. Phosphodiesterases (PDEs) are enzymes that regulate cyclic nucleotide levels, including cAMP. As reviewed by Hannah-Shmouni et al., inactivating mutations and other functional variants in PDE11A and PDE8B, encoding cAMP-binding PDEs, predispose to ACTs. Seidel and Scholl review the molecular differences between aldosterone- and cortisol-secreting adrenal cortical adenomas (APAs and CPAs) leading to primary aldosteronism and Cushing's syndrome, respectively. In both cases, acute stimulation leads to increased hormone production, and chronic stimulation causes hyperplasia of the respective zones. The authors review some of the pathologies (APAs and CPAs) caused by channel mutations

(referred to as channelopathies), for example, mutations in the  $K^+$  channel gene, *KCNJ5*, or mutations in the  $Ca^{2+}$  channel gene, *CACNA1D*, which directly lead to increased calcium influx. Some CPAs carry a recurrent gain-of-function mutation in the *PRKACA* gene, which causes constitutive PKA activity. The contribution by Leccia et al. reviews the mouse models recapitulating human ACTs, highlighting their limitations in replicating human diseases. Development of mouse models is a crucial step to firmly establish the functional significance of candidate genes, to dissect mechanisms leading to tumors and endocrine disorders and to provide *in vivo* tools for therapeutic screens. In the article, the authors provide an overview on the existing mouse models of ACTs by focusing on the role of PKA and Wnt/ $\beta$ -catenin pathways in this context. They present the advantages and limitations of models that have been developed and point out to the necessary improvements for the development of the next generation of mouse models of adrenal diseases.

Two examples of pathological stressful situations due to ACTH and glucocorticoids excess are given in the reviews by Annane and Moeller et al. Annane revisits the role of ACTH and glucocorticoids in septic shock and sepsis. Sepsis is a common disorder associated with high morbidity and mortality. It is now defined as an abnormal host response to infection, resulting in life-threatening dysfunction of organs. Numerous experimental and clinical data have established the paramount importance of an appropriate activation of the HPA axis to respond to severe infection. Clinical trials in sepsis found variability in beneficial effects of corticosteroids on survival, although most trials demonstrated faster resolution in shock and organ dysfunction, suggesting that physicians should consider corticosteroids mainly in septic shock who do not respond rapidly to fluid therapy and vasopressors. Moeller et al. examine how glucocorticoids regulate the food choice behavior in humans with evidence from Cushing's syndrome. The review is an attempt to elucidate the contribution of glucocorticoid to food-seeking behavior and reward processing, aiming to understanding the mechanistic of weight fluctuations associated with oral glucocorticoid therapy and/or chronic stress.

Even if many questions have been answered since the seminal pioneering work by Selye, including the cloning of the ACTH receptor, MC2R, in 1992 (7) and the discovery of MRAP, about 10 years ago, in 2005 (8), many of the aspects addressed in this Research Topic still represent a stimulus for future studies. These topics reinforced the evidence that the adrenal cortex, through the steroid hormones it secretes, occupies a central position in many metabolic functions where if its homeostasis disrupted have profound deleterious effects. An in-depth investigation of the mechanisms underlying these pathways will be invaluable in developing new therapeutic tools and strategies.

## AUTHOR CONTRIBUTIONS

As guest editor, I and my co-editors, AL and AM, have read all the contributions, selected external reviewers, reviewed the forum discussion, and finalized decisions regarding final acceptability for publications.

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# Functional Zonation of the Adult Mammalian Adrenal Cortex

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The standard model of adrenocortical zonation holds that the three main zones, glomerulosa, fasciculata, and reticularis each have a distinct function, producing mineralocorticoids (in fact just aldosterone), glucocorticoids, and androgens respectively. Moreover, each zone has its specific mechanism of regulation, though ACTH has actions throughout. Finally, the cells of the cortex originate from a stem cell population in the outer cortex or capsule, and migrate centripetally, changing their phenotype as they progress through the zones. Recent progress in understanding the development of the gland and the distribution of steroidogenic enzymes, trophic hormone receptors, and other factors suggests that this model needs refinement. Firstly, proliferation can take place throughout the gland, and although the stem cells are certainly located in the periphery, zonal replenishment can take place within zones. Perhaps more importantly, neither the distribution of enzymes nor receptors suggest that the individual zones are necessarily autonomous in their production of steroid. This is particularly true of the glomerulosa, which does not seem to have the full suite of enzymes required for aldosterone biosynthesis. Nor, in the rat anyway, does it express MC2R to account for the response of aldosterone to ACTH. It is known that in development, recruitment of stem cells is stimulated by signals from within the glomerulosa. Furthermore, throughout the cortex local regulatory factors, including cytokines, catecholamines and the tissue renin-angiotensin system, modify and refine the effects of the systemic trophic factors. In these and other ways it more and more appears that the functions of the gland should be viewed as an integrated whole, greater than the sum of its component parts.

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## INTRODUCTION—THE STANDARD MODEL

The nature and significance of the zonation of the mammalian adrenal cortex has attracted considerable interest during the fifteen decades following the first description of its three main zones, zona glomerulosa, zona fasciculata, and zona reticularis, by Arnold (1866). Later, Gottschau (1883) was the first to suggest that adrenocortical cells originate in the outer part of the gland, and migrate centripetally, thus becoming phenotypically glomerulosa, then fasciculata and reticularis in sequence. Zwemer et al. (1938) illustrated a direct lineage of cortical cells from the connective tissue capsule. Before the functions of the gland were understood, studies were directed to further descriptions of adrenocortical morphology and development, in mammals and other vertebrates (Dostoiewsky, 1886; Rabl, 1891; Wiesel, 1902). By the 1940s it was clear that the adrenal cortex, as well as the medulla, is crucially involved in the response to stress, and further, that secretions

of the cortex fell into three functional classes: androgens, and what Selye and Jensen were the first to call mineralocorticoids and glucocorticoids (Selye, 1946; Selye and Jensen, 1946). Coupled with the observable changes in appearance and abundance of the zones in different physiological situations it then became apparent that the different zones had different functions, and androgens, mineralocorticoids, and glucocorticoids were now thought to be products of the zona reticularis, zona glomerulosa, and zona fasciculata respectively (Vines, 1938; Swann, 1940; Chester Jones, 1957; Deane, 1962). Moreover, their regulation was different, and though the inner adrenocortical zones, fasciculata and reticularis, were dependent on an intact pituitary and the secretion of ACTH, the glomerulosa was not (Chester Jones, 1957; Deane, 1962; Vinson, 2003). Later, the essential role of the renin-angiotensin system in the regulation of aldosterone was elucidated (Gross, 1958; Laragh et al., 1960; Carpenter et al., 1961), though other factors, possibly many, are also involved (Vinson et al., 1992b; Ehrhart-Bornstein et al., 1998; Mulrow, 1999; Vinson, 2003).

Together, such evidence has led to the development of what may be thought of as the Standard Model of adrenocortical zonation:

- Adrenocortical cells arise in the outer part of the gland and migrate centripetally, with changes in phenotype as they progress from the glomerulosa through the fasciculata to the reticularis, where they undergo apoptosis.
- Each of the three main differentiated zones secretes a specific profile of steroid hormones.
- Each of the zones is under separate and independent regulatory control.

There have, however, always been gaps in interpretation that have scarcely been addressed. Among these is the challenge of unraveling the mechanisms underlying the profound changes of phenotype that the cells undergo during their lifespan. Again, but less frequently considered, what drives cell migration in the mature gland? Additionally, and rarely addressed: what is the significance of the zonal arrangement—what advantage is conferred by the configuration of the different zones as concentric shells?

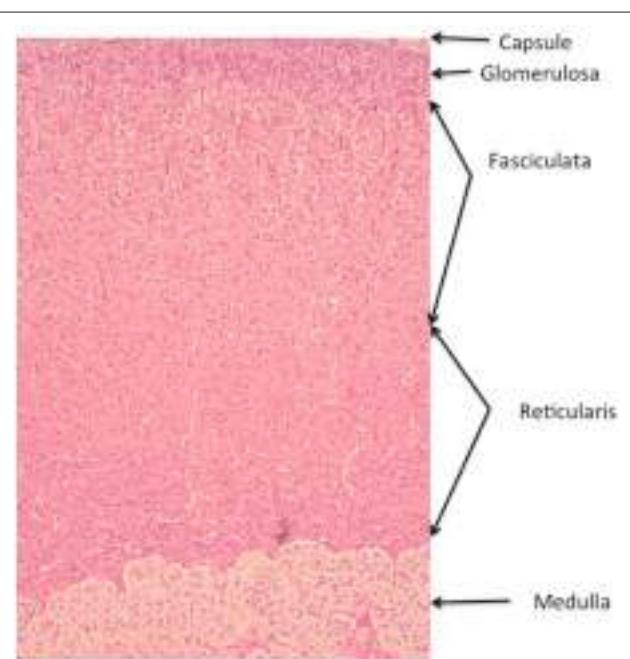
It is the purpose of this review to examine both whether the Standard Model remains compatible with the literature of the last few years, and also whether there are now clues to these additional problems.

## WHAT DO WE MEAN BY ADRENOCORTICAL ZONATION?

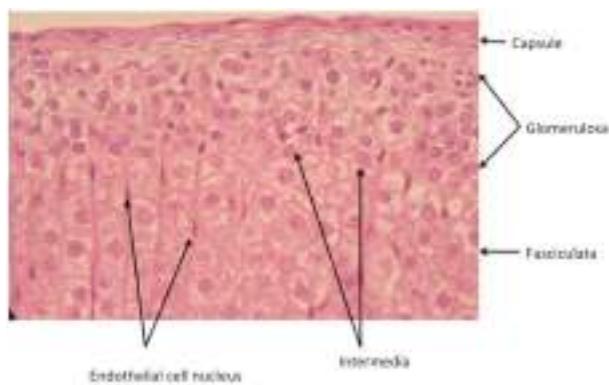
It is important to define the terms we use. Zonation is a descriptive term, which has been applied by different observers to mean sometimes quite different things. Without a strict, generally adhered-to definition, it is most appropriate to go back to the pioneers of the terminology, and follow their usage. Since the earliest descriptors of adrenocortical zonation were morphological and topographical, and these alone were used for 70–80 years, it follows that,

without a contrary formal decision, agreed perhaps by a caucus of international exponents of the field, zonation remains defined by cellular morphology and topology alone (Figures 1, 2).

The names of the three main zones are descriptive of the arrangement of their cells. Thus, the zona glomerulosa is so called because the whorls or baskets of cells that compose it somewhat recall the glomeruli of the kidney (Latin *glomerulus*, ball) though this is more apparent in some species (e.g., the dog) than others (rat, human). The cells of the zona fasciculata lie in parallel radial cords, and the name stems originally from the Roman symbol of office, the *fascies* or bundle of birch rods (etymologically the same origin, incidentally, as *fascist*), cf. the anatomical term *fascicle* (bundle of nerves or muscle fibers), while the cords of cells of the reticularis form a network (Latin: *rete*). Essentially, the smaller, more densely staining glomerulosa cells are characterized by their lamelliform cristae, whereas the larger and paler fasciculata cells have tubulo-vesicular cristae. The reticularis cells retain tubulo-vesicular cristae, and are again more densely staining and smaller than fasciculata cells. Lipid distribution varies with the state of stimulation, but in general is greater in the inner zones. These criteria have been reviewed on many occasions (Chester Jones, 1957; Deane, 1962; Neville and O'Hare, 1981; Vinson et al., 1992b).



**FIGURE 1 | Zonation of the Wistar rat adrenal cortex.** The main zones of the cortex. The arrangement of the concentric bands of cells is representative of most mammal species, though there is some species variation. In the adult human gland, the glomerulosa is sparse and confined to discrete islets, whereas in others, such as the dog, the glomerulosa is much more marked, with large whorls of cells justifying the comparison with the renal glomerulus. Additional zones may occur in other species, including the X zone in the mouse gland, and the fetal zone in the developing and newborn human gland (See Chester Jones, 1957; Deane, 1962; Neville and O'Hare, 1981; Vinson et al., 1992b).



**FIGURE 2 | Higher power view of the capsule/glomerulosa/outer fasciculata view of the rat adrenal cortex.** Note that in this tissue from an ~2 month old animal, the zona intermedia is very sparse. There is a marked differentiation between the glomerulosa and the fasciculata, emphasizing the precision with which the signals that determine positioning of the different cell types must operate (see text).

Thus, zonation is not described by function, nor by the presence of this or that steroid product, or molecular marker. As it happens, and as this article will make clear, function follows morphology/topology only partially. For example, in the rat adrenal, only outer zona glomerulosa cells express CYP11B2 (see Table 1 for abbreviations) in sodium sufficient animals, and the inner glomerulosa does not. The non-CYP11B2-containing glomerulosa region was first called the white or undifferentiated zone (ZU) (Mitani et al., 1994, 1995), while others have called it the “zona intermedia” (Engeland and Levay-Young, 1999; Ulrich-Lai et al., 2006). This is confusing, because the term zona intermedia was previously applied to a morphologically distinct flattened cell type, quite different from the glomerulosa, that is limited to a very few cells at the glomerulosa-fasciculata border (Cater and Lever, 1954; Chester Jones, 1957; Deane, 1962; Nussdorfer, 1986). Indeed Cater and Lever (Cater and Lever, 1954) report it as absent from 2 month old rat adrenals, while it is very marked in older animals. It is sparsely evident in the illustration in Figure 2. It should be noted that these descriptions apply to the rat adrenal, and the presence of a discrete ZU in other species is not clear (see below).

Similarly, others have interpreted, and used, CYP11B2 as a “highly specific zona glomerulosa marker” (Freedman et al., 2013). It isn’t, since (as noted above) it is expressed by only a small population of glomerulosa cells.

In yet another re-writing of the literature on adrenocortical zonation, some authors appear to define the zona reticularis, not on the basis of its morphology, but as the site of DHEA and androstenedione production. Defined in this way, the rat adrenal, it is claimed, does not contain a zona reticularis (de Joussineau et al., 2012; Pihlajoki et al., 2015), when it quite obviously does!—see Figure 1—moreover reticularis cells have been isolated from rat adrenals, their steroidogenic potential analyzed, and even shown to produce androstenedione, albeit in small amounts (Bell et al., 1978, 1979), see also (Pignatelli et al., 2006).

**TABLE 1 | Abbreviations used.**

ACTH	Adrenocorticotropic hormone, corticotrophin
AgRP	Agouti-related peptide
AKR1B7	Aldo-keto reductase family 1 member B7
AKR1C3	Aldo-keto reductase family 1 member C3, type 5 17 $\beta$ -HSD
ANP	Atrial natriuretic peptide
AP-1	Activator protein 1 (c-fos, c-jun heterodimeric transcription factor)
APCC	Aldosterone producing cell clusters
ARNTL	Aryl hydrocarbon receptor nuclear translocator-like protein 1, =BMAL
AsP	Adrenal secretory protease
AT1R	Angiotensin II type 1 receptor
AZ1	Adrenocortical zonation factor
BMAL	basic helix-loop-helix/PER-ARNT-SIM (bHLH/PAS) transcription factor, = ARNTL, MOP3
BMP	Bone morphogenetic protein
3 $\beta$ -HSD	3 $\beta$ -hydroxysteroid dehydrogenase
11 $\beta$ -HSD	11 $\beta$ -hydroxysteroid dehydrogenase
20 $\alpha$ -HSD	20 $\alpha$ -hydroxysteroid dehydrogenase
18-OH-DOC	18-hydroxydeoxycorticosterone
BrdU	Bromodeoxyuridine
c-AMP	cyclic adenosine monophosphate
c-fos	See AP1
c-jun	See AP1
CLOCK	Circadian Locomotor Output Cycle Kaput gene
CRF	Corticotrophin releasing factor
Cry2	Cryptochrome circadian clock 2 gene
Cyt B5	Cytochrome B5
CYP2D16	Cytochrome P450 <sub>2D16</sub>
CYP11A	Cytochrome P450 <sub>sox</sub> (cholesterol side chain cleavage)
CYP11B1	Cytochrome P450 <sub>11B1</sub> (11 $\beta$ -hydroxylase)
CYP11B2	Cytochrome P450 <sub>11B2</sub> (aldosterone synthase)
CYP17	Cytochrome P450 <sub>17</sub> (17-hydroxylase)
CYP21	Cytochrome P450 <sub>21</sub> (21-hydroxylase)
Dab-2	Disabled homolog-2, = DOC2, C9, p96/p67
DACH-1	Dachshund family transcription factor
DAX1	Dosage sensitive reversal adrenal hypoplasia critical region, chromosome X, = NrOb1
DHCR24	24 dehydrocholesterol reductase, seladin-1
DHEA	Dehydroepiandrosterone
Eph	Ephrin receptor
ERK	Extracellular signal regulated kinase, = MAPK
bFGF	basic Fibroblast growth factor
GATA	GATA sequence binding transcription factor
Gi1	Glioblastoma 1
Grx	Glutaredoxin
GPCR	G protein coupled receptor
IGIF	Interferon- $\gamma$ inducing factor = IL 18 or IL-1 $\gamma$
IGF	Insulin-like growth factor
IL	Interleukin
IP3	Inositol trisphosphate
IZA	Inner zone antigen, = PGRMC-1, Sigma 2 receptor
Ki67	Antigen K67, = MKI67

(Continued)

**TABLE 1 | Continued**

LEF1	Lymphoid enhancer-binding factor 1
LHR	Luteinizing hormone receptor
MC2R/MC5R	Melanocortin receptor 2/5 (ACTH/ melanotrophin receptors respectively)
MAPK	Mitogen activated protein kinase, =ERK
MnSOD	Manganese superoxide dismutase
MRAP	Melanocortin-2-receptor accessory protein
$\alpha$ -MSH	$\alpha$ -melanocyte stimulating hormone
Nek2	NMA (never in mitosis gene a) related expressed kinase 2
NGFIB	nerve growth factor induced clone B;
NPY	Neuropeptide Y
Notch1,2	Notch ( <i>Drosophila</i> ) homolog 1,2
NOV	Nephroblastoma overexpressed, = CCN3
NURR-1	Nur-related factor 1
OAT	Organic anion transporter
OATP	Organic anion transporter polypeptide
ODC	Ornithine decarboxylase
Per	Period component of Per/Tim (timeless) heterodimer
Pik3c2g	Phosphatidylinositol-4-phosphate 3-kinase C2 domain-containing gamma polypeptide gene
PKC	Protein kinase C
Prkar1 $\alpha$	Type 1 $\alpha$ -regulatory subunit of cAMP dependent protein kinase
PPAR $\gamma$	Peroxisome proliferator activated receptor
Pref1	Preadipocyte factor 1, = dlk1 (delta like homolog 1)
Prx	Peroxiredoxin
ROS	Reactive oxygen species
R26 YFP	Rosa 26 yellow fluorescent protein
Rev-Erb $\alpha$ )	Nuclear receptor subfamily 1, group D, member 1, NR1D1
SCN	Suprachiasmatic nucleus
SF1	Steroidogenic factor 1 (also AD4BP, NR5A1)
Shh	Sonic hedgehog (hedgehog signaling pathway component)
SLC27A2	Solute carrier family 27 (fatty acid transporter), member 2
StAR	Steroidogenic acute regulatory protein
SULT2A1	Steroid sulfotransferase
TASK1,3	TWIK-related acid-sensitive K <sup>+</sup> channels, KCNK3 and 9 (members of family of two-pore domain potassium channels)
TGF $\beta$	Transforming growth factor
TNF	Tumor necrosis factor
Trx	Thioredoxin
TrxR	Thioredoxin reductase
TSPAN12	Tetraspanin 12
Ucn3	Urocortin 3
VIP	Vasoactive intestinal peptide
Wnt	Wingless related integration site
ZU	Undifferentiated zone

## ORIGIN OF THE ADRENAL CORTEX, CELL PROLIFERATION, AND MIGRATION

The adrenal cortex originates from a section of the coelomic epithelium, firstly as part of the SF1-expressing adrenogonadal primordium, then separating as the adrenal anlage into approximately its ultimate location, ventrolateral to the dorsal

aorta. At this site, it is invaded by cells from the neural crest, and eventually becomes encapsulated as the discrete adrenal gland (Xing et al., 2015). An interplay between the transcription factors SF1 and Dax1 determines the extent to which steroidogenic enzymes are induced, and adrenocortical cells become differentiated: Dax 1, as a co-repressor of SF1, sustains the pluripotential stem cell-like phenotype (Xing et al., 2015).

In the established cortex, the central concept that there is a stem cell population located in the capsule or immediate subcapsular region (Zwemer et al., 1938; Lombardo and Cortesini, 1988) has largely been supported by evidence from several types of experiment that track the fate of labeled cells.

Earlier studies followed labeled cells after the pulse administration of tritiated thymidine in the rat (Wright, 1971; Wright et al., 1973; Zajicek et al., 1986), latterly BrdU was used in mice (Chang et al., 2013). These gave broadly similar results, showing initial labeling in the outer part of the gland, with centripetal migration and phenotype change at a rate of 13–20  $\mu\text{m}$  per day in the mouse adrenal (Chang et al., 2013), reaching the medulla after 104 days from initial labeling in the rat gland (Zajicek et al., 1986). Others have used transgenic mice bearing the 21OH/LacZ gene (Morley et al., 1996) or other constructs (Walczak and Hammer, 2015). Using 21OH/LacZ transgenic mice gives typical results in that mice bearing this transgene develop stripes of beta-gal staining cells in the adrenal extending from the capsule to the medulla, consistent with the concept of an outer region of proliferation with subsequent inward migration. It has to be said that the staining is not consistent with the distribution of 21-hydroxylase in the normal mouse adrenal, which is present only as a trace in the glomerulosa, though strongly expressed in the adrenal inner zones (Chang et al., 2013). Similar radial distribution of labeled cells has shown such lineages in mice bearing for example *shh*<sup>gfpcre</sup>;R26-YFP cells: *shh* is highly transcribed in the outer cortex in the mouse (though only in the ZU in the rat), so this parallels the results obtained with 21OH/LacZ. A similar cell lineage is seen in mice with the *Gli1-creT2 R26-X* label: *Gli1* is normally transcribed in the inner capsule in cells that apparently represent stem cells. Since the capsular expression of *Gli1* is induced by *shh* signaling, this suggests what King and co-authors call a bootstrapping loop, by which the recruitment of new cortical cells from the capsule is stimulated, and both *shh* and *Gli1* cells are transformed into other cell types throughout the cortex (King et al., 2009). In fact this process is reinforced by the glomerulosa specific Dlk1 (also known as Pref-1), a member of the Notch family, which is co-expressed with *shh* in the rat, and, like *shh*, also signals to the capsule, inducing Gli-1 transcription and cortical cell recruitment (Guasti et al., 2013b; Finco et al., 2015). In mouse adrenals regenerating after dexamethasone treatment, fasciculata cells were similarly shown to be formed directly from CYP11B2-Cre-bearing zona glomerulosa cells in an SF-1 dependent process (Freedman et al., 2013), although the possibility of an intermediate *shh*-expressing form does not seem to be excluded.

There is often some ambiguity in such studies in that although the Standard Model is supported, alternative lineages are not

necessarily excluded. Thus, fasciculata cells may be formed in the mouse adrenal from sources independent of the glomerulosa (Freedman et al., 2013). That proliferation may occur at various sites in the gland (Payet et al., 1980; Kirillov and Popova, 1985; McEwan et al., 1996, 1999) does not of itself counter the view that the stem cells are capsular or subcapsular. However, there is some additional complexity revealed by the timed appearance of BrdU labeled cells following pulse labeling in the mouse adrenal cortex that suggests there may be variation in the fates of individual cells (Chang et al., 2013), for example, some cells may be retained in the glomerulosa. Perhaps this may be expected from the evidence of the expanded glomerulosa, and CYP11B2 expression, that results from a low sodium diet or angiotensin II treatment (McEwan et al., 1996, 1999). In control mice, Ki67 staining showed proliferation predominantly in the glomerulosa and also in the outer fasciculata, where it was significantly increased by a pulse of ACTH. These fasciculata cells also expressed 21-hydroxylase, whereas the BrdU and Ki67 stained glomerulosa cells were steroidogenically inert, confirming that proliferation occurs in differentiated as well as partially differentiated cells (Chang et al., 2013).

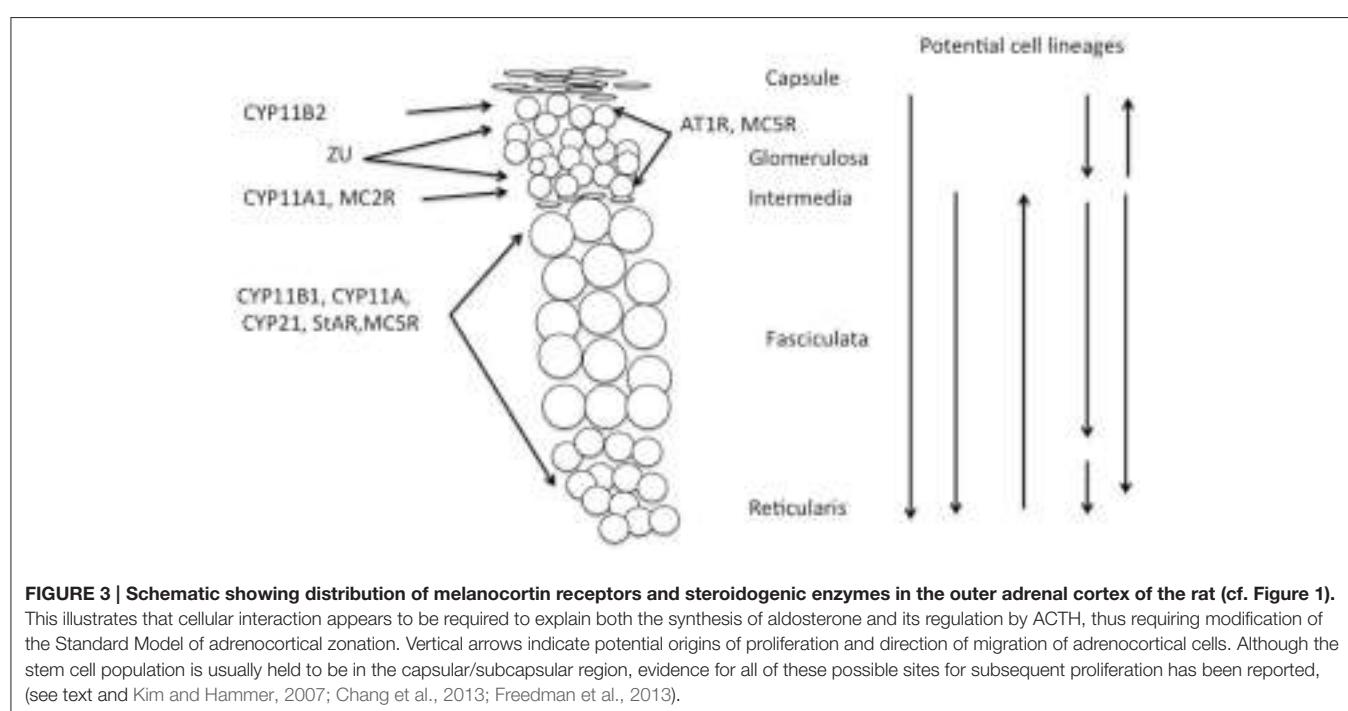
Another example of such ambiguity is found in rats, where the compensatory hypertrophy following unilateral adrenalectomy may depend on proliferation in the fasciculata (Engeland et al., 2005) thus 5 days after enucleation proliferation may occur in both the glomerulosa and the inner part of the regenerating fasciculata, and after 10–28 days in the outer fasciculata (Ennen et al., 2005). Different sources of cells may also arise in other experimental conditions, for example in the Prkar1a knockout mouse, there is a strong ACTH-independent centrifugal expansion of corticosterone secreting cells, apparently

from the X-zone (Sahut-Barnola et al., 2010; de Joussineau et al., 2012). All these potential sites for proliferation and migration of cells are indicated in **Figure 3**.

The assumption that, since adrenocortical cells originate in the outer part of the gland, and migrate centripetally, apoptosis is essentially a reticularis event, also requires re-examination, and it now appears that apoptosis occurs throughout all three zones in humans (Wolkersdorfer and Bornstein, 1998), and in the rat (Petrovic-Kosanovic et al., 2013). Like the cortex-wide proliferation that has been described, this presumably confers flexible tissue remodeling at all levels in the gland. ACTH inhibits apoptosis, thus stabilizing cortical structure *in vivo* (Carsia et al., 1996; Thomas et al., 2004), and *in vitro* according to some authors (Carsia et al., 1996) although not according to others, who describe ACTH-induced apoptosis in cultured rat cells (Mattos et al., 2011).

## MAINTENANCE OF ZONAL DIFFERENTIATION

Here zonal differentiation implies the anatomical and functional features that distinguish the cells of the different zones. As emphasized above, and will be seen in **Table 2**, functional differences are not always definitive, many properties may be shared. Nevertheless, it is remarkable how the differentiation between glomerulosa and fasciculata, for example, is maintained (cf. **Figures 1, 2**). The selection and maintenance of cellular phenotype clearly depends on location within the gland. Thus, the three adrenocortical zones always occupy the same relative position, glomerulosa outermost, reticularis innermost, adjacent



**TABLE 2 | Characteristics of zonal function in (a) rat (b) mouse, and (c) human adult adrenal glands.**

(a) Rat				
Factor/Gene	Glomerulosa	Fasciculata/	Reticularis	References
<b>STEROIDOGENESIS</b>				
CYP11A	+/-?	+++		Roskelley and Auersperg, 1990; Halder et al., 1998; Mitani et al., 1999; Guasti et al., 2013b; but see Engeland and Levay-Young (1999)
CYP11B1	-	+++	+/-	Ho and Vinson, 1993; Halder et al., 1998; Mitani et al., 1999
CYP11B2	+	-		Halder et al., 1998; Peters et al., 1998; Mitani et al., 1999
CYP21	+/-	+++		Chang et al., 2011, 2013
3 $\beta$ -HSD	+	+++		Dupont et al., 1991; Engeland and Levay-Young, 1999; Pignatelli et al., 1999
11 $\beta$ -HSD1	-	-	+	Shimojo et al., 1996
11 $\beta$ -HSD2	-	++	++	Smith et al., 1997; Atanassova and Koeva, 2012
StAR	-	+++		Peters et al., 1998, 2007
<b>RECEPTORS</b>				
ATR1	+++	-	-	Lehoux et al., 1997
MC2R	+/- Inner ZU,	+++	+/-	Gorriaga et al., 2011
MRAP	+/- see text	+++	+/-	
MRAP2	=/-	+/-	+/-	
MC5R	+++	+		Griffon et al., 1994; van der Kraan et al., 1998
<b>ZONAL HOMEOSTASIS</b>				
Shh	+++	-		Guasti et al., 2011, 2013b
Pref-1/dlk1	+++	-		Halder et al., 1998; Raza et al., 1998; Whitworth and Vinson, 2000
<b>NEUROTRANSMITTER</b>				
VIP	+	-		Holzwarth et al., 1987; Bernet et al., 1994
NPY	+	-		Holzwarth et al., 1987; Bernet et al., 1994; Renshaw and Hinson, 2001
Catecholamines	+	-		Holzwarth et al., 1987; Bornstein and Ehrhart-Bornstein, 1992
<b>CYTOKINES</b>				
IGIF	-	+++	++	Conti et al., 1997
*IL-6	+			Judd and MacLeod, 1992
*TNF	+			Judd and MacLeod, 1995
<b>GROWTH FACTORS</b>				
Prorenin	+	++	+	Ho and Vinson, 1998
bFGF	+	+		Chambaz et al., 1994; Ho and Vinson, 1995, 1997
IGF	+	+		
TGF $\beta$	-	-		
<b>SIGNALING</b>				
ANP	+++	-		Lai et al., 2000
AgRP	-	+++	+++	Bicknell et al., 2000
Prorenin	-	+		Ho and Vinson, 1998
MAPK:ERK-1, ERK-2	++	-		McNeill et al., 1998; McNeill and Vinson, 2000; Vinson et al., 2000; Guasti et al., 2013b
SF-1	++	++		Raza et al., 1998
Dab2	+++	-		Romero et al., 2007
Nek 2b	+	+++	-	de Mendonca et al., 2014
Notch1	+++	++	+	
Notch2	+capsule	-	-	
Notch3	+++	++	+	
<b>DETOXIFICATION</b>				
**MnSOD	-	++		Raza and Vinson, 2000
<b>CLOCK GENES</b>				
PER 1	(?)	+++ (reticularis)		Fahrenkrug et al., 2008
PER 2 Bmal 1				

(Continued)

**TABLE 2 | Continued**

Factor/Gene	Glomerulosa	Fasciculata/	Reticularis	References
<b>OTHER</b>				
IZA	—	+++	++	Barker et al., 1992; Halder et al., 1998; Vendeira et al., 1999; Raza et al., 2001
OAT1	—	++		Beery et al., 2003
Oatp1,2	—	++		
Oatp3	++	—		
AsP	+++	+		Bicknell et al., 2001
EphA2, EphA2, EphA3	++	—		Brennan et al., 2008
Spexin	+++	++	++	Rucinski et al., 2010

\*Release from incubated glomerulosa cells \*\*immunoblotting

**(b) Mouse**

Factor/Gene	Glomerulosa	Fasciculata/Reticularis	X-Zone	References
<b>STEROIDOGENESIS</b>				
CYP11B1	—	+++	— (?)	Mukai et al., 2003; King et al., 2009
CYP11B2	+++	—		
20 $\alpha$ -HSD	—	—	++	Hershkovitz et al., 2007
3 $\beta$ -HSD	+++	+++	—	
CYP21	+/-	+++		Chang et al., 2013
<b>RECEPTORS</b>				
ATR1	+++	—	—	Huang et al., 2013
<b>SIGNALING</b>				
Shh	+++	—		King et al., 2009
Dax1	+++	—		Kim et al., 2009
GATA4/GATA6 GATA4 in outer capsule only	+++			Mukai et al., 2002; Scheys et al., 2011
$\beta$ -catenin	+++	+/-		Tevosian et al., 2015 Pihlajoki et al., 2015
<b>CLOCK GENES</b>				
Bmal1, CLOCK, Cry2	+++	++		Oster et al., 2006
Per1, Per2, Per3, Rev-Erb $\alpha$				
Pik3c2g	—	—	+++	Pihlajoki et al., 2013
<b>DETOXIFICATION</b>				
AKR1B7	—?	++		Aigueperse et al., 2001; Martinez et al., 2001
Grx 1	+/-	+++	+	Godoy et al., 2011
Grx 2,5	++	+	++	
Prx 1,4,5	+	+++	+	
Prx 3	+	+	++	
Trx 1,2	+	+++	+	
TrxR2	+	—	+	
Other				
AZ-1	+++	+(?)	+(?)	Mukai et al., 2003; Bastida et al., 2007; Li et al., 2007
ODC	+++	++	—	

**(c) Human**

Factor/Gene	Glomerulosa	Fasciculata	Reticularis	References
<b>STEROIDOGENESIS</b>				
CYP11B1	—	+++	+++	Nishimoto et al., 2010
CYP11B2	+	—	—	

(Continued)

**TABLE 2 | Continued**

Factor/Gene	Glomerulosa	Fasciculata/	Reticularis	References
<sup>†</sup> CYP11A		+++	+++	Rege et al., 2014
CYP17		+++	+++	Nishimoto et al., 2010
CYPB5		+	+++	Rainey et al., 2002
SULT2A1		+	+++	Rainey and Nakamura, 2008
3 $\beta$ -HSD	+	+++	+/-	Nishimoto et al., 2010; but see Rainey et al. (2002)
<sup>†</sup> 3 $\beta$ -HSD2		+++	+	Rege et al., 2014
<sup>†</sup> AKR1C3		+		
<b>ZONAL HOMEOSTASIS</b>				
DACH1	+	+++		Zhou et al., 2015
BMP4	+++	++	+	Rege et al., 2015
<b>SIGNALING</b>				
* <sup>†</sup> LEF1		+++	+/-	Rege et al., 2014
* <sup>†</sup> NOV		+++	+/-	
<sup>†</sup> SLC27A2		+	+++	
<sup>†</sup> TSPAN12		+	+++	
NGFIB	+++	++		Bassett et al., 2004b
Nurr-1	+++	+		
PPAR $\gamma$	++	+		Urano et al., 2011
Ucn3	+	+		Fukuda et al., 2005
CRF1	+/-	++	++	
CRF2	+/-	++	++	
LHR	+/-		+	Pabon et al., 1996
<b>DETOXIFICATION</b>				
Seladin	-	+++	+++	Battista et al., 2009
<sup>†</sup> AKR1C3		+	+++	Rege et al., 2014

This table lists some of the genes and gene products that have been used to define the different functions of the zones of the adrenal cortex, mostly using either immunocytochemistry (shown in normal text) or *in situ* hybridization (*in italics*). It is by no means exhaustive, many more components have been listed, notably when cDNA arrays have been used, (e.g., de Mendonca et al., 2014; Nishimoto et al., 2014; Tyczewska et al., 2014). Plus signs (+) indicate presence, minus signs (-) absence. The number of + signs gives a rough indication of the abundance of signal under control conditions, i.e., with no special treatment. Naturally all of these values may change, sometimes enormously, if neutral physiological regimes are perturbed.

\*Also reported in glomerulosa, but data not provided.

<sup>†</sup> Transcriptome profiling.

to the medulla, and the fasciculata between. How can this be achieved? As discussed further below, differentiated zonal function is maintained by systemic factors, including (in experimental animals at least) angiotensin II, potassium ions and  $\alpha$ -MSH in the glomerulosa, and ACTH in the fasciculata and reticularis. At the same time these activators are clearly not the originating stimuli for differentiation since their receptors are already zonally distributed in order for them to have a zonally specific response—they therefore reinforce the actions of existing morphogens. An example of how this can be disturbed is given by experiments in which the disruption of the tandem potassium channels TASK1 and TASK3 gives rise to extended expression of CYP11B2 in the mouse fasciculata, resulting in aldosterone secretion that is insensitive to sodium intake, though sensitive to ACTH regulation. This is only true of females, so this unprecedented chaotically unzoned hypersecretion of aldosterone is remediable by testosterone treatment (Heitzmann et al., 2008). In contrast to the action of testosterone, which tends to restore the normal zonal, i.e., exclusively glomerulosa, distribution of CYP11B2, inactivation of the Cav2.3 calcium

channel in TASK1<sup>-/-</sup> mice reduces aldosterone to normal levels, but leaves CYP11B2 in the fasciculata (El Wakil, 2013).

So, if systemic factors cannot be responsible for localization of cell type and the organization of the zones, these must instead depend on factors within the gland. It may be speculated what such factors might be. We have previously considered these to be of three types, morphogens or paracrine agents produced within the gland, growth factors that essentially amplify the morphogenic signal, and transcription factors (Vinson and Ho, 1998; Vinson, 2003). In essence this is illustrated by the action of shh in upregulating Gli1, or the Wnt/catenin systems, described more fully below.

The concept that morphogens, secreted by a fixed source, diffuse along a concentration gradient to inform more or less remote cells of their position, and trigger appropriate responses, is sometimes difficult to envisage, because of the randomness of diffusion, and its susceptibility to local variation due for example to tissue inhomogeneity (Lander, 2013), and, in the case of the adrenal, also because of the relatively massive centripetal

blood flow (Vinson et al., 1985b; Vinson and Hinson, 1992). The actions of morphogen gradients have been most widely studied in the context of limb development and regeneration in mice and other vertebrates. In mice, for example, bone morphogenetic proteins, BMPs, which are closely related to the TGF family, stimulate local growth and regeneration (Urist, 1965; Reddi, 1998; Bandyopadhyay et al., 2006; Tsuji et al., 2006; Yu et al., 2012) and have been primarily recognized for their roles in stimulating chondrogenesis and osteogenesis. However, they are also important in other tissues including the prostate (Thomas et al., 1998) breast and prostate cancer (Buijs et al., 2010), and in the pituitary and other components of the reproductive system (Shimasaki et al., 2004). Here wnt and shh signaling is crucial, as in the early development of the adrenal (below). In the limb, induction of shh is brought about by retinoic acid, which generates positional information at various stages in development (Tickle, 2006). This is achieved with a robustness that is remarkable, given the inherent variation in retinoic acid availability, depending as it does on dietary sources. But retinoic acid gradients may not depend solely on release and diffusion, but additionally on sites of degradation, as in the zebrafish brain (White et al., 2007; Schilling et al., 2012). It is possible that this applies to other morphogens, which could be critical when not only morphogen concentration but the duration of target cell exposure is critical, as in the mouse limb response to shh (Tickle, 2006). Crucially, it is thought that sharp boundaries between different cell types, such as found in the adrenal cortex, can still be achieved in the presence of a fluctuating and shallow morphogen gradient, by means of various mechanisms that depend in part on cell plasticity and in part on intercellular signaling. In other systems, these may include for example the retinoic acid-sensitive autoactivating genes Krox 20 and Hoxb1 that are mutually inhibitory in the zebrafish hindbrain (Zhang et al., 2012). Other mechanisms of noise-induced switching have been described (Schilling et al., 2012). Nevertheless, interfering with gap junctions affects positional clarity (Tickle, 2006), so direct cell-cell signaling is also involved (Lander, 2013).

With regard to the adrenal, it is clear that the same morphogens may play a role as in vertebrate limb regeneration, notably Wnt, Shh and BMPs. BMPs are expressed in all zones of the human adrenal cortex, but particularly the glomerulosa (see **Table 2**). Additionally, in H295R cells, BMP4 suppresses CYP17 RNA and protein, and DHEA secretion (Rege et al., 2015). In contrast, overexpression of the transcription factor DACH1 activates TGF $\beta$  and canonical Wnt signaling pathways, thereby suppressing CYP11B2 and aldosterone secretion (Zhou et al., 2015). Considerable advances in recent years have highlighted the particular importance of the Wnt/catenin system (MacDonald et al., 2009). Secreted Wnt glycoproteins bind to GPCRs of the *frizzled* family, bringing about increased cell content of the coactivator  $\beta$ -catenin, now known to be essential for the differentiation of SF1 positive adrenocortical cells from the adrenal capsule, and for their proliferation (Kim et al., 2008, 2009). The Wnt/catenin signaling system is involved in adrenocortical development in other ways also, by interaction with the nuclear receptor Dax1 and inhibin, both of which modulate adrenal/gonadal selection. Dax1 is induced by SF1, but

inhibits SF1 stimulated gene transcription (Ito et al., 1997; Yu et al., 1998). DAX1 is prominent in the subcapsular region in the mouse adrenal (but completely absent from the X-zone) (Mukai et al., 2002), together with  $\beta$ -catenin (Pihlajoki et al., 2015). Inhibin- $\alpha$ , which suppresses gonadal phenotype, is induced by SF1 and  $\beta$ -catenin (Gummow et al., 2003). The overall outcome of these effects in combination, is both to establish the adrenocortical fate of the subcapsular cells, while maintaining their further developmental plasticity (Wood and Hammer, 2010; Simon and Hammer, 2012). This, in effect, defines the glomerulosa (Berthon et al., 2012). SF1 is absolutely required for the development of the gland, and for the cell redifferentiation associated with centripetal migration (Freedman et al., 2013).

The search for stimulators of migration has however so far produced meager results. It is possible that the extracellular matrix may be involved: in bovine adrenal cells, laminin secretion is regulated by ACTH, and, in Boyden chamber experiments, laminin acted as a chemoattractant in promoting adrenal cell migration. However, the distribution of laminin is uniform throughout the cortex, and therefore seems unlikely to promote adrenocortical cell cellular migration (Pellerin et al., 1997). Other extracellular matrix proteins, including fibronectin and collagens I and IV, have been shown to affect steroidogenic enzyme expression in rat adrenal cells in culture, and in particular to modulate basal and ACTH stimulated steroid secretion (Otis et al., 2007). Additionally, rat glomerulosa cells secrete fibronectin, which promotes proliferation, while angiotensin II interferes with this process at the level of integrin binding, thus inhibiting proliferation while enhancing steroidogenesis (Otis et al., 2008).

Equally, how the inward migration of cells is achieved while the position of the zones relative to each other remains constant also continues to be a matter of some mystery. It is possible to speculate that various mechanisms might play a role. It is entirely possible that structures within the gland external to the adrenocortical cells themselves provide positional information. Such structures could include the neural network or elements of the vasculature, which though not necessarily visible in conventional microscopy are nevertheless important components both of which affect adrenocortical function (**Figure 4**; Vinson and Hinson, 1992; Vinson et al., 1994) and may be independent of ACTH (Bocian-Sobkowska et al., 1997). Alternatively, the relative positions of the capsule, external to the cortex, and of the inner medulla might suggest that a morphogenetic landscape could be generated by signals from these two poles. Certainly medullary products can affect inner cortical function (Ehrhart-Bornstein et al., 2000; Haidan et al., 2000). Or, another possibility is that positional information is provided by the adrenocortical cells themselves. Cortical cells, arranged as they are in cords traversing the cortex, themselves provide a polarity, indeed each cell may have an apical and a basal pole. Clearly this is true at least of some of them: cells of the outermost glomerulosa layer (which express both CYP11B2, AT1R, and MC5R) about the capsule at one pole, and other, perhaps less differentiated cells at the base. The innermost glomerulosa cells in the ZU abut either true zona intermedia or outer fasciculata cells, while the innermost reticularis cells



**FIGURE 4 | General arrangement of the vasculature and innervation of the rat adrenal cortex.** Most nerves are located in the capsular (c) and subcapsular region, with arborization around the vasculature, including arterioles (a) and around the cells of the glomerulosa (zg). Nerve fibers are rare in the fasciculata (zf), but some traverse the width of the zone, often associated with medullary arteries (ma, 6–8 per gland), which specifically supply the medulla. Most of the blood reaching the medulla passes centripetally through the sinuses, which, in contrast to the thicker walled medullary arteries, are bordered by a single layer of very attenuated endothelial cells (cf. **Figure 2**). Near the medulla (m) a few short nerve fibers reach the reticularis (zr) from islets of chromaffin cells (i) in the inner cortex and from the medulla itself. There is now considerable evidence that products from both neural tissue and vasculature can affect corticosteroid secretion. They may also provide positional information for migrating and transforming cortical cells (see text). Drawing by Bridget Landon. Reproduced with permission from Vinson et al. (1994).

lie directly adjacent to the medulla in the rat; in other species, such as the postpubertal male mouse there may be a band of connective tissue around the medulla. Such cell-cell contact is known to be important in positional information in other tissues (Tickle, 2006; Lander, 2013). The Eph/ephrin forward-reverse signaling system provides a possible mechanism for such cellular orientation and migration within the zones, and elements of the system have been demonstrated in the rat adrenal cortex. In

particular, EphA2 is strongly transcribed in the rat glomerulosa, and responds to the physiological stimuli of a low sodium diet, captopril, or betamethasone treatment in a manner that suggests association with cell phenotype (Brennan et al., 2008).

## FUNCTIONAL ZONATION OF THE ADRENAL CORTEX

A summary of some of the components expressed in the zones of the rat mouse and human cortex is given in **Table 2**. The Standard Model of adrenocortical zonation, as summarized in the Introduction, interprets functional zonation in terms of steroid hormone output alone. It is quite evident that in fact many other adrenocortical cell products are zone specific, and functional zonation is not just about steroids. Many of these other zonally organized functions, such as receptors and signaling pathways that transduce trophic hormone action, can be considered to complement the steroidogenic function. Others, such as clock genes, or enzymes metabolizing xenobiotics, may not be quite so easily accommodated in this way.

Although the same corticosteroids, cortisol, corticosterone, and aldosterone (though not usually all three) appear throughout the non-mammalian vertebrates, functional zonation of the adrenal cortex is a universal feature in mammals alone. While the appearance of the zones in different mammalian species may vary, aspects of their function do not. Thus, it is clear that the CYP11B2 catalyzed production of aldosterone is invariably limited to a population of cells in the zona glomerulosa, and CYP11B1 generates cortisol (or corticosterone in rats, mice, and some other species) predominantly in the fasciculata, and to a lesser extent, in the reticularis. Though this is consistent with the Standard Model, it is nevertheless now clear that some qualification is required.

The qualification arises because the extent of *de novo* steroidogenesis that can be unequivocally associated with each zone is limited. The human adrenal, for example, is complex. Here the well-defined CYP11B2-expressing glomerulosa seen in young individuals gives way in advancing years to a sparse glomerulosa generally confined to scattered islets (Neville and O'Hare, 1981; Aiba and Fujibayashi, 2011), interspersed by cells that may be analogous to those of the rat ZU [and are called the “progenitor zone” by Aiba and Fujibayashi (2011)], but which express 3 $\beta$ -HSD. Later, CYP11B2 expressing cell clusters (APCC) develop at the glomerulosa/fasciculata border, and extend into the fasciculata (Aiba and Fujibayashi, 2011; Nishimoto et al., 2015); these appear from the age of about 30 years. These additional sites of aldosterone production, arising as a consequence of somatic mutation of ion channels, are common in normal subjects, but the authors hypothesize that they predispose to primary aldosteronism (Nishimoto et al., 2015).

In the rat zona glomerulosa, starkly, of the steroidogenic enzymes only CYP11B2 is at all strongly expressed, and that only in the outermost glomerulosa: other required components for complete steroidogenesis, including CYP11A and CYP21 are barely detectable (**Table 2**), as is StAR according to some authors

(Peters et al., 1998, 2007), but not others (Lehoux et al., 1998; Tyczewska et al., 2014).  $3\beta$ -HSD appears to be present in all zones in the rat, as it is in the mouse (**Table 2**) though it is not expressed in the glomerulosa of the bovine adrenal (Ishimura and Fujita, 1997) while in humans it is relatively sparse in the glomerulosa according to some authors (Nishimoto et al., 2010), but not others (Rainey et al., 2002). Guasti et al. (2013a) located CYP11A in the inner undifferentiated zone (ZU) of the rat adrenal zona glomerulosa, quite clearly—indeed by definition—not a site of CYP11B2 expression, which, as noted, is normally confined to a very few cells in the outer glomerulosa. Notably, this site of CYP11A expression coincides with an intense signal for MC2R mRNA, otherwise apparently lacking in the rat glomerulosa (Gorriaga et al., 2011). The intensity of MC2R mRNA staining in this region is not inconsistent with the observations of Gallo-Payet and Escher (1985) who found greater ACTH binding in the glomerulosa than other zones, but who did not distinguish between CYP11B2 expressing and non-expressing glomerulosa cells. Also contrary to expectation, StAR and CYP11A are not always co-expressed. Thus not only does the inner ZU site of CYP11A not coincide with CYP11B2, with the caveats above in mind, it does not here coincide with StAR either: Peters and colleagues have consistently demonstrated StAR expression throughout the fasciculata, but, in untreated animals, hardly at all in any of the morphologically distinct smaller glomerulosa cells in the rat, including the ZU (Peters et al., 1998, 2007).

SF1 though is very definitely present throughout the adrenal cortex (Morohashi et al., 1994; Raza et al., 1998). Although SF1 is absolutely required for the development of the gland and its steroidogenic capacity (Lala et al., 1992; Parker and Schimmer, 1993; Parker et al., 1995; Freedman et al., 2013), its role must be very different in the cells of the different zones. Thus, although exposure to high SF1 inhibits CYP11B2 expression (Ye et al., 2009), the presence of SF1 in the glomerulosa is required not only for functional CYP11B2, but also for the glomerulosa-fasciculata cell type transformation that is a feature of adrenal cell centripetal migration and redifferentiation (Freedman et al., 2013).

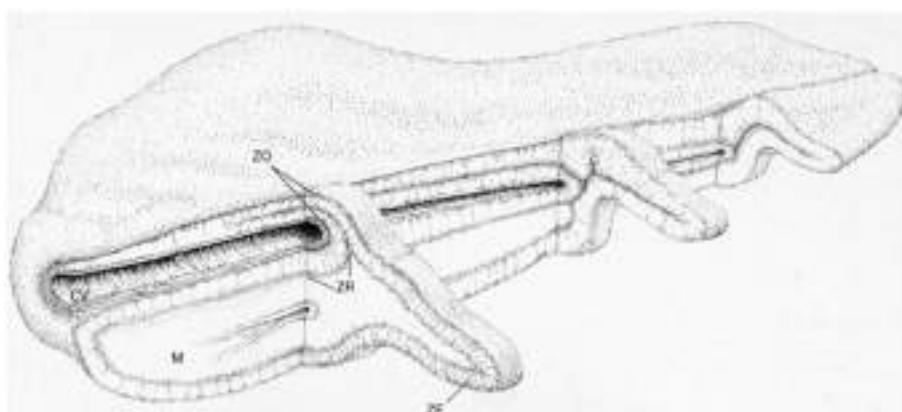
In contrast to the glomerulosa, all fasciculata cells strongly express all of the enzymes required for glucocorticoid production enzymes, CYP11A1, CYP21, CYP11B1,  $3\beta$ -HSD, and StAR, together with, in human (and other species') glands, CYP17 as well. These components are also present in the reticularis though (with the possible exception of CYP17) in lower abundance.

This then raises the question: how is aldosterone produced in the rat and human gland, if the entire required suite of enzymes and StAR are not co-expressed? Twenty years ago, we suggested that (at least) two cell types are required for aldosterone synthesis and secretion in the rat adrenal (Vinson et al., 1995; Vinson, 2004). The evidence for this is primarily that in the rat the efficient production of aldosterone *in vitro* requires an intact gland, and as it is disrupted, from whole tissue, first to separated zones, then to enzyme dispersed cells, aldosterone yields *in vitro* are progressively diminished, whereas corticosterone output is unchanged, and 18-OH-DOC, a major product of the fasciculata in the rat (Tait et al., 1970; Nonaka and Okamoto, 1991; Okamoto and Nonaka, 1992), is

increased (Vinson et al., 1985a). Together with other evidence this suggested 18-OH-DOC might be an aldosterone precursor in this species (Vinson et al., 1992a, 1995; Vinson, 2004). Objections may be raised on the basis that the hemiketal structure of 18-OH-DOC is stable, and not easily converted, that the blood flow is centripetal, hence precluding steroid movement from inner zone to the glomerulosa, and that conversion of exogenous precursors is invariably inefficient. However, tracer 18-OH-DOC is certainly converted to aldosterone by rat glomerulosa tissue, and is sensitive to sodium status (Fattah et al., 1977). How it could reach the glomerulosa *in vivo* remains open to speculation, but if we accept that shh and wnt diffuse centrifugally (see above), there seems no reason why other substances should not. Or there may be cell-cell transport: it is not the proteolytic enzymes used in cell dispersal methods that diminishes aldosterone output, but the physical disruption of the capsular tissue (Raven et al., 1983). Finally, in the rat 18-OH-DOC circulates at concentrations sufficient to be utilized as substrate (Vinson, 2004).

It is not suggested that the same mechanism would necessarily operate in any species other than the rat, which seems to be unique in that 18-OH-DOC is a major product of the fasciculata. Nevertheless, in this connection it is intriguing that in the human gland there exist more obvious mechanisms for the transfer of steroid substrate or other products from the inner zones to the glomerulosa. First, there are arteriovenous loops which derive from the cortical arterioles in the capsule region, pass through the glomerulosa and fasciculata, but then loop back to the outer cortex to a site near their origin. Secondly, as the adrenal vein leaves the gland, the cortex is inverted and wraps around the vein as the cortical cuff, which retains its zonation, (though inverted). Here the blood supply is from the arteriae comitantes in the wall of the vein. Drainage from this region is through vessels from the medulla through the cortex (i.e., from reticularis to the fasciculata and glomerulosa), thence to the central vein (**Figure 5**) (Dobbie and Symington, 1966; Neville and O'Hare, 1981). Finally, in older subjects, CYP11B2 expressing aldosterone producing cell clusters project into the fasciculata (Nishimoto et al., 2015), providing the possibility of much more intimate contact between the different cell types.

A more specific function for the reticularis has sometimes seemed to be less clear cut, especially in those species in which 17-hydroxylase (CYP17) is sparse, but in the human, and some other species, it is the major source of C<sub>19</sub> steroids, especially dehydroepiandrosterone, both as the free steroid and as the sulfate. This general concept has been strengthened by methods developed to separate individual cell types, and assess their steroid output *in vitro*, and most recently by microdissection and transcriptome profiling (see **Table 2**). Notably, these methods have shown that CYP11A1 (side chain cleavage) and CYP17 (17-hydroxylase) gene transcripts are similar in the human fasciculata and reticularis, but Cyt B5A, sulfotransferase SULT2A1, and 17 $\beta$ -HSD (aldo-ketone reductase AKR1C3) are significantly more prominent in the reticularis (Rainey et al., 2002; Rege et al., 2014). The combined activity of these components accounts for the characteristic secretion of large amounts of DHEAS by the reticularis: Cyt B5 has been recognized as an important regulator of CYP17, and its presence is required for the efficient side chain



**FIGURE 5 | General structure of the human adrenal gland showing the cortical cuff.** The inward folding adjacent to the central vein results in a doubling of the cortical thickness and the juxtaposition in this region of the inner zona glomerulosa and the central vein. C, cortex; M, medulla; ZG, zona glomerulosa; ZF, zona fasciculata; ZR, zona reticularis; CV, central vein. Drawing by B. Landon. Figure reproduced with permission from Vinson and Hinson (1992).

cleavage activity of CYP17, though not for its  $17\alpha$ -hydroxylase function (Hall, 1991; Lee-Robichaud et al., 1995; Soucy and Luu-The, 2002). Cyt B5 acts through allosteric action rather than (as originally thought) provision of the second electron required for side chain cleavage (Auchus et al., 1998; Akhtar et al., 2005). It is the heightened secretion of DHEA and DHEAS by the reticularis that incurs the onset of adrenarche in humans (Belgorosky et al., 2008).

How then is androstenedione, the second most abundant androgen secreted by the human adrenal, actually synthesized? As we now see, in the human gland, the fasciculata cell expresses  $3\beta$ -HSD, but the reticularis only a trace. On the other hand, the fasciculata expresses relatively little Cyt B5, required for  $C_{21}$  steroid side chain cleavage, whereas the reticularis expresses a lot. Perhaps interaction between the cell types, as postulated for aldosterone biosynthesis above, provides an answer, with transfer of DHEA from the reticularis to the fasciculata for the final stage of  $3\beta$ -HSD catalyzed dehydrogenation/isomerization to give the  $\Delta^4$ -3-ketone configuration of androstenedione.

Zonation in the adrenal cortex of the mouse is more complex than the broad divisions into glomerulosa, fasciculata, and reticularis, usually taken as the mammalian norm, because of the additional presence of an inner zone, the X-zone, that is only apparent in the prepubertal animals of both sexes, and in the nulliparous adult female. It does not appear to be responsive to ACTH, and it has been suggested that it is supported by LH (Chester Jones, 1955). It involutes at puberty in the male mouse, and at first pregnancy in the female leaving a band of connective tissue between the reticularis and the medulla. Its involution is exquisitely sensitive to testosterone, and although from more recent studies it appears that involution precedes the increase in blood testosterone associated with puberty in the male (Hershkovitz et al., 2007), involution does not occur in castrates, and castration of adult males results in the development of a secondary X-zone lying outside the connective tissue residue of the original (Chester Jones, 1955, 1957). The function of

the X-zone has remained largely obscure, though it is known to express  $20\alpha$ -hydroxysteroid dehydrogenase, but not  $3\beta$ -hydroxysteroid dehydrogenase, which occurs widely throughout the rest of the cortex (Hershkovitz et al., 2007). To some extent the lack of data regarding this zone is compounded by the wide variation in its appearance, indeed in some inbred laboratory animals, notably the DDD strain, the extent of X-zone vacuolation suggests the presence of little functional tissue at all (Suto, 2012).

Other components that have a clear zonal distribution are enzymes that are thought to protect the gland against toxic products associated with a high degree of cytochrome P450 activity. Thus the aldo-keto reductase-like enzyme, AKR1B7 disposes of isocaproic aldehyde, the byproduct of CYP11A1 mediated cholesterol side chain cleavage, and manganese superoxide dismutase may protect against free radicals arising from leaky mitochondrial cytochrome P450 activity. Both of these enzymes are expressed in inner adrenocortical zones (in mouse and rat respectively) (Sahut-Barnola et al., 2000; Martinez et al., 2001; Raza et al., 2005), but not in the glomerulosa. In this context, seladin-1 (DHCR24), highly expressed in human and rat fasciculata cells, may be involved in oxidative stress management, and use of a specific seladin-1 inhibitor enhances ROS formation in response to ACTH (Battista et al., 2009). It may also be involved in specific regulation of DHEA secretion (see below).

The multifunctional protein that we originally called the Inner Zone Antigen (IZA) because it is present in the rat adrenocortical fasciculata and reticularis but not glomerulosa (Laird et al., 1988; Barker et al., 1992) was later identified with the so-called progesterone receptor membrane component 1 (PGRMC1) (Min et al., 2004; Cahill, 2007), and is now also designated as the sigma 2 receptor (Xu et al., 2011; Zeng et al., 2016). In the rat adrenal this protein specifically supports 21-hydroxylation (Laird et al., 1988; Min et al., 2005). The inner zone distribution of all three of these components, AKR1B7, MnSOD, IZA/PGRMC1/Sigma2,

and their absence from the glomerulosa is entirely consistent with the view that the glomerulosa is not a significant site of *de novo* steroidogenesis.

Another metabolic function of the adrenal has been described that has a distinct zonal distribution, this is the capacity for xenobiotic metabolism conferred by CYP2D16, a member of the CYP2D cytochrome P450 subfamily, in the reticularis of the guinea pig (Martin and Black, 1983; Black et al., 1989; Jiang et al., 1996; Yuan et al., 1998, 2001). CYP2D16 increases with age, and is more abundant in males than in females, there are also strain differences (Huang et al., 1996). In contrast to CYP17, which is enhanced, CYP2D16 is diminished by ACTH treatment (Yuan et al., 1998), as is 21-hydroxylase in the reticularis, but not in the outer zones (Colby et al., 1992). It is also bimodally distributed in that it is abundant in the majority of individual animals, but low in others (Colby et al., 2000). CYP2D isoforms themselves have 21-hydroxylase activity however, and are responsible for this function in the rat brain (Kishimoto et al., 2004). Type 5 17 $\beta$ -HSD, another member of the aldo-keto reductase family, also designated AKR1C3, is prominently transcribed in the human reticularis (Rege et al., 2014). Its function, presumably, is the reduction of 17-ketosteroids, such as androstenedione to testosterone (Nakamura et al., 2009). Conceivably it may also be protective, perhaps, like CYP2D16, metabolizing xenobiotics or (like AKR1B7) metabolizing isocaproic aldehyde. Interestingly, in other cell types, AKR1C3 also suppresses cell differentiation (Desmond et al., 2003).

Also not directly part of the corticosteroid synthesizing apparatus are the clock genes, which are zonally distributed, though quite differently, in rat and mouse adrenals. Thus *Per 1*, *Per 2*, and *Bmal 1* are most strongly expressed in the reticularis in rats, but, together with other clock genes (see Table 2) in glomerulosa and fasciculata in mice. Their expression depends on clock time, and is instrumental in maintaining the circadian rhythm inherent in corticosteroid secretion, in conjunction with hypothalamic/pituitary input (and in turn its regulation by the SCN clock system) and SCN regulated autonomic innervation (Ishida et al., 2005; Chung S. et al., 2011; Leliavski et al., 2015).

The secretion of endogenous ouabain is also zonal, in that bovine glomerulosa cells secrete several times as much as fasciculata cells, both under control conditions, and also when stimulated by angiotensin II or ACTH (Laredo et al., 1995). The presence of signaling molecules NOV, SCL27A2, and TSPAN12, not previously associated with adrenal function, and their differential transcription in the zones of the human adrenal cortex (Rege et al., 2014; see Table 2) also add to the view that our understanding of zonation is far from complete.

## REGULATION OF ADRENOCORTICAL ZONATION

### Trophic Regulation of Zonal Function

In part, the maintenance of the differentiated cortex follows the activation of specific signaling pathways. Glomerulosa steroidogenic function, that is the expression of CYP11B2 and the final stage of aldosterone synthesis, for example, is induced by

(among other factors) angiotensin II stimulation, via angiotensin type 1 receptors (ATR1a and b) resulting in the activation of calcium mediated signaling pathways, whereas the fasciculata, CYP11B1 expression and cortisol/corticosterone production are ACTH dependent, modulated by the melanocortin type 2 receptor (MC2R) acting through cAMP formation.

### Regulation of the Glomerulosa

Angiotensin II, the potassium ion and ACTH are the most widely studied regulators of aldosterone secretion and glomerulosa function. They have both acute effects and longer term trophic actions.

There is ample evidence that angiotensin II supports glomerulosa structure and aldosterone secretion in a manner which parallels that of dietary sodium restriction: this topic has been reviewed on many occasions, and need not be repeated here (Mulrow, 1999; Hattangady et al., 2012; Bollag, 2014). The potassium ion also has direct stimulatory actions, both on acute secretion of aldosterone (Haning et al., 1970; Aguilera and Catt, 1978), and, longer term, in induction of CYP11B2 (Tremblay et al., 1992; Yagci et al., 1996). Its action is mediated through voltage gated calcium channels (Haning et al., 1970; Aguilera and Catt, 1986; Kenyon et al., 1991) but also may require the presence of angiotensin II, perhaps generated by the intra-adrenal RAS, as well (Yamaguchi et al., 1990; Tremblay et al., 1992; Gupta et al., 1995; Vinson et al., 1998), certainly the actions of angiotensin II and potassium ions are linked (Shepherd et al., 1991).

ACTH, has long been recognized as an acute stimulator of aldosterone secretion (Haning et al., 1970; Aguilera and Catt, 1978; Braley et al., 1992; Williams, 2005; Hattangady et al., 2012; Bollag, 2014). In the rat, this presents an enigma, how is this achieved?—given what we now know about the distribution of MC2R and CYP11A expressing cell types, which in the glomerulosa are seemingly confined to the inner ZU and do not coincide with CYP11B2 (Gorrigan et al., 2011; Guasti et al., 2013a). But glomerulosa preparations, such as used by many authors (Haning et al., 1970; Aguilera and Catt, 1978; Braley et al., 1992; Williams, 2005) clearly will contain both CYP11B2 and CYP11A/MC2R-expressing cell types, as well as, usually, some fasciculata cells as well. This raises the possibility of cell-cell interaction, developed further below.

Chronically, on the other hand, ACTH does not support the glomerulosa cell phenotype, but instead downregulates CYP11B2, induces CYP11B1, and in these and other ways imposes the fasciculata phenotype instead (Aguilera et al., 1981; Ho and Vinson, 1993; Ni et al., 1995; Mitani et al., 1996). Though aldosterone secretion is also sensitive to ACTH stimulation (Hattangady et al., 2012), MC2R inactivated mice have normal glomerulosa, but greatly involuted fasciculata (Chida et al., 2007), whereas POMC knock-out may disrupt the adrenal entirely (see below). This may be attributed to the additional loss of  $\alpha$ -MSH which in rats acts specifically on the glomerulosa in which MC5R abound (van der Kraan et al., 1998; Liakos et al., 2000):  $\alpha$ -MSH acting on MC5R is 10 times more potent than ACTH (1-39), and 100 times more than  $\gamma$ -MSH in stimulating cAMP (Griffon et al., 1994). There is however a problem in that  $\alpha$ -MSH stimulation of aldosterone production by the rat adrenal

is not convincingly linked to cAMP, which is only stimulated at high  $\alpha$ -MSH concentrations, whereas IP3 and PKC pathways are activated at lower levels (Kapas et al., 1992, 1994); others have suggested that a further MSH receptor remains to be discovered (Bicknell et al., 2000). In hypophysectomised rats,  $\alpha$ -MSH, not ACTH is the POMC product that restores aldosterone secretion and its response to sodium depletion (Shenker et al., 1985), cf. (Vinson et al., 1980; Costa et al., 2011).

Downstream of the increased intracellular calcium evoked by potassium or angiotensin II, the orphan nuclear receptors Nurr1 and NFGFIB are both implicated in CYP11B2 expression, which also responds to COUP, SF-1 and CREB through promoter sites (Bassett et al., 2004b,c). NFGFIB, somewhat more widespread through the (human) cortex than Nurr1 (see Table 2), also mediates the expression of 3 $\beta$ -HSD2, which converts the  $\Delta^5,3\beta$ -hydroxy configuration of 17 $\alpha$ -hydroxypregnенolone or DHEA to the corresponding  $\Delta^4,3$ -ketones (Bassett et al., 2004a,d). Angiotensin II also inhibits CYP17 expression, via PKC and src-kinase mediated pathways.

Many other factors have been found to modulate aldosterone secretion, at least under experimental conditions, these include particularly the neurotransmitters (Torda et al., 1988; Janossy et al., 1998; Vinson, 2003; Whitworth et al., 2003) cytokines, which are also produced within the gland (Judd and MacLeod, 1992; Ehrhart-Bornstein et al., 1998; Judd, 1998; Call et al., 2000), vascular products, including endothelins, nitric oxide, and adrenomedullin (Hinson and Kapas, 1998; Delarue et al., 2004), and adipokines (Willenberg et al., 2008).

## Actions of ACTH on Adrenal Proliferation and Zonation

The earliest observations on the effects of hypophysectomy and ACTH treatment on adrenal form and function, showing the absolute dependence of the inner adrenocortical zones, but not the glomerulosa, on an intact functioning pituitary, was borne out by later experiments using specific gene knockout methods. First, the elimination of POMC products, including ACTH, was shown to prevent the complete development of the adrenal gland. In one study, the adrenals of POMC $^{-/-}$  mice were not macroscopically discernable, and both corticosterone and aldosterone were undetectable (Yaswen et al., 1999). However, in another report, although both fasciculata and reticularis/X-zone were greatly reduced in the mutant animals, the glomerulosa was relatively unaffected, and this was reflected in the milder effect on circulating aldosterone, while corticosterone was virtually eliminated (Coll et al., 2004). The administration of ACTH restored both circulating corticosterone (but not aldosterone) and adrenocortical structure to normal (Coll et al., 2004). In a later study, MC2R $^{-/-}$  animals were also shown to have normal glomerulosa structure, but greatly reduced inner adrenocortical zones, combined with undetectable plasma corticosterone while aldosterone was reduced by two thirds (Chida et al., 2007). These effects, of POMC [at least in the (Coll et al., 2004) study] and MC2R knockout, appear accurately to reproduce the actions of hypophysectomy (Chester Jones, 1957; Deane, 1962; Vinson, 2003). It is curious that the rat adrenal also expresses AgRP, an

$\alpha$ -MSH antagonist (Yang et al., 1999) but only in the fasciculata and reticularis, and not the glomerulosa (Bicknell et al., 2000): in the mouse, the agouti locus is associated with X-zone, but not reticularis, morphology (Tanaka et al., 1994, 2006).

Other studies suggest that even within the zona fasciculata different areas may respond differently to chronic stress, which induced hyperplasia in the outer fasciculata, and hypertrophy in the inner fasciculata, while glomerulosa cell size was reduced (Ulrich-Lai et al., 2006).

Peptides derived from pro- $\gamma$ -MSH, a 16kD fragment from the N-terminus of the POMC molecule secreted by the pituitary, that do not contain the  $\gamma$ -MSH sequence are thought to have a role in stimulating adrenocortical proliferation (Estivariz et al., 1982, 1988) and POMC 1-28 in particular has been shown to have this activity. An adrenal serine protease, designated AsP, which is thought to generate mitogenic peptides from pro- $\gamma$ -MSH at the adrenocortical cell surface, has been characterized (Bicknell et al., 2001; Bicknell, 2003). Support for this concept has been provided by some subsequent authors, who found that POMC 1-28 stimulated adrenocortical proliferation in dexamethasone or hypophysectomised rats, though primarily in the glomerulosa (Torres et al., 2010; Mendonca and Lotfi, 2011), but others found it had no effect on adrenal growth in POMC $^{-/-}$  mice, although there was a good response to ACTH (Coll et al., 2006). In contrast, *in vitro*, rat POMC 1-28 stimulated proliferation in cultured rat glomerulosa and fasciculata/reticularis cells, but ACTH was inhibitory (Mattos et al., 2011). The discrepancy between the stimulatory actions of ACTH on adrenal cell proliferation *in vivo* and its inhibitory actions *in vitro* is difficult to resolve. It is possible that other tissue components, such as the vasculature (Figure 4), absent from cell cultures, are required for the proliferative actions of ACTH *in vivo* (Thomas et al., 2004).

Although dependence on ACTH is paramount, other factors are known to modulate fasciculata cell secretory activity, including cytokines, which are differentially produced throughout the cortex, and have specific actions on individual cell types (Spangelo et al., 1995; Judd, 1998; Koldzic-Zivanovic et al., 2006; Woods and Judd, 2008), and neurotransmitters (Ehrhart-Bornstein et al., 2000). The possibility of medullary, neural and vascular regulation of cortical function is emphasized by the intimate relationship between these adrenal gland components (Figure 4).

## Regulation of the Reticularis

The control of adrenal androgen secretion is problematic, in that although supported by ACTH, secretion of androstenedione and DHEA is dissociated from cortisol under some conditions, and particularly during adrenarche which has led some authors to postulate additional pituitary or other factors (McKenna et al., 1997; l'Allemand and Biason-Lauber, 2000), and, more recently, melatonin was found to stimulate DHEA release from cultured adrenals of the solitary hamster *Phodopus sungorus* (Rendon et al., 2015). However, the factors causing the increase of adrenal androgen secretion during adrenarche are largely unknown, as are those causing the later decline, or adrenopause (Dharia and Parker, 2004; Bird, 2012) leading some authors to postulate the importance of intra-adrenal factors; these could include growth

factors (l'Allemand and Biason-Lauber, 2000), cytokines or even steroids. Cortisol stimulates DHEA secretion in H295R cells by inhibiting 3 $\beta$ -HSD (Topor et al., 2011), DHEA modulates chromaffin cell proliferation and differentiation (Chung K. F. et al., 2011) and LH receptors are present in human fasciculata and reticularis, albeit at a low level (Pabon et al., 1996; Rao, 2010). However, another provocative finding in this regard is that inhibition of seladin-1, which is strongly expressed in the fasciculata and reticularis of the human adrenal, specifically inhibits the ACTH induced secretion of DHEA in human adrenal cells, while having no effect on cortisol output (Battista et al., 2009). This awaits physiological interpretation.

## THE SIGNIFICANCE OF ADRENOCORTICAL ZONATION—HOW DOES THE STANDARD MODEL STAND UP?

That adrenocortical cells arise in the capsular or subcapsular region of the gland and migrate inwards is now virtually unchallenged. That proliferation can occur throughout the gland is also clear, but does not affect the main thesis, even given there appear to be exceptional situations in which migration may be reversed.

It is also clear that the distribution of enzymes and other components between the zones show sharp phenotypic differences between the glomerulosa and the fasciculata, and slightly less sharp differences between the fasciculata and the reticularis, hence the inwardly migrating cells undergo more or less profound phenotypic changes in their lifespan. What remains unclear is how this is achieved, although recent advances in our understanding of the role of morphogens such as wnt and shh give some powerful guides: these go a long way to explaining how the glomerulosa becomes differentiated from the capsule. Remaining to be determined is how the fasciculata becomes distinct from the glomerulosa, or the reticularis from the fasciculata. What morphogens are involved here?

As well as the speculation that they could conceivably be products of neural or vascular components of the gland (**Figure 4**), one school of thought holds that it could be the steroid products themselves. From *in vitro* studies, and on the basis that there may be a gradient of steroids from the outer to the inner part of the cortex, Hornsby and colleagues postulated that this results in high concentrations of steroids in the inner zones, which, by acting as pseudosubstrates, could directly compromise cytochrome P450 structure and function, in this way perhaps inhibiting hydroxylase activity in the reticularis, for example (Hornsby, 1987). Though this is contrary to the usual concept of morphogen diffusion which posits a decrease in concentration with distance from the source, it acquires new life, and a new mechanism, with the characterization of GR in the human adrenal cortex, particularly in the reticularis (Paust et al., 2006).

That the individual zones have defined steroidogenic roles is clear. Whether they are completely autonomous is less so. Rather, because of the paucity of other steroidogenic enzymes in the CYP11B2 expressing cells of the glomerulosa, it is arguable that

the substrate for CYP11B2 must come from the fasciculata, and the different ways this could occur are via cell-cell contact, or by uptake from the general circulation, as presumably is the case for extra-adrenal CYP11B2 thought by some to be expressed in the vasculature and other tissues (Hatakeyama et al., 1996; Taves et al., 2011; though not by others, MacKenzie et al., 2012) or by diffusion. Similarly, the production of androstenedione by the human gland would seem, on the basis of the zonal distribution of enzymes possibly to be a reflection of the availability of DHEA from the reticularis to 3 $\beta$ -HSD in the fasciculata.

If that is the case, then the regulation of these secretory products must also reflect co-operation between zones. Given the distribution of mRNA coding for MC2R in the rat adrenal, in the inner ZU, and fasciculata/reticularis but not in the CYP11B2 region of the glomerulosa (Gorrigan et al., 2011), the well-recognized stimulation of aldosterone secretion by ACTH is otherwise difficult to explain. Of course it is still possible that it is the MC5R in the glomerulosa to which ACTH binds. The actions of angiotensin II and  $\alpha$ -MSH on aldosterone secretion in the rat are equally difficult to understand, because the appropriate receptors, AT1R and MC5R respectively, do not appear to coincide with CYP11A1, thus cholesterol side chain cleavage, the recognized site in the pathway for stimulation of steroid hormone secretion, seemingly cannot be directly affected by these agents (**Figure 3**).

Taken together, all this evidence seems to suggest it may be mistaken to think of the adrenal cortex, or even the whole adrenal gland, as simply a collection of different types of cells, each independently performing its role in isolation. Rather we should think of these different cell types as acting in concert. We have understood for some time now that there are intraadrenal mechanisms that modify and refine the signals from the systemic regulators. These comprise medullary products, cytokines, the adrenal renal-angiotensin system, among others, and have been reviewed elsewhere (Ehrhart-Bornstein et al., 1998). Extending this has been conceptually problematic in view of the apparently confounding effect of the blood flow through the gland, which would seemingly limit the action any cell might have on other than its closest neighbors by secreted products.

We now know that secreted products from adrenocortical cells can indeed pervade the gland through diffusion, and this has been demonstrated by the wnt-catenin and shh-Gli signaling systems. So if it is possible for these products then there is no reason why it cannot be the case for others, including perhaps catecholamines, cytokines, and the steroids themselves. This may give a partial explanation for the architecture of the gland, one of the problems posed at the start of this article—why are the zones arranged as concentric spheres? One answer may be that this has the potential to maximize the likelihood of diffused products arriving at their targets. If we take as an example the secretion of shh from its origin in the ZU, random walk diffusion to its target in the inner capsule is better achieved when the capsule completely envelops the glomerulosa than it would be if the system were more open, or composed of sheets, like a mesentery or secretory epithelium.

In addition, there is absolutely no reason to suppose the steroids themselves do not move between the zones, as substrates

or modifiers. How else can we explain how aldosterone or androstenedione are synthesized? And, given that MC2R and CYP11A1 are expressed in the ZU and fasciculata, but not the CYP11B2 expressing cells of the rat glomerulosa, how else can we explain how ACTH has its undoubted action on aldosterone secretion, other than by the enhanced provision of steroid substrates from the ZU or the fasciculata to the CYP11B2 cells in the glomerulosa?

Like the human body itself, the function of the adrenal gland is an integrated whole, much greater than the sum of its parts. We should aim to think of it that way.

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## AUTHOR CONTRIBUTIONS

GV researched and wrote this article.

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# Molecular Mechanisms of Stem/Progenitor Cell Maintenance in the Adrenal Cortex

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The adrenal cortex is characterized by three histologically and functionally distinct zones: the outermost zona glomerulosa (zG), the intermediate zona fasciculata, and the innermost zona reticularis. Important aspects of the physiology and maintenance of the adrenocortical stem/progenitor cells have emerged in the last few years. Studies have shown that the adrenocortical cells descend from a pool of progenitors that are localized in the subcapsular region of the zG. These cells continually undergo a process of centripetal displacement and differentiation, which is orchestrated by several paracrine and endocrine cues, including the pituitary-derived adrenocorticotropic hormone, and angiotensin II. However, while several roles of the endocrine axes on adrenocortical function are well established, the mechanisms coordinating the maintenance of an undifferentiated progenitor cell pool with self-renewal capacity are poorly understood. Local factors, such as the composition of the extracellular matrix (ECM) with embedded signaling molecules, and the activity of major paracrine effectors, including ligands of the sonic hedgehog and Wnt signaling pathways, are thought to play a major role. Particularly, the composition of the ECM, which exhibits substantial differences within each of the three histologically distinct concentric zones, has been shown to influence the differentiation status of adrenocortical cells. New data from other organ systems and different experimental paradigms strongly support the conclusion that the interactions of ECM components with cell-surface receptors and secreted factors are key determinants of cell fate. In this review, we summarize established and emerging data on the paracrine and autocrine regulatory loops that regulate the biology of the progenitor cell niche and propose a role for bioengineered ECM models in further elucidating this biology in the adrenal.

**Keywords:** adrenal cortex, paracrine signaling, stem cells, organogenesis, extracellular matrix, tissue homeostasis

## INTRODUCTION

In vertebrates, adrenal steroid hormones are effectors of different adaptive responses to oscillations in the organism's internal and external environment, broadly referred as *stress*. Adrenal steroid hormones serve to modulate a wide range of processes that are central to physiologic response to

stress, including energy metabolism, immune response, electrolyte homeostasis, and fluid balance. The three main classes of adrenal steroid hormones, mineralocorticoids, glucocorticoids, and androgens, are produced by the adrenal cortex under the tight regulation of distinct and independent endocrine regulatory loops: the renin–angiotensin–aldosterone system (RAAS) and the hypothalamus–pituitary–adrenal (HPA) axis. The ability of the adrenal gland to respond independently to these endocrine signals is dependent upon subpopulations of steroidogenic cells with distinct morphological and functional characteristics that are localized in specific concentric compartments (zones) of the cortex. In humans, three histologically distinct zones are evident: the outermost *zona glomerulosa* (zG), the intermediate *zona fasciculata* (zF), and the innermost *zona reticularis* (zR), which are responsible for the production of mineralocorticoids, glucocorticoids, and androgens, respectively (1). Although the morphological and physiological aspects of the adrenal cortex have been relatively well characterized, the regulatory mechanisms responsible for the establishment and maintenance of the three zones are not fully understood.

In the last few years, sophisticated molecular techniques, such as lineage tracing, and genetically modified animals have significantly contributed to our understanding of the embryonic development and homeostasis of the adrenal cortex, illuminating key molecules and signaling pathways that are implicated in these processes (discussed below in Sections “Progenitor Populations in the Adrenal Gland” and “Signaling Pathways and Adrenal Progenitors”). Accordingly, the Wnt and the hedgehog pathways have emerged as major paracrine factors that regulate both organogenesis and homeostasis of the gland. Both are essential for the establishment and maintenance of an undifferentiated population of steroidogenic precursor cells in the periphery of the organ that continuously replenish the cortical cells of the three zones throughout life (discussed below in Sections “Progenitor Populations in the Adrenal Gland” and “Signaling Pathways and Adrenal Progenitors”). While the effectors of the RAAS and the HPA axis [angiotensin 2 and adrenocorticotropic hormone (ACTH), respectively] are considered primary endocrine mediators that promote activity of adrenocortical steroidogenic cells (2–4), recent data support that the *microenvironment*, comprised of the extracellular matrix (ECM), its associated proteins, and several paracrine and autocrine factors, are also major determinants of cell behavior and fate (discussed below in Sections “Signaling Pathways and Adrenal Progenitors” and “The Adrenocortical Progenitor Niche”). Emerging data support an important role for non-steroidogenic/mesenchymal-like cells that lie in the adrenal capsule in the maintenance of the adrenocortical progenitor pool (5–8). This review focuses on the role of microenvironmental factors and signaling pathways that contribute to the maintenance of the adrenal cortex. We will discuss emerging data on the role of specific paracrine and autocrine loops between capsular/stromal cells and cortical cells in establishing a specialized microenvironment that supports and maintains the adrenal progenitors throughout the life—the *niche*—and how this signaling contributes to the anatomical and functional zonation of the adrenal cortex.

## DEVELOPMENTAL ORIGINS OF THE ADRENAL CORTEX

The adrenal cortex originates from cells of the celomic epithelium, which in the mammalian embryo is composed of a single squamous cell layer that covers the external surface of the viscera and the inner surface of the body wall. Around embryonic day 9.5 (E9.5) in mice, and during the fourth to sixth weeks of human gestation, a thickening of the celomic epithelium between the urogenital ridge and the dorsal mesentery forms the adrenogonadal *primordium* (AGP). At the eighth week of human gestation (E10.5 in mice), the AGP divides into dorsomedial and ventrolateral portions, giving rise to the adrenal and gonadal *primordia*, respectively (1, 9–11).

Between the weeks 8 and 9 of human gestation (E13 in mice), the *adrenal primordium* is invaded by cells of the neural crest that coalesce centrally to form the adrenal medulla (12). Subsequently, the *adrenal primordium* is surrounded by mesenchymal cells, which will ultimately form the adrenal capsule (13). At this point, compartmentalization of the adrenal cortex into two structurally distinct areas is evident: a central area, comprised of large polyhedral eosinophilic cells referred as the “fetal zone,” and a peripheral zone adjacent to the newly formed capsule comprised of small and basophilic cells, referred to as “the definitive zone.” While in mice this compartmentalization is subtle, in humans, the fetal zone predominates over the definitive zone, constituting up to 80% of the adrenal mass by the end of the gestation (9, 14). In addition, ultrastructural studies in humans have demonstrated the presence of a third zone, referred as the “transitional” or “intermediate zone,” which has intermediate morphologic characteristics between the fetal and the definitive zones (9). It has been suggested that after mid-gestation the transitional zone has the capacity to synthesize cortisol (9, 15). By the week 30 of human gestation, the definitive and the transitional zones have morphological features that resemble the adult zG and zF, respectively (16). In humans, the fetal cortex starts to regress by apoptosis soon after birth, completely disappearing after a few weeks (9). In mice, definitive evidence about the presence of a transient fetal zone was provided by the identification of the fetal adrenal-specific enhancer (FAdE), which is only active during early fetal development (see below). The differentiation process of the human adrenal cortex continues until the onset of puberty, when the definitive cortex completes its organization into the three distinct histologic zones that characterize the adult cortex of human and higher primates (14). While the zG and the zF are evident at birth, the androgen-producing zR only starts to form a few years later, marking the onset of the adrenarche, which is the earliest stage of sexual maturation and a precursor of puberty (17).

A transcription factor critical for adrenocortical development and homeostasis is steroidogenic factor 1 (SF1, also known as adrenal four-binding protein or nuclear hormone receptor Ad4BP, encoded by the gene *NR5A1*). All cells that belong to steroidogenic lineages of the adrenal and gonads express SF1, including subpopulations of long-term retained progenitor cells in each organ (8, 18). Therefore, SF1 expression defines the identity of these cells and commitment to steroidogenic

differentiation (14, 19, 20). The expression of SF1 is detectable early in fetal life, between the AGP formation and the ultimate establishment of the adrenal *primordium* (14, 18). Genetic loss of *Nr5a1* or its upstream transcriptional regulators *Pbx1*, *Wt1*, and *Cited2*, interferes with AGP formation leading to various degrees of adrenal hypoplasia in mice (12, 14, 21–23). While *Nr5a1* is continuously expressed from the time of *adrenal primordium* formation throughout the adult life, during embryonic stages and early fetal life in mice, the *Nr5a1* expression is driven by the fetal adrenal-specific enhancer (FAdE), which becomes inactive when the definitive cortex forms, suggesting that distinct mechanisms sustain *Nr5a1* expression in the fetal and in the definitive cortex (14).

While the enhancer that is responsible for initiation and maintenance of *Nr5a1* expression in the definitive zone is currently not known, lineage-tracing experiments performed by Zubair et al. have shown that FAdE-driven-*Ad4bp*-expressing cells are indeed precursors of most, if not all, SF1-expressing cells of the definitive cortex (14). It has since been demonstrated that after extinguishing FAdE-dependent SF1 expression, some cells become embedded within the coalescing capsule and later reemerge as FAdE-independent SF1-expressing cells during the formation of the definitive cortex (24).

Steroidogenic factor 1 not only defines the identity/specification of the cells of the definitive cortex but also serves as a key regulator of hormone-dependent steroidogenesis, the hallmark of a differentiated adrenocortical cell. In this regard, the effects of ACTH on differentiation and steroidogenesis are partially mediated by an SF1-induced transcription of steroidogenic enzymes that defines a cortisol-producing cell (25). It remains unclear, however, how a subpopulation of SF1-expressing cells are spared from the pro-differentiation effects of ACTH, preserving their undifferentiated state. One clue to such regulation comes from studies of DAX1, an atypical nuclear receptor that is encoded by the gene *NR0B1* and expressed preferentially in the peripheral/subcapsular cortex. DAX1 functions as a repressor of SF1-mediated transcription and has been shown to have an important role in the maintenance of the undifferentiated state (lack of steroidogenesis) of the adrenocortical progenitor population (26). Studies examining the consequences of genetic loss of DAX1 in the adrenal revealed an unexpectedly hyper-functional zF in younger mice, characterized by an increase in cell proliferation and steroid production. Surprisingly, as the animals aged, they developed adrenal hypofunction consistent with an abnormal premature differentiation of the progenitor cell pool that ultimately resulted in its exhaustion (27, 28). In the next section, we will briefly summarize studies that have begun to explore the molecular and cellular processes that underlie the homeostasis of the progenitor cell population.

## PROGENITOR POPULATIONS IN THE ADRENAL GLAND

One of the first reports regarding adrenal regeneration in rats describes the restoration of the adrenal cortex 6 weeks after unilateral adrenal enucleation, a process that involves the removal of

the inner content of the adrenal gland (including the medulla), while leaving the capsule and underlying subcapsular cells intact. This observation suggested the presence of an adrenocortical stem and/or progenitor cell population located in the periphery of the adrenal gland (29). The notion of a renewing progenitor population in the adrenal cortex was corroborated by the observation that transplants of bovine adrenocortical cells underneath the kidney capsules of adrenalectomized immunocompromised mice can give rise to adrenocortical tissue with steroidogenic properties (30, 31).

The presence of a progenitor cell population in the periphery in the adrenal gland that gives rise to all the differentiated cell types within the different cortical zones is consistent with a homeostatic model of centripetal migration and differentiation. This idea was first hypothesized in the 19th century, based on histological observations of gradual changes in morphology observed in cells between the capsule and the medulla (32, 33) [reviewed in Ref (34)]. Over the years, different studies have shown that, under physiological conditions, proliferating cells are located preferentially in the outermost layers of the cortex, in the subcapsular region (35, 36). Also, it has been demonstrated that peripheral cortical cells are centripetally displaced until they reach the cortical–medullary boundary and become apoptotic (35, 37). Moreover, studies performed in chimeric and transgenic mosaic rats and mice have shown a radial variegated pattern of the reporters extending from the outer cortex to the cortico-medullary boundary. This observation is consistent with a peripheral clonal origin (38–40). Definitive genetic evidence for a centripetal conversion of adrenocortical cells between concentric zones was finally provided by cell-lineage tracing (8, 41). In one of these studies, transgenic mice were created with a Cre recombinase gene inserted at the *Cyp11b2* locus, in which Cre was expressed only in terminally differentiated zG cells (41). When these mice were crossed with mice expressing the *R26R<sup>mt/mG</sup>* reporter, GFP-positive cells that occupied the zG upon *Cyp11b2-Cre* expression, eventually populated the entire cortex. Over time, an increasing number of centripetally located *Cyp11b1*-expressing cells were found to express GFP, indicating that peripheral zG cells underwent lineage conversion to more centripetal zF cells. In 12-week old mice, nearly the entire cortex was comprised of GFP-positive cells. Although these existing data strongly support the existence of a progenitor population localized in the periphery of the gland that gives rise to all the steroidogenic cell types of the cortex through a process of centripetal displacement/migration and differentiation, the molecular mechanisms that govern this process are not completely understood.

Further studies have provided clues on the molecular fingerprint of the progenitor cell populations and the signaling pathways that regulate their renewal and differentiation. Specific paracrine/autocrine signaling pathways are activated in zonally restricted patterns that reflect the different cell subpopulations that are hypothesized to play an active role in organ homeostasis. The most important and well characterized are the Wnt and the hedgehog pathways. Additionally, a cross-talk between cortical cells and capsular stromal cells regulates the activity of these pathways. Below, we briefly summarize these findings.

## SIGNALING PATHWAYS AND ADRENAL PROGENITORS

### Wnt Signaling Pathway

The mammalian wingless-type MMTV integration site (Wnt) signaling pathway is one of the most studied pathways in developmental biology, playing important roles in organogenesis, homeostasis, and stem cell biology (42). Wnt ligands represent a large family of highly conserved morphogens that are characterized by repetitive cysteine residues. Secreted Wnt ligands bind to receptors of the Frizzled and lipoprotein receptor-related protein families on the cell surface. Through several cytoplasmic components, the signal is either transduced through  $\beta$ -catenin, which enters the cell nucleus and complexes with T-cell factor/lymphocyte enhancer factor (TCF/LEF) family of transcription factors to activate transcription of Wnt target genes (canonical pathway), and/or activates the non-canonical Wnt pathway to regulate planar cell polarity and calcium signaling (42). In the mouse adrenal gland,  $\beta$ -catenin expression is first observed at E12.5 in Sf1+ cells of the definitive cortex in a scattered fashion beneath the newly formed capsule (43). After E18.5 and throughout life,  $\beta$ -catenin protein expression becomes more enriched in cells of the zG, which have also been demonstrated to bear active canonical Wnt signaling (43, 44).

Canonical Wnt signaling plays several roles in the adrenal cortex. Recent evidence demonstrates that the population of Wnt-responsive cells is heterogeneous and contains small clusters of Shh-producing cells (considered to be adrenal progenitors, as it will be discussed later) and separate differentiated Cyp11b2-expressing cell clusters (44). Interestingly, despite the well-known role of Wnt signaling pathway in promoting cell proliferation in a variety of organ systems, the Wnt-responsive cells do not appear to be heavily proliferating, implying that any role of Wnt signaling in adrenal homeostatic growth might involve a potential cell non-autonomous mechanism (44).

The Wnt pathway is essential for the establishment and maintenance of the subcapsular progenitor cell population. However, it does not appear to play a role in the early formation of the AGP since expression of *Ctnnb1* is first detectable in mesothelial cells overlying the indifferent gonad at around E11.5 and in the adrenal primordium at around E12.5, following their separation from the AGP (43, 45). Studies performed in a *Nr5a1/Cre*-mediated *Ctnnb1* conditional knockout mouse model (*Ctnnb1tm2kem* mice crossed into *Sf1/Cre-high* mice), in which canonical Wnt signaling to adrenal primordium SF1-expressing cells from around E12.5 is reduced or absent reveal a marked decrease in cortical cell proliferation which ultimately results in the complete regression of the adrenal gland by E18.5, despite normal formation of the adrenal primordium at earlier time points (43, 46). Loss of  $\beta$ -catenin in approximately 50% of Sf1-expressing cells and hence a resultant ~50% decrease in  $\beta$ -catenin-mediated Wnt signaling (induced by a low efficiency *Sf1/Cre-low* driver that allows for a subset of cells to escape recombination) results in a histologically normal adrenal through 15 weeks of age, after which the adrenal cortex undergoes progressive thinning over time and ultimate adrenal failure (43, 46). Consistent with a primordial role of the Wnt signaling in maintaining the progenitor

cell population, it has been demonstrated that *Nr0b1* (*Dax1*) is a transcriptional target of  $\beta$ -catenin, suggesting that both proteins synergistically act to maintain the undifferentiated state that characterizes the progenitor cell population (28). Together, these evidences suggest that canonical Wnt signaling has a role in establishing and maintaining a pool of adrenocortical progenitors throughout life.

### Hedgehog Signaling Pathway

The hedgehog signaling pathway (Hh) is a conserved evolutionary pathway that is essential for embryonic development and adult tissue maintenance, renewal, and regeneration. Three secreted hedgehog proteins have been described: sonic hedgehog (SHH), desert hedgehog, and Indian hedgehog. These proteins function in a concentration- and time-dependent fashion, controlling several processes ranging from survival and proliferation to cell fate specification and differentiation (47).

Shh is the only Hh family member that is present in the murine adrenal gland, being detected as early as E12.5 and later expressed in a restricted manner in a subpopulation of cells in the outer zG (8). These cells are Sf1-positive and do not express Cyp11b2 or Cyp11b1, the enzymes necessary for terminal reactions that lead to aldosterone and corticosterone production, respectively. While in the mouse adrenal the Shh+ cells are localized in clusters under the capsule, in the rat these cells form a continuous layer localized between the zG and the zF, known as the *undifferentiated zone* (zU) (48).

Lineage-tracing studies that mark all Shh-expressing cells and their descendants reveal that, by postnatal day 12 (P12), virtually all cells of the cortex are derived from *Shh*-expressing cells, consistent with a role for Shh in the peripheral stem/progenitor cells (8). Studies utilizing an inducible *Shh-Cre* recombinase have demonstrated that shortly after recombination in adult mice, Shh-expressing cells, and their immediate descendants are restricted to the periphery of the cortex. Over time, these cells and their descendants form centripetally expanding radial stripes, supporting the hypothesis that the Shh-expressing cells are a progenitor population that gives rise to all other cortical cell populations (8). BrdU-labeling experiments carried out on rat adrenals identified two sites of proliferation in the outer zF and inner zU and between the zG and zU, suggesting that cell proliferation at the periphery of the zU provides cells to both zG and zF (36). However, the proliferative capacity of these cells is more consistent with their identification as progenitors rather than as stem cells as suggested by the authors. Another study on mouse adrenocortical cell proliferation used BrdU pulse-chase labeling to identify a small population of quiescent non-steroidogenic cells located in the outer cortex that may represent a quiescent progenitor population, which is prompted to divide following ACTH stimulation (35). Additionally, this study suggested that two distinct subpopulations of rapidly cycling cells emerged from the progenitor population—the first constituted by cells that proliferate in response to ACTH and migrate inwards and the second constituted by cells that migrate outwards and are less responsive to ACTH. The authors have suggested that these distinct subgroups of cells are committed transient-amplifying cells that are responsible for the maintenance of the zF and zG, respectively (35). Interestingly,

another study has shown that Cyp11b2-expressing cells in the zG derived from the Shh-expressing cells undergo lineage interconversion, differentiating into Cyp11b1-expressing cells in the zF as they migrate inwards, suggesting that cells from both zones are in fact derived from the same lineage (41).

In addition to serving as a progenitor cell population in the cortex, Shh-positive cells presumably signal to Gli1-expressing cells that lie within the capsule (48, 49). Interestingly, the capsular Gli1-expressing cells are a mesenchymal-like population that do not express Sf1 (Gli1+/Sf1-) but descends from the fetal FAdE-utilizing Sf1+ cells of the adrenal *primordium* (24). The fate of the descendants of these *Gli1*-expressing cells has been investigated in a transgenic mouse model with an inducible *Gli1-CreERT2* allele. It has been demonstrated that during adrenal development, *Gli1*+ capsular cells behave as stem or precursor cells since they give rise to Sf1+ cells of the early definitive cortex that later become the Sf1+/Shh+ progenitor cell pool (8).

## Other Signaling Pathways

In addition to the Gli+/Sf1- cell population, other mesenchymal-like cell lineages have also been identified in the capsule and the stroma. These cells may also have important roles in the adrenal gland maintenance. Bandiera and colleagues observed a long-retained pool of cells expressing the Wilms tumor protein homolog (WT1) in the adrenal capsule that originates from capsular mesenchymal cells and is characterized by AGP features (50). WT1 is a transcriptional regulator that has important roles in organogenesis of different organs, including the adrenals and gonads. Using a *Wt1-Cre* recombinase line crossed with a reporter line, Bandiera et al. demonstrated that WT1-expressing cells could give rise to steroidogenic cortical cells in the adult adrenal and the Gli1+ population as well, lending support for a role for WT1 in the activation of *Gli1* transcription.

Wood et al. identified a population of cells in the mouse adrenal that starts to express the transcription factor 21 (*Tcf21*, also known as *Pod1*/capsulin/epicardin) at E9.5 onward. After E14.5, *Tcf21* expression becomes predominately restricted to the capsule and further diminishes over time, leaving only a small population of capsular *Tcf21*+ cells through adulthood. Lineage-tracing experiments revealed that before adrenal encapsulation, *Tcf21*-expressing cells and/or their descendants give rise to both non-steroidogenic capsular cells and steroidogenic cortical cells. On the other hand, the population of *Tcf21*-expressing capsular cells only gives rise to a population of Sf1- stromal cells that express collagen, desmin, and *Pdgfra* and persist throughout adult life within the cortex (24).

Taken together, these studies provide evidence that the capsule is a complex niche comprised of multiple long-term retained cell lineages. The roles of each of these populations in the adult adrenal remain unknown, but recent evidence suggests that signaling coming from the capsule is required for the long-term maintenance of the adrenocortical progenitor cells.

## THE ADRENOCORTICAL PROGENITOR NICHE

The proper balance of progenitor proliferation and differentiation is crucial, as dysregulation of the mechanisms that regulate the

differentiation, proliferation, and self-maintenance of progenitor cells throughout life can result in organ failure. Stem and progenitor cells from different organs and tissues are embedded within a specialized microenvironment, termed the *niche*. Niches are protective locations that support stem cell residence and maintenance. Several components of the niche have been identified, including bioactive compounds, such as Wnt ligands, growth factors [e.g., fibroblast growth factors (FGFs) and epidermal growth factor], chemokines, and a distinct ECM composition that sequesters growth factor signaling and spatially restricts cell movement for appropriate cell-cell interactions. While well-defined niches characterized in different organs vary in size and complexity, most are maintained by a small set of fundamental regulatory signaling loops (51). Although several studies have revealed the importance of these signaling pathways in the adrenal biology as coordinators of stem/progenitor cell specification and differentiation, the formal structural and molecular definition of an adrenocortical progenitor niche remains elusive (52). ECM components are predicted to provide additional structural context to zone-specific signaling pathways discussed in the previous section. Below, we discuss these regulatory mechanisms in further details.

## Components of the Adrenocortical Niche Soluble Factors

### *Wnt Ligands*

As previously discussed, the canonical Wnt/β catenin signaling pathway is considered to be a crucial pathway for the maintenance of the subcapsular stem/progenitor pool (44, 46, 53). While the source and the contribution of different Wnt ligands to Wnt signaling are not currently known, previous observations based on adrenal tumors expression profiles, human clinical syndromes, and mouse models suggest that *WNT4* is an important Wnt regulatory paracrine factor in the adrenal cortex. In humans, *WNT4* mutations underlie the defects in Serkla syndrome, an autosomal recessive disorder characterized by multiple malformations including adrenal hypoplasia and male-to-female sexual reversion (54). In mice, while *Wnt4* expression is observed in the developing adrenal glands as early as E11.5, by E14.5 it is restricted to the outer cortex (46, 55). Additionally, a mouse model of *Wnt4* inactivation suggests that this ligand is required for proper zG differentiation and aldosterone production. In these mice, genetic loss of *Wnt4* decreases *Cyp11b2* expression, leading to a diminished production of aldosterone (55). Interestingly, the anterior tips of the developing gonads of these mice exhibit a steroidogenic enzyme expression pattern similar to adrenocortical cells, suggesting an abnormal differentiation process or a defect in adrenal cell specification during the separation of the AGP (55, 56).

*WNT4* is a known transcriptional target of Wnt/β catenin signaling (55). In the adrenal cortex, *WNT4* expression is restricted to the zG, precisely where canonical Wnt pathway is known to be active. Additionally, according to our own analysis on a recently published molecular profiling study on adrenocortical carcinomas (57), samples with activating exon 3 *CTNNB1* mutations exhibit increased expression of *WNT4* in comparison to samples without somatic alterations in components of the Wnt pathway (fold change = 17.5, FDR-adjusted *p*-value <0.001). More recently, it has been demonstrated that the expression levels

of canonical Wnt target genes are significantly reduced in the adrenals of a mouse model of *Sf1-Cre* mediated *Wnt4* knockout (*Sf1:Cre; Wnt4Fl/Fl*) (58). Interestingly, it has also been shown that the activation of the protein kinase A pathway enables a zG-to-zF lineage conversion by antagonizing canonical Wnt signaling, which is partially mediated by repression of *Wnt4* expression (58). Together, these data support a model whereby WNT4 serves as an autocrine activator of Wnt signaling, amplifying canonical ( $\beta$  catenin-dependent) Wnt activation within the zG, which is essential for proper zonation. However, whether WNT4 downstream signaling also induces a non-canonical ( $\beta$  catenin-independent) effect is currently not known. In other systems, WNT4 is described preferentially as a non-canonical ligand (59, 60). Last, the roles and the sources of other Wnt ligands that are expressed in the adrenal are yet to be determined.

#### **Other Wnt Pathway-Related Soluble Factors**

Bone Morphogenetic Protein-4 (BMP4) is a ligand of the TGF- $\beta$  superfamily that plays essential roles in embryonic development, stem cell biology, and tissue regeneration (61). Recently, Rege et al. have demonstrated that this protein, its receptors, and downstream molecules are expressed and have functional roles in the human adrenal cortex and in the H295R adrenocortical carcinoma cell line. Interestingly, mRNA expression of *BMP4* follows a zonal distribution gradient, with the highest levels in the zG, suggesting that *BMP4* is a Wnt target gene. *In vitro*, BMP4 actively inhibits the expression of 17,20-lyase and DHEA secretion, possibly serving to prevent a zR fate (62). Consistent with the observed zonal distribution in the adrenal cortex, *BMP4* has been reported as a Wnt target gene in a colorectal cancer cell line (63). Moreover, according to our own analysis on two independent datasets (57, 64), *BMP4* mRNA is upregulated in ACC samples with nuclear immunostaining for  $\beta$ -catenin or with an activating mutation of *CTNNB1* in comparison to those with membranous immunostaining or wild-type for *CTNNB1* somatic mutations (fold change  $>3.21\text{--}6.76$ , FDR-adjusted *p*-value  $<0.001$ ). These observations suggest that *BMP4* is a paracrine factor regulated by the Wnt pathway that may have a role in functional zonation by inhibiting a zR fate.

Coiled-coil domain containing 80 (*CCDC80*) has recently been described as a novel Wnt target gene in the adrenal cortex. *In situ* hybridization has shown that it is expressed in cells within the zG. Functional studies have shown that *CCDC80* decreases steroidogenesis *in vitro*, suggesting, as for *BMP4*, a role in cell differentiation or fate. The molecular pathway by which *CCDC80* regulates steroidogenesis is yet to be determined in the adrenal gland (44). In the chick embryo, the *CCDC80* homolog equarin regulates FGF signaling by increasing its local availability and by facilitating the interactions of FGF ligands with FGF receptors and proteoglycans (65). Further studies in *CCDC80* null mice aiming to characterize the overall mechanism of action of *CCDC80* in the adrenal gland are ongoing.

#### **Sonic Hedgehog**

Sonic hedgehog-expressing cells serve as a *bona fide* progenitor population that contributes to the adrenal cortex homeostasis in adult life, as previously described. Mice with global genetic

deletion of *Shh* exhibit severe adrenal hypoplasia during embryonic development due to a reduction in both cortical and capsular cell proliferation (8, 66, 67). However, these animals also have severe malformations in other organs, including pituitary defects that impair ACTH production, making the adrenal phenotype difficult to interpret. Moreover, targeted deletion of *Shh* in *Sf1*-expressing cells in mice also causes adrenal abnormalities, indicating that *Shh* is indeed essential for intrinsic (ACTH-independent) adrenal development. While AGP separation, neural crest migration, and encapsulation occur normally, these animals have a hypoplastic adrenal cortex and diminished mitotic activity by E13.5. As age increases, progressive cortical thinning with an apparent defect in steroidogenesis is observed as reflected by a compensatory increase in ACTH levels. Also, an overall thinner capsule is also seen (66). The mechanisms by which *Shh* deficiency causes this capsular phenotype are not known, but is predicted to result from a defective cortical *Shh* signaling to the capsular Gli1-expressing cells.

*Shh* is a secreted ligand that binds to the cell-surface receptor patched homolog 1 (Ptch1). *Shh* binding relieves Ptch1-mediated inhibition of Smoothened homolog (Smo). Smo stimulates intracellular downstream *Shh* signaling that, when activated, inhibits the otherwise proteolytic degradation of Gli transcription factors, resulting in Gli-mediated transcriptional activation of Hh target genes (8, 47, 66, 67). Whether a secreted factor produced by *Shh*-responsive Gli+ capsular cells is required for maintaining the subcapsular progenitor population is currently unknown. Together, these observations indicate that the *Shh*-expressing subcapsular progenitor cells and the capsular Gli-expressing cells are integral components of the adrenocortical progenitor cell niche.

#### **Fibroblast Growth Factors**

The FGFs are a family of secreted signaling proteins that bind to a subclass of membrane-bound tyrosine kinase-coupled receptors (FGFRs 1–4) and intracellular non-signaling proteins (iFGFs) that work as cofactors for other membrane-associated proteins. With few exceptions, FGFs are ubiquitous paracrine/autocrine factors with essential roles in embryogenesis, organogenesis, and tissue homeostasis. FGFs regulate fundamental cellular processes, including proliferation (both promoting and inhibiting), survival, differentiation, migration, and metabolism. FGFs routinely bind to heparin sulfate proteoglycans, which limit their diffusion through the ECM and serve as cofactors that regulate FGF specificity and affinity to different types of FGFRs (68, 69).

Early studies have demonstrated that FGFs exhibit a potent mitogenic effect both in the murine-derived Y1 adrenocortical cell line and primary cultures of bovine and human adrenocortical cells. Interestingly, this effect can be antagonized by ACTH, which promotes cell differentiation by inducing cell cycle arrest and steroidogenesis *in vitro* (70–74). *In vivo*, different combinations of FGF ligands and receptors are expressed in the adrenal capsule and cortex, consistent with a proposed role in homeostasis. RT-PCR analysis from laser capture microdissected adrenals from E15.5 mouse embryos have shown that *Fgf1*, *Fgf2*, and *Fgf9* are the only FGF ligands that are expressed in the embryonic adrenal gland. While *Fgf1* is expressed in the cortex, *Fgf2* and *Fgf9* are expressed preferentially in the capsule. Additionally,

*Fgfr1-IIIc*, *Fgfr2-IIIb*, and *Fgfr2-IIIc* are expressed in cells of both the capsule and the cortex. Interestingly, the cortical cells that express *Fgfr2* are also Shh-positive (48, 75, 76).

The importance of FGF signaling in adrenal maintenance has been revealed through several *in vivo* studies. FGF2 encapsulated in poly-lactic-co-glycolic acid enhances the growth of adrenocortical cells implanted under the kidney capsule of mice by fivefold to eightfold (74). Additionally, engineered deletion of the FGF ligand, FGF2 or the FGF receptor *Fgfr2*, results in various degrees of adrenal hypoplasia after birth. The global *Fgfr2-IIIb* knockout results in embryonic lethality due to severe malformations, including adrenal hypoplasia (7). The same adrenal phenotype was recapitulated by a *Sfl*-driven specific knockout of both *Fgfr2-IIIb* and *Fgfr2-IIIc* (77). Evaluation of the embryonic adrenal gland of *Fgfr2-IIIb* global knockout mice at E15.5 revealed a marked capsular defect, characterized by thickening and disorganization of the mesenchymal capsule, with increased mitotic activity and increased number of Gli1-positive cells. Meanwhile, the underlying cortex was hypoplastic with a decrease in both steroidogenic differentiation and mitotic activity. An unexpected, decrease in capsular delta-like protein 1 (Dlk1) suggests that, together with Shh and FGFs, Dlk1 is a mediator of the proposed homeostatic cross-talk between cortical and capsular cells (75).

### Delta-Like Protein 1 (DLK1)

Delta-like protein 1 (also known as preadipocyte factor 1—*Pref1*) is a paternally expressed imprinted gene that encodes an epidermal growth factor repeat-containing transmembrane protein. Dlk1 is cleaved by the TNF-alpha-converting enzyme (TACE or ADAM17) to generate a soluble, secreted, and biologically active protein (78, 79). In adipose tissue, DLK1 negatively regulates proliferation and terminal differentiation of adipocyte progenitors (80–83). Mechanistically, DLK1 interacts with fibronectin and facilitates downstream integrin signaling, which includes MEK/ERK activation (80). While the human adrenal cortex expresses high levels of the *DLK1* transcript (the highest levels among all human tissues according to the GTEx portal database), little is known about its downstream signaling and overall function in adrenocortical cells (84). However, *in vivo* studies in rat reveal that *Dlk1* expression is markedly decreased in the regeneration process following enucleation, suggesting a role in adrenal remodeling and zonation. The Dlk1 expression is restricted to a subcapsular population in the rat zU (6, 85, 86). Indeed, the Dlk1+ cells do not express Cyp11b1 and are rarely positive for Cyp11b2. They do, however, express Shh, indicating that they are indeed the progenitor population (48, 87). A low-sodium diet induces a marked downregulation of both Dlk1 and Shh, with a proportional increase in the expression of Cyp11b2. Blocking the RAAS with captopril induces the opposite effect—expansion of Dlk1 and Shh-expressing cells. Additionally, a population of capsular cells responds directly to Dlk1 signaling by increasing the levels of both p-ERK and Gli1, consistent with a role in regulating hedgehog signaling (6). These results predict that in the rat adrenal, Dlk1 is expressed by the cortical progenitor cell population and acts in concert with Shh to activate capsular Gli1, modulating differentiation, remodeling, and zonation (6). However, it should be noted that these conclusions are based on studies in rat, which

express Dlk1 in the subcapsular cells whereas, in mice, Dlk1/*Pref1* is expressed in the capsule (6, 75).

### R-Spondin Family Member 3 (RSPO3)

R-spondins are secreted proteins that have been recently described as important positive regulators of the canonical Wnt pathway (88). R-Spondins are paracrine factors that exhibit a very low diffusion gradient since these proteins firmly binds to certain ECM components such as syndecan-4, remaining close to its secretory source (89). In organs, such as the liver and the gastrointestinal tract, RSPO3 gradients are a critical determinant of regions of active canonical Wnt signaling, being a crucial regulator of the stem/progenitor cell compartment, cell differentiation, and functional compartmentalization (90). R-Spondins interact with members of the leucine-rich-repeat-containing G-protein-coupled receptor (Lgr), which have recently been described as surface stem cell markers (91). Clinical evidence suggests an important role of R-Spondins in stem cell biology and morphogenesis since inactivating mutations in R-Spondin genes are associated with syndromes characterized by stem cell failure and developmental abnormalities (89). The interaction of R-Spondins with the Lgr receptors results in the inactivation of the U3-ubiquitin ligases RNF43 and ZNRF3, which are negative regulators of Wnt signaling by promoting internalization of the *Frizzled* receptors. Consequently, in the presence of R-Spondins, the *Frizzled* receptor will remain on the membrane allowing for Wnt signaling activation (91, 92). A recent paper explores the roles of R-Spondins in the regulation of the canonical Wnt signaling, functional zonation, and progenitor cell activity in the adrenal cortex (5). By using *in situ* hybridization, the authors demonstrated the expression of *Rspo1* and *Rspo3* in the adrenal capsule of the mouse from E12.5 onward. While *Rspo1* co-localized with the *Wt1* mesodermal-like expressing cells, *Rspo3* was preferentially co-expressed with *Nr2f2* and *Gli1*-expressing cells. Additionally, the mRNA abundance of *Rspo3* was significantly higher than *Rspo1*. Interestingly, while the genetic loss of *Rspo1* had no observable effects on the adrenals, loss of *Rspo3* was associated with remarkable phenotypes. By using different crosses of a *Rspo3*<sup>fl/fl</sup> allele and several CreERT drivers, the authors demonstrated that (a) loss of *Rspo3* results in cortical atrophy both during development and postnatally; (b) these changes are accompanied by a dramatic decrease in canonical Wnt signaling, which results in loss of the expression of canonical Wnt target genes such as *Axin2* and *Wnt4*; (c) loss of a functional zG, characterized by profound morphological changes and loss of expression of zG markers (while retaining zF differentiation); (d) loss of cortical Shh-expressing cells and capsular Gli1-expressing cells; and (e) a significant decrease in the mitotic activity of the cortex. The authors concluded that capsular-derived *Rspo3* is a key regulator of both Wnt and Hedgehog signaling in the adrenal cortex, having determinant roles in the establishment and the maintenance of the stem/progenitor cell populations, and in functional zonation (5).

These above findings support a general model whereby several autocrine/paracrine mediators from different sources play regulatory roles in the major signaling pathways that control the progenitor cell compartment and fate determination in the

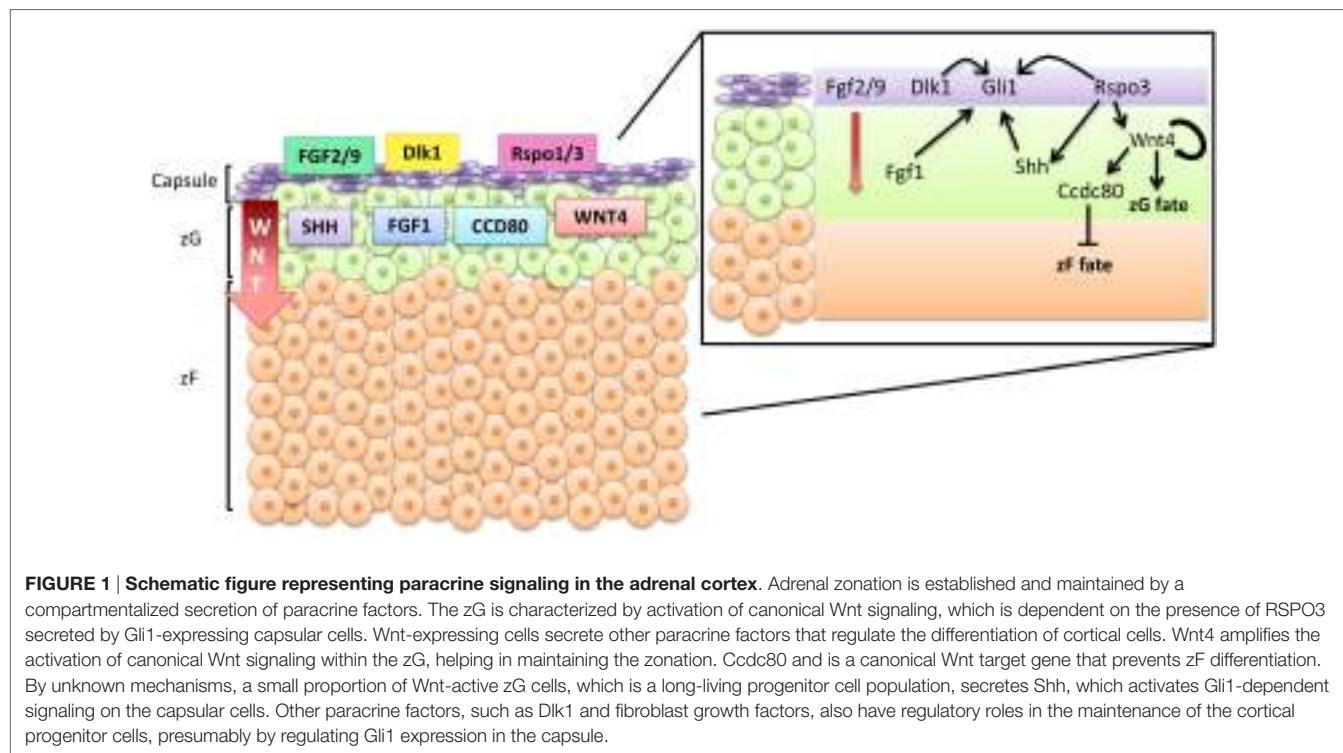
adrenal cortex (**Figure 1**). Some are known to interact with ECM components, indicating that the ECM composition may play a role in these regulatory loops. However, several gaps in the knowledge about the role of the adrenal ECM in paracrine/autocrine signaling and cell fate determination remain. In the following sections, we summarize the main findings that support a regulatory role for the adrenal ECM.

### The ECM and Associated Proteins

The ECM is a three-dimensional meshwork of extracellular proteins and polysaccharides that supports cells in each tissue (93). Among its components are included fibrous proteins such as collagens, laminin, fibronectin, and several types of bioactive compounds, such as latent growth factors, enzymes, chemoattractants, and morphogens (94). Therefore, in addition to its role in providing the physical scaffold that shapes the different tissues in an organism, the ECM is a reservoir of several biologically active compounds of different sources that are delivered to the cells in a spatially controlled manner (93, 94). Cells attach to the ECM through a special type of membrane-bound adhesion molecule known as integrins, which are comprised of two interacting subunits. The vertebrate integrin family contains 18  $\alpha$  and 8  $\beta$  subunits that can assemble into 24 different receptor complexes, each with unique binding properties for different ECM components (93). In the context of the stem cell niche of a variety of organ systems, the interactions between ECM proteins and integrins are fundamental for establishing a balance between self-renewal and differentiation. By forming complexes with different membrane receptors and ECM components, integrins facilitate a strict compartmentalization of cellular responses to different factors on a

cell-by-cell basis. Such highly precise control is fundamental to the process of asymmetric division, a fundamental characteristic of self-renewing cells. The complexes that contain integrins, ECM components, and other receptors induce the establishment of cell polarity, restricting fate-determinant molecules to one pole and directing the plane of cell cleavage in such a way that signaling molecules will be asymmetrically distributed between the daughter cells (95). In fact, certain types of integrins have been recognized as crucial for stem cell maintenance in different tissues (96). Supporting this observation, genetic ablation of  $\beta 1$  integrin favors a symmetric pattern of cell division in the mammary stem cell niche, leading to exhaustion of the stem cell compartment (97). Finally, biophysical and biochemical properties of the substrate, such as shape, stiffness, and protein composition, can trigger different transcriptional programs that support either stem cell maintenance or differentiation (98, 99).

Despite the emerging importance of ECM in tissue homeostasis and stem cell biology, few studies have addressed its roles in the adrenal. Early studies in different species have shown that several ECM components and integrins follow a zonal distribution in the adrenal cortex (100, 101). Furthermore, *in vitro* studies on primary adrenal cultures grown on different substrates suggest that different ECM components affect cellular responses to ACTH. For instance, it has been demonstrated in primary cultures from human fetal adrenals that laminin exerts an inhibitory effect on basal and ACTH-induced steroidogenesis, and a positive effect on cell proliferation (102). On the other hand, collagen IV and fibronectin increased cortisol and DHEAS production, respectively (102). While collagen IV is expressed throughout the human fetal adrenal gland, laminin and fibronectin follow a



zonal distribution, being preferentially expressed in cells of the definitive and the fetal zones, respectively (101). Therefore, the differences in cellular responses *in vitro* induced by these different ECM components parallel the functional zonation observed in the fetal gland: laminin suppresses differentiation and promotes proliferation (consistent with the cellular phenotype of the newly forming definitive zone), collagen IV promotes *HSD3B2* expression and cortisol production (resembling the fetal adrenal transitional zone), and fibronectin supports *CYP17A1* expression and DHEAS production. Indeed, primary cultures from zG and zF cells from the adult rat adrenal grown on laminin-coated plates exhibited increased proliferation and reduced basal and ACTH-stimulated aldosterone and cortisol production, respectively (103). Taken together, these results support the hypothesis that the ECM composition modulates cellular responses to hormone and growth factor stimulation, providing an additional mechanism by which the niche itself regulates the balance between self-renewal and differentiation.

## CONCLUDING REMARKS AND FUTURE PERSPECTIVES

In the last decade, several important aspects of the physiology of the adrenal cortex have been characterized, including the developmental origins of the different progenitor populations and the molecular pathways that are essential for self-renewal, organ remodeling, and differentiation. Additionally, several paracrine/autocrine bioactive compounds that initiate and maintain these signaling pathways have been identified. However, while the progenitor niche populations of other tissues, such as the skin and the gastrointestinal tract, are clearly defined, the progenitor niche of the adrenal cortex is just beginning to be characterized. Many features of the molecular mechanisms that govern the fate of the adrenocortical progenitor cells are still unknown. As discussed in the previous sections, several studies involving transgenic animal models have illuminated important observations and concepts central to the field. However, these studies are complex and time-consuming. Furthermore, studying such a small yet complex organ like the adrenal gland imposes additional challenges. In the near future, innovative techniques that allow investigators to engage in more complex *in vitro* studies have the potential to move the field forward greatly. Among these, organoid culture and engineered ECM are promising approaches.

Organoid culture has emerged in recent years as a valuable tool to study several aspects of stem cell biology, tissue morphogenesis, and lineage specification (104, 105). Organoids are self-organizing three-dimensional structures that are derived from stem cells and exhibit organotypic anatomic and functional features, including spatially restricted lineage commitment (106, 107). Organoids can be grown *in vitro* from pluripotent stem cells (embryonic stem cells and induced pluripotent stem

cells) or organ-specific stem cells (105). Organoid cultures have been established for different mouse and human tissues, including intestines, stomach, lungs, mammary gland, and prostate (108–114). A crucial requirement for establishing an organoid culture is the supplementation of organ-specific and well-defined stem cell niche factors in the culture medium. For example, to culture intestinal organoids, culture medium is supplemented with Wnt3a, EGF, Noggin, and R-spondin 1 (108). Organoids have been proven to be valuable models for studying different aspects developmental biology and stem cell research such as tissue morphogenesis, organogenesis, differentiation, heterotypic interactions between different cell types, and the effects of the ECM on cell differentiation and behavior. However, a current limitation of organoid cultures is the organoid dependence on a suitable three-dimensional matrix. Among the most commonly used scaffolds are collagen and Matrigel. These substrates, however, feature variable compositions and physical properties, which limits studies aiming to characterize the influence of the microenvironment on the organoid properties (105). Furthermore, these substrates pose risks of immunologic reactions and pathogen transfer and are, therefore, unsuitable for clinical applications. Engineered synthetic ECM has emerged as an attractive approach to circumvent these limitations (115).

Engineered ECM, constructed as an artificial substrate, acts to mimic the original three-dimensional microenvironment of a given organ or tissue. Increasingly, sophisticated scaffolds comprised of materials, such as synthetic hydrogels and nanofibers, are frequently employed as artificial ECM substitutes. In addition to biochemical interactions in cell-cell and cell-ECM signaling, mechanical stimuli such as substrate stiffness, and tension forces can also be modeled in engineered ECM (115). In a recent publication Gjorevski et al. studied the effects of stiffness on the dynamics of single cell-derived intestinal organoid formation (116). While intermediate to high stiffness and the presence of fibronectin sustained the expansion of intestinal LGR5-positive stem cells, differentiation and organoid formation required softer matrices and the presence of laminin.

We believe that, in the near future, organoid culture and tissue engineering techniques will increase our power to understand the molecular interactions that regulate the adrenal cortex stem cell niche. Protocols to establish normal adrenocortical and cancer organoid cultures in bioengineered matrices are currently under development in our laboratory.

## AUTHOR CONTRIBUTIONS

All authors equally contributed to this manuscript.

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# Comparative Effect of ACTH and Related Peptides on Proliferation and Growth of Rat Adrenal Gland

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Pro-opiomelanocortin (POMC) is a polypeptide precursor known to yield biologically active peptides related to a range of functions. These active peptides include the adrenocorticotropic hormone (ACTH), which is essential for maintenance of adrenal growth and steroidogenesis, and the alpha-melanocyte stimulation hormone, which plays a key role in energy homeostasis. However, the role of the highly conserved N-terminal region of POMC peptide fragments has begun to be unraveled only recently. Here, we review the cascade of events involved in regulation of proliferation and growth of murine adrenal cortex triggered by ACTH and other POMC-derived peptides. Key findings regarding signaling pathways and modulation of genes and proteins required for the regulation of adrenal growth are summarized. We have outlined the known mechanisms as well as future challenges for research on the regulation of adrenal proliferation and growth triggered by these peptides.

**Keywords:** adrenal growth, ACTH, N-POMC, proliferation, cell cycle

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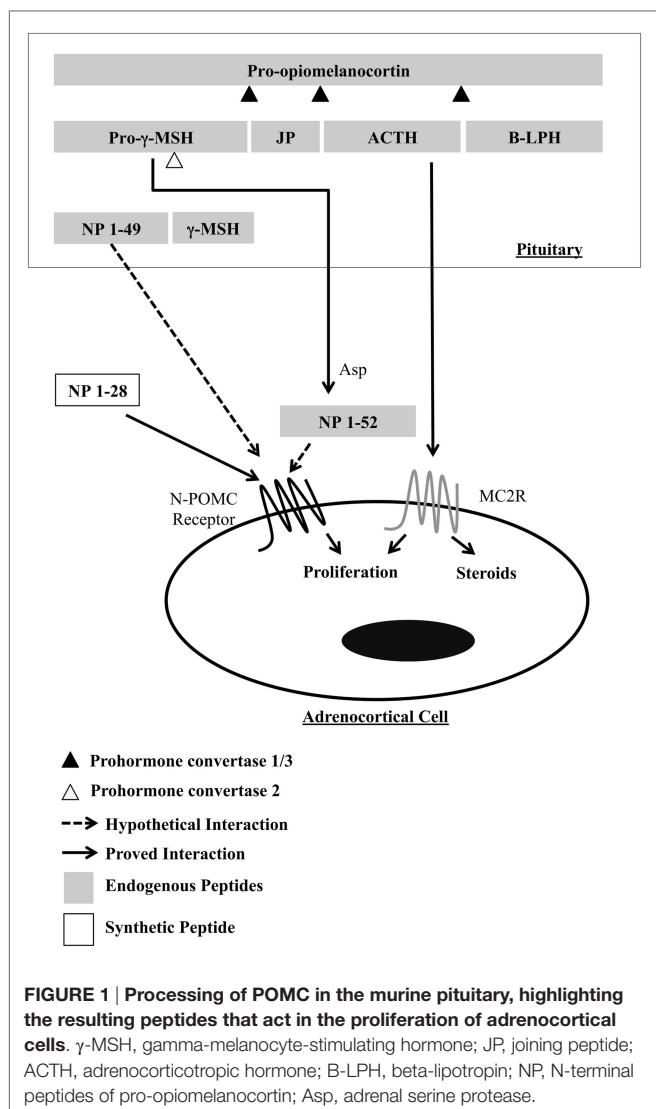
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## INTRODUCTION

The primary function of the adrenal cortex is to produce steroids. Each zone of the adrenal cortex synthesizes different steroids in response to endocrine and paracrine stimuli. Adrenal function and maintenance of adrenal size are associated with regulation of adrenocortical growth, a topic that has been covered by other studies (1, 2). This review summarizes our understanding of growth regulators of the murine adrenal and highlights the action of adrenocorticotropic hormone (ACTH) and N-terminal peptides of pro-opiomelanocortin (N-POMC) in the control of proliferation and maintenance of the adrenal cortex.

## PRO-OPIOMELANOCORTIN IN MURINE

*Pomc* is a gene that belongs to the opioid/orphanin family. It is a highly conserved gene found from agnathan fish to mammals (3). In murines, this gene encodes a prohormone of 235 amino acids produced mainly by corticotrophic cells in the pituitary gland. Post-translational processing at specific sites results in production of various smaller peptides, including peptide hormones with a range of physiological functions (Figure 1). In addition to the pituitary gland, POMC peptides are found in a diverse range of tissues, including the hypothalamus, skin, lung, gut, and pancreas (4). POMC transcripts found in these tissues are not full length, resulting in low levels of protein (5), and its function is not clear. POMC peptides in the circulation are derived mainly from the pituitary, and thus, the peptides produced in peripheral tissues act in an autocrine or paracrine way. The enzymes



**FIGURE 1 |** Processing of POMC in the murine pituitary, highlighting the resulting peptides that act in the proliferation of adrenocortical cells.  $\gamma$ -MSH, gamma-melanocyte-stimulating hormone; JP, joining peptide; ACTH, adrenocorticotrophic hormone; B-LPH, beta-lipotropin; NP, N-terminal peptides of pro-opiomelanocortin; Asp, adrenal serine protease.

responsible for cleavage of POMC are called prohormone convertases (PC) and are of two types, PC1 and PC2. In the anterior lobe of the pituitary, the action of PC1 generates the main four POMC-derived peptides: the N-terminal peptide 1–74 (N-POMC 1–74 or pro- $\gamma$ -MSH), the joining peptide (JP), ACTH, and beta-lipotropin ( $\beta$ -LPH). In the intermediate lobe, ACTH is cleaved by PC2 to produce alpha-melanocyte-stimulating hormone ( $\alpha$ -MSH) and the corticotrophin-like intermediate peptide (CLIP);  $\beta$ -LPH is completely processed to  $\gamma$ -LPH and  $\beta$ -endorphin; and pro- $\gamma$ -MSH is cleaved to generate  $\gamma$ 1- or  $\gamma$ 3-MSH and N-POMC 1–49.  $\gamma$ 1-MSH is found in humans but not in murines, as the cleavage site (a dibasic residue pair required for processing  $\gamma$ 3-MSH into  $\gamma$ 1-MSH) is missing in rodents (6).  $\gamma$ -MSH appears to potentiate the steroidogenic effect of ACTH in the adrenal gland, but the exact form of the peptide ( $\gamma$ 1,  $\gamma$ 2, or  $\gamma$ 3) that produces this effect is still unclear (7). Currently, the concept of tissue-specific cleavage of POMC is acceptable, at least in the adrenal gland, where a serine-protease has been cloned and is responsible for cleaving the pro- $\gamma$ -MSH into a 52-residue

peptide (8). The family of G-protein-coupled receptors named melanocortin receptors (composed of five members) is responsible for intermediating the action of POMC peptides.

## PROLIFERATIVE ADRENAL CORTEX RESPONSES TO ACTH

The 39-amino acid peptide ACTH is the primary regulator of adrenal gland growth, maintenance, and function. Due to the actions of corticotropin-releasing hormone (CRH), arginine vasopressin, and other secretagogues, ACTH stimulates the pituitary corticotroph cells to release ACTH (9). ACTH binds to specific high-affinity receptors [melanocortin receptor 2 (MC2R)] located on the surface of adrenal cortical cells, stimulating the production of cortisol and corticosterone in murines, which in turn suppresses ACTH-releasing factors. ACTH increases rat adrenal weight by inducing both hyperplasia and hypertrophy in specific zones. In fact, administration of chronic exogenous ACTH in rats induces hyperplasia and hypertrophy in the outer and inner zona fasciculata, respectively (10), and results in a 70% increase of adrenal mass in rats (11). This phenomenon is also seen in knockout mice for both glucocorticoid and dopamine receptors showing elevated levels of circulating ACTH (12, 13). On the other hand, low levels of ACTH, such as those seen in animals submitted to hypophysectomy (14) or treated with dexamethasone (15), result in adrenal atrophy. When adrenal growth occurs to compensate for unilateral adrenalectomy in hypophysectomized rats, neither a decrease in circulating corticosterone nor elevated ACTH levels are observed (16), suggesting the action of neural mediation or other POMC-derived peptides.

## MOLECULAR MECHANISMS IMPLICATED IN ACTH ADRENOCORTICAL GROWTH

Abundant data relating to the signaling triggered by ACTH have been provided by experiments performed in cultured normal and tumoral adrenocortical cells. However, the *in vitro* action of ACTH on signaling pathways involved with adrenocortical growth is controversial and seems to depend on the cell type, the state of the responding cell, and other environmental signals from extracellular matrices (17, 18).

### ACTH *In Vitro*

In support of the mitogenic or antimitogenic action of ACTH, there are studies analyzing the regulation of ERK/MAPK and related pathways, in different cell types. In quiescent Y1 mouse adrenocortical tumor cells, the molecular mechanisms of cell cycle control comprise two contrasting control pathways for 1-nM ACTH treatment: (1) a mitogenic effect *via* induction of the *fos* and *jun* gene families and weak activation of ERK/MAPK; and (2) a cAMP/PKA-mediated antimitogenic mechanism comprising Akt pathway deactivation, cMyc degradation, and p27<sup>Kip1</sup> induction (17, 19, 20). However, the ability of activate the ERK/MAPK was not interrupted in the cAMP-resistant mutant Y1 cells (Kin-8 cells) stimulated by ACTH, indicating the PKA not mediate the mitogenic action of ACTH (21). Arola and

collaborators (22) also found an ACTH-inducible biphasic growth effect in rat adrenocortical cells in primary culture, in which a 7–70 nM ACTH-mitogenic effect transduced through the cAMP-mediated system and an ACTH-antimitogenic took place *via* a cAMP-independent pathway.

In another study performed with Y1 cells, the authors observed the inhibition of ERK/MAPK and c-Jun N-terminal kinases pathways through a PKC and  $\text{Ca}^{2+}$ -dependent pathway (23), which favors an antimitogenic action of ACTH. In agreement, it was demonstrated by Bey and collaborators (24) that in Y1 cells, MAPK phosphatase-1 is a component of the ACTH signaling cascade, suggesting that ACTH can downregulate MAPKs. The antimitogenic and pro-apoptotic action of ACTH was reinforced in normal adrenal cells. In rat adrenocortical cells in primary culture, treatment for 3 days with 1-nM ACTH-induced apoptosis, activation of PKA/CREB but not ERK, and expression of c-Fos protein (25, 26). Also in support to antimitogenic action of ACTH, in bovine adrenocortical cells, angiotensin II activate MAPK after 5 min of treatment ( $\text{EC}_{50} = 0.1 \text{ nM}$ ), whereas ACTH does not stimulate ERK (27). Moreover, in rat adrenal zona glomerulosa cells, ERK activation blocked cell proliferation (28, 29).

In another widely used cellular model, the H295 human adrenocortical tumor cell line, which shares similarities with cells of the zona glomerulosa, ACTH stimulates ERK/MAPK signaling. However, in H295 cells, it has been described that MAPK stimulation by 100-nM ACTH depends on receptor internalization (30). On the other hand, in MC2R-transfected human embryonic kidney cells (31), 1-nM ACTH induces ERK phosphorylation that is partially PKA dependent. However, arrestin-coupled internalization does not involve any level of ACTH-dependent ERK phosphorylation (32). In summary, the analysis of signaling pathways involving the action of ACTH on different cell types and *in vitro* conditions gives support for an antimitogenic action of ACTH.

## ACTH *In Vivo*

In research involving depletion of the hypothalamic–pituitary–adrenal (HPA) axis using *in vivo* models with various approaches such as enucleation-induced adrenal regeneration (33), dexamethasone (Dex) treatment, and hypophysectomy, most but not all of the evidence converges on existing signals and pathways related to a mitogenic effect of ACTH.

Early response genes in the Fos and Jun gene families that form the transcriptional factor AP-1 and stimulate cellular proliferation (34) are induced by both ACTH and FGF2 infused in the rat adrenal gland *in situ* or in the adrenal cortex of hypophysectomized rats (35, 36). In enucleation-induced rat adrenal gland regeneration, the Fos gene was unregulated in the first 2 days of regeneration, while after 5 days of enucleation, downregulation of the Fos and Jun genes was observed (37).

Although it has been proposed that ACTH induces SAPK/JNK signaling activation and ERK/MAPK inhibition *in vivo* (23), results of chronic ACTH treatment in Dex-treated rats showed that ACTH is able to induce a sustained and progressive increase in ERK activation and proliferating cell nuclear antigen (PCNA) expression in all adrenal zones (38).

Other findings link the proliferative action of ACTH in Dex-treated rats with regulation of the cyclin-dependent kinase inhibitors (CDKIs) p27Kip1 and p57Kip2 in a time- and site-specific manner. A study shows that after Dex treatment, most of the cells expressed p27Kip1 but not p57Kip2. Subsequent ACTH treatment suppressed p27Kip1 expression and induced p57Kip2, while PCNA-expressing cells appeared mainly around the zona glomerulosa (39). Other cell-cycle regulators are also implicated in ACTH adrenocortical growth of Dex-treated rats. Besides increasing p27Kip1 expression, inhibition of the HPA axis downregulates cyclin D2 and D3 expression in the adrenal cortex. ACTH increases cyclin E and D3 expression, while it reduces expression of p27Kip1 protein in the outer and inner fraction preparations of adrenal cortex, respectively (40, 41). Moreover, the cell-cycle regulation is time dependent and zone specific. More recently, the Nek2 gene and its protein, together with the Notch gene, have also been shown to be involved in the cell cycle regulation triggered by ACTH (42).

The extracellular matrix (ECM) contributes to the regulation of cell proliferation and cell differentiation and therefore has a role in embryonic development and adult tissue homeostasis. Feige and colleagues have described the composition and expression of ECM components in the adult adrenal gland (43, 44). From the periphery to the center of the gland, the authors observed differential expression of fibronectin and laminin, which can be associated with specific activities of the cell components of the zones. However, studies conducted by the Gallo-Payet group show that ECM modulates basal and ACTH-induced cell functions, with fibronectin and collagen I and IV favoring steroid secretion, while laminin promotes proliferation (18). These findings illustrate the importance of the morphological changes associated with ACTH.

Despite the *in vivo* evidence that ACTH is the only factor that stimulates adrenal growth, other studies point in a different direction. As briefly described above, there are considerable data showing that ACTH inhibited growth of adrenal cells *in vitro*. In addition, Rao and colleagues (45) showed that rats treated with antiserum against ACTH had significant reduction of blood corticosteroids levels but did not exhibit adrenal atrophy. These and other results, which have been described in comprehensive reviews of the last 60 years of POMC research (46, 47), suggest that another factor distinct from ACTH has the ability to promote adrenal growth.

## PROLIFERATIVE ADRENAL CORTEX RESPONSES TO N-POMC PEPTIDES

In 1980, Estivariz and colleagues (48) extracted and purified pro-gamma-MSH from human pituitaries and showed that this peptide could not prevent adrenal atrophy in hypophysectomized rats. However, smaller N-POMC peptides (without the  $\gamma 3$ -MSH portion) produced by trypsin digestion of pro-gamma-MSH or extracted from pituitary glands proved to be potent mitogens both *in vivo* and *in vitro*. In this section, we present information on the proliferative effect of the most important N-POMC peptides.

## N-POMC 1–28

N-POMC 1–28 was first isolated from human pituitary glands and later characterized as an extraction artifact (49). Even though N-POMC 1–28 is not an endogenous peptide, it has been extensively used to show the mitogenic effect of the N-POMCs. Moreover, the first 28 amino acids of the N-terminal portion of POMC have been shown to be essential to the triggering of adrenal cell proliferation. The mitogenic activity of N-POMC 1–28 has been demonstrated *in vivo* in murine models (40, 50, 51) and *in vitro* in Y1, NCI-H295R, and rat primary culture cells (26, 52, 53). Peripheral delivery of this peptide in *Pomc* KO mice does not promote any alterations in the adrenal gland (54) but may prevent atrophy of regenerating adrenal glands after hypophysectomy (55). These findings mean that besides promoting mitosis, this peptide may prevent apoptosis of adrenal cells. Indeed, our group has shown the anti-apoptotic effect of N-POMC 1–28 in adrenal glands of hypophysectomized rats (51). However, the molecular mechanisms underlying this effect are not clear. The positions of two disulfide bridges (between cysteine residues 2–24 and 8–20) seem to be essential to its biological activity (56).

## N-POMC 1–49

N-POMC 1–49 is an endogenous peptide produced and secreted by the intermediary lobe of the pituitary. It is one of the products from the cleavage of pro-gamma-MSH into smaller peptides. *In vitro* studies have shown that this peptide may promote proliferation of Y1 and NCI-H295R cells (52, 53). However, *in vivo* studies have shown that N-POMC 1–49 does not increase adrenal weight in fetal sheep when infused for 48 h (57). Interestingly, the presence of an O-linked glycan seems to be crucial for its proliferative effect. However, cleavage of pro-gamma-MSH in the pituitary occurs only if the O-linked glycan is not present in the molecule, resulting in an N-POMC 1–49 without the glycan [reviewed in Bicknell and Lowry (58)]. Clearly, the present data are controversial, and more assays must be done before it can be concluded that N-POMC 1–49 is the natural N-POMC peptide involved in adrenal proliferation and maintenance.

## Pro-Gamma-MSH

Pro-gamma-MSH is considered to be an active fragment found in the bloodstream at the same levels as ACTH (59). When infused into sheep fetus, this N-POMC peptide increased adrenal weight (57). However, as mentioned before, when administered in hypophysectomized rats, no effect on the adrenal weight was observed (60). Since the mitogenic peptides are located in the N-terminal portion of pro-gamma-MSH, a hypothesis of post-secretional cleavage occurring at the level of specific tissues has emerged. Indeed, Bicknell and collaborators (8) characterized a serine protease they named AsP (adrenal serine protease) that is present in the ECM of adrenal cells and is responsible for cleaving pro-gamma-MSH. The cleavage releases a peptide of 52 residues that induces proliferation of adrenal cortical cells. Moreover, Asp is capable of cleaving small basic substrates (e.g., arginine–arginine, lysine–arginine, etc.), generating N-POMC 1–49. These findings suggest the existence of an endogenous mitogenic N-POMC peptide, but no consensus about its identity has been reached.

## THE MOLECULAR MECHANISM INVOLVED IN THE N-POMC PROLIFERATIVE EFFECT

The proliferative effect of N-POMC peptides has been established since the beginning of the 1980s, but its mechanism has begun to be unveiled only recently. The first study on this topic, conducted by Fassnacht and colleagues (52), concluded that N-POMC 1–28 promotes cell proliferation in NCI-H295R, Y1, and primary cultures of bovine adrenocortical cells by triggering a rapid activation of the ERK/MAPK but not the APK/JNK or p38 pathways. Pepper and Bicknell (53) corroborated those findings and showed that the upstream ERK regulators c-RAF and MEK were activated in Y1 and NCI-H295R cells treated with N-POMC 1–28 or N-POMC 1–49. In 2011, Mattos and collaborators (26) showed that ERK1/2 was activated in primary cultures of rat adrenocortical cells treated with N-POMC 1–28.

In 2014, we performed a PCR array to evaluate the effect of N-POMC 1–28 on the expression of key genes related to the control of the cell cycle. The genes *Nek2* and *Notch* were upregulated after treatment, suggesting that the proliferative effect of this peptide might be mediated by these genes (42). Additional research is needed to further elucidate the molecular mechanisms involved in the proliferative effect of N-POMC.

A fundamental question that has yet to be definitively answered is the identification of the receptor through which N-POMC peptides elicit their effects on adrenal growth. There have been two unsuccessful attempts to identify such a receptor (53, 58). In 2014, we joined efforts with Bicknell's group and proposed a new approach to identifying this receptor. We cloned the most expressed orphan G-protein-coupled receptors in the rat adrenal gland and performed a magnetic cell separation assay using the N-POMC peptide attached to magnetic beads. A likely candidate for the N-POMC receptor was identified, confirmed by ligand-binding assays, and shown to be capable of activating the ERK pathway after stimulation with N-POMC. Further experiments are now being conducted to characterize *in vivo* and *in vitro* this potential adrenal N-POMC receptor. Final confirmation of the identity of the adrenal N-POMC receptor is essential for the understanding of cell proliferation in adrenocortical cells.

## CONCLUSION

In this paper, we summarize the current state of knowledge of the roles of ACTH and N-POMC in the proliferation of murine adrenal cells. We identify gaps in knowledge and describe conflicting results that need to be further investigated in order to fully understand the biology of this phenomenon. Examples of such urgently needed studies include gene array assays and pathway analysis to provide more data on the molecular mechanisms triggered by N-POMC peptides as well as to confirm the identity of the natural endogenous mitogenic N-POMC peptide. A holistic and interdisciplinary approach will be required, as none of these peptides or hormones act alone in nature. On the contrary, they trigger a net of responses and activate dozens of pathways

simultaneously. When we begin to examine this phenomenon from a holistic perspective, we may come to truly understand the proliferative effect of these peptides.

## AUTHOR CONTRIBUTIONS

The authors CL and PM contributed to the conception and design work.

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# Transcriptome Profile of Rat Adrenal Evoked by Gonadectomy and Testosterone or Estradiol Replacement

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Sex differences in adrenal cortex structure and function are well known in different species. In the rat, they are manifested as larger adrenal cortex and higher corticosterone secretion by females compared with males. These sex differences depend, among others, on functioning of the hypothalamic-pituitary-adrenal axis (HPA). In this aspect, it is widely accepted that testosterone exerts an inhibitory and estradiol stimulatory effect on the said axis. The molecular bases of these sex-related differences are poorly understood. Therefore, we performed studies aimed to demonstrate the effect of testosterone and estradiol on the expression of differentially regulated genes in rat adrenal gland. The classical method applied in the study—gonadectomy and gonadal hormone replacement—allows obtaining results suggesting a physiological role of the tested hormone (testosterone or estradiol) in the regulation of the specific genes. Adult male and female rats were either gonadectomized or sham operated. Half of orchectomized rats were replaced with testosterone while ovariectomized ones with estradiol. Transcriptome was identified by means of Affymetrix® Rat Gene 2.1 ST Array. Differentially expressed genes were analyzed by means of DAVID web-based bioinformatic tools and confirmed by means of Gene Set Enrichment Analysis. For selected genes, validation of the results was performed using QPCR. Performed experiments have provided unexpected results. Contrary to expectations, in orchectomized rats, testosterone replacement stimulates expression of numerous genes, mainly those associated with lipids and cholesterol metabolism. However, in ovariectomized animals, estradiol replacement inhibits the expression of genes, mainly those involved in intracellular signaling pathways. The physiological relevance of these findings awaits further research.

**Keywords:** rat, adrenal gland, transcriptome, global gene profiling, functional annotation clustering, gonadectomy, testosterone, estradiol

## INTRODUCTION

Sex-related differences in the structure and function of the adrenal cortex of mature rats are well recognized. These differences are the result of the action of sex hormones on the various components of the hypothalamic-pituitary-adrenal axis (HPA). The results of studies on the effects of gonadectomy and sex hormone replacement on the rat adrenal cortex suggest that estrogens stimulate and

androgens inhibit the HPA axis function. The results of these studies are presented in many comprehensive monographs and textbooks (1–7). It is important to note that in gonadectomized animals sex hormones exert generalized response, not limited to HPA axis only. For example, they regulate the levels of corticosteroid-binding globulins (CBG) (8–11) as well as corticosterone biological half-time (12–14). CBG is involved in the mechanism of steroid hormone action by keeping steroids in circulation, and therefore controlling the availability of unbound hormones for their targets. In the rat, CBG levels are notably higher in females. Orchectomy (ORX) resulted in 60–70% increase in CBG levels, while ovariectomy (OVX) had no effect (8, 15). Of interest, in the rat, estradiol effect on CBG is mediated via thyroid gland. Furthermore, biological half-life of corticosterone in adult male rats is ca. two times longer than in females (12, 16). Also, the metabolic clearance rate of corticosterone is markedly higher in female than male rats (17, 18).

The above-described sex differences in the structure and functions of the rat adrenal cortex probably depend on differences in the gene expression of this gland. However, to our knowledge, no studies are available on the effects of estradiol or testosterone on transcriptome profile of the rat adrenal gland. Regarding this, recently we have shown that in adult rat adrenal cortex, the differentially expressed genes that are significantly enriched included genes involved in steroid hormone metabolism, and their expression levels in females were significantly higher compared with those in the male rat (19). Generally, if compared with males, in the female rats higher expression levels of genes involved directly in steroid hormone synthesis were accompanied by lower expression levels of genes regulating basal cell functions. To our knowledge, only one publication on global gene profiling refers to the effect of testosterone on the expression of genes in the adrenal gland and it relates to the mouse (20). In this research, however, authors administered testosterone into intact female mice. Comparison between females and females treated with testosterone revealed 94 differentially regulated genes, with 23 being upregulated and 71 downregulated. The observed changes in gene expression authors combine with the disappearance of the X zone of the mouse adrenal cortex. It should be emphasize that this experimental model is not a physiological one.

In the present study, to demonstrate the effect of estradiol and testosterone on the expression of differentially regulated genes in rat adrenal gland, we applied experimental model considered in endocrinology as the “gold standard.” Dual control, it means removal of the gonads and replacement with the appropriate hormone allows obtaining results suggesting a physiological role of the tested hormone (estradiol or testosterone) in the regulation of the specific genes. Performed experiments have provided unexpected results. Contrary to expectations, in orchectomized rats, testosterone replacement stimulates expression of numerous genes, mainly associated with lipids and lipid metabolism. However, in ovariectomized animals, estradiol replacement inhibits the expression of genes, mainly those involved in intracellular signaling pathways.

In the present study, to demonstrate the effect of estradiol and testosterone on the expression of differentially regulated genes in rat adrenal gland, we applied the classical method used to study

endocrine glands, it means “remove and replace” (gonadectomy and gonadal hormone replacement). Performed experiments have provided unexpected results. Contrary to expectations, in orchectomized rats, testosterone replacement stimulates expression of numerous genes, mainly associated with lipids and lipid metabolism. However, in ovariectomized animals, estradiol replacement inhibits the expression of genes, mainly those involved in intracellular signaling pathways.

## MATERIALS AND METHODS

### Animals and Experiments

Adult female and male Wistar rats (12 weeks old; body weight: 120–150 g) were obtained from the Laboratory Animals Breeding Center, Department of Toxicology, Poznan University of Medical Sciences, Poznan, Poland. Animals were kept under standardized conditions of light (14:10 h light-dark cycle, illumination onset 06.00) at constant temperature (23°C) with free access to standard food pellets and water. All experiments were carried out between 10 and 11 am. All procedures described herein were approved by the Local Ethics Committee for Animal Research (Poznan, Poland), permission number: LKE—11/2015. All possible efforts have been done to minimize the number of animals and their suffering.

Rats were gonadectomized or sham operated under ketamine (100 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.) anesthesia. ORX was performed via scrotal access while OVX by two dorsolateral incisions. Fourteen days after surgery, half of ORX rats was replaced with testosterone (s.c. injection of Testoviron-Depot, Schering AG, Berlin, 5 mg/100 g body weight), while half of OVX animals with estradiol (s.c. injection of Estradiol-Depot, Jenapharm, 0.5 mg/100 g body weight). Doses of administered depo hormones were based on previous reports (21–24). It is believed that from both compounds either testosterone or estradiol are liberated slowly, providing a physiological hormone levels in gonadectomized rats. As emphasized by Schulte-Beerbühl and Nieschlag (24), increasing the dose of injected testosterone esters appears not to influence the maximal concentrations of testosterone in the blood but rather the duration of the effect. Moreover, administration of depo compounds allows to avoid the stress evoked by daily administration of the tested substances. After 2 weeks (4 weeks post surgery), rats were decapitated. Adrenal glands were collected to RNAlader and stored in –70°C for further analyses. Seminal vesicles and uteri were also collected and weighed.

### Corticosterone, Cholesterol, and Lipoproteins Detection

Serum corticosterone levels were determined by means of ELISA kit (ELISA Demeditec kit). Serum total cholesterol, lipoproteins, and triglycerides concentrations were evaluated by means of Roche Cobas Integra 400+ system.

### RNA Extraction

Total RNA was extracted from samples of entire adrenals using TRI Reagent (Sigma, St. Louis, MO, USA) and RNeasy MinElute

cleanup Kit (Qiagen, Hilden, Germany). The amount of total mRNA was determined from the optical density at 260 nm, and the RNA purity was estimated using the 260/280 nm absorption ratio (higher than 1.8) (NanoDrop spectrophotometer, Thermo Scientific, ALAB, Poland). The RNA integrity and quality were checked in a Bioanalyzer 2100 (Agilent Technologies, Inc., Santa Clara, CA, USA). The resulting RNA integrity numbers were between 8.5 and 10 with an average of 9.2 (Agilent Technologies, Inc., Santa Clara, CA, USA). Each sample was diluted to the RNA concentration of 100 ng/ $\mu$ l, at the OD260/OD280 ratio of 1.8/2.0. From each RNA sample, 100 ng of RNA was taken for microarray experiments. The remaining amount of isolated RNA was used for RT-qPCR study.

## Microarray Expression Analysis and Statistics

The Affmetrix procedure and methods of analyzes were described previously (19, 25–27). Total RNA (100 ng) from each sample was subjected to two rounds of sense cDNA amplification (Ambion® WT Expression Kit) (Ambion, TX, USA). The obtained cDNA was used for biotin labeling and fragmentation using AffymetrixGeneChip® WT Terminal Labeling and Hybridization kit (Affymetrix, Santa Clara, CA, USA). Biotin-labeled fragments of cDNA (5.5  $\mu$ g) were hybridized to Affymetrix® Rat Gene 2.1 ST Array Strip (48°C/20 h). Then, microarrays were washed and stained according to the technical protocol, using Affymetrix GeneAtlas Fuidics Station. The array strips were scanned employing Imaging Station of GeneAtlas System. The preliminary analysis of the scanned chips was performed using AffymetrixGeneAtlasTM Operating Software. The quality of gene expression data was checked according to quality control criteria provided by the software. Obtained CEL files were imported into downstream data analysis.

All analyzes were performed using BioConductor software, based on the statistical R programming language. For background correction, normalization, and summation of raw data, the Robust Multiarray Averaging algorithm implemented in “affy” package of BioConductor was applied (28). Biological annotation was taken from BioConductor “oligo” package where annotated data frame object was merged with normalized data set, leading to a complete gene data table (29).

The selection criteria of a significantly changed gene expression were based on expression fold difference higher than abs. 2 and adjusted  $p$  value <0.05. The result of such a selection was presented as volcano plots, where total number of up- and downregulated genes has been shown.

Data files were also deposited in the Gene Expression Omnibus (GEO) repository at the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/geo/>) under the GEO accession number GEO: GSE93726.

## Assignment of the Genes Regulated by Sex Hormones to Relevant Gene Ontology Terms

Sets of female and male genes regulated by sex hormones were also subjected to functional annotation clusterization using DAVID

web-based bioinformatics tools (Database for Annotation, Visualization, and Integrated Discovery) (30). Gene symbols for up- or downregulated genes from each of the compared groups were loaded to DAVID by “RDAVIDWebService” BioConductor package (31). Functional annotation charts generated by DAVID with overrepresented gene annotations in gene ontology biological process database (BO BP FAT) were shown as box plot. Interested gene sets from DAVID functional annotation charts were also subjected to hierarchical clusterization algorithm and presented as a heatmaps. Such analyses were carried out separately for male and female data sets. Arbitrary signal intensities from selected genes were represented by colors (green—higher; red—lower expression value). Log2 signal intensity values for any single gene were resized to Row Z-score scale (from -1.5, the lowest expression, to +1.5, the highest expression for single gene). Additionally, gene names and calculated fold values were also shown.

## Gene Set Enrichment Analysis (GSEA)

Gene Set Enrichment Analysis is a computational method used for testing *a priori* defined gene sets (GO terms, pathways) for association with one of the two compared biological groups. The method uses Kolmogorov-Smirnov (K-S) statistical test for identification of significantly enriched or depleted groups of genes (32). GSEA analysis has been conducted using GSEA Java Desktop Application from Broad Institute (<http://software.broadinstitute.org/gsea/index.jsp>). Normalized data from all of genes were transformed to an appropriate format and imported to application. Then, a predefined gene sets database named Hallmark was selected from Molecular Signatures Database (33). Genes belonging to the selected set were ranked according to the difference in their expression level using signal-to-noise ratio with 1,000 times permutation. By walking down the ranked list of genes, the enrichment score (ES) was calculated for each selected gene set. It was done by sum statistic when a gene was present in the gene set and decreasing it when it was not (34). ESs were normalized by their gene set size, and false positive finding were corrected by FDR.

## QPCR

QPCR was performed by means of the Lightcycler 2.0 instrument (ROCHE) with the 4.05 software version. SYBR green detection system was applied as described earlier (19, 25–27, 35–37). Every 20  $\mu$ l reaction mixtures contains 2  $\mu$ l template cDNA (standard or control), 0.5  $\mu$ M of specific primer, and a previously determined optimum MgCl<sub>2</sub> concentration (3.5  $\mu$ M for each reaction). LightCyclerFastStart DNA Master SYBR Green I mix (ROCHE) was used. The real-time PCR program included 10 min denaturation step to activate the Taq DNA Polymerase, followed by a three-step amplification program: denaturation at 95°C for 10 s, annealing at 56°C for 5 s, and extension at 72°C for 10 s. Specificity of reaction products was checked by determination of melting points (0.1°C/s transition rate). Expression of studied genes was related to B2m, only in case of Hcrtr2 to PBGD.

The primers used were designed by Primer 3 software (Whitehead Institute for Biomedical Research, Cambridge, MA, USA) (Table 1). They were purchased from the Laboratory

**TABLE 1 |** Primers used for QPCR validation of selected genes.

cDNA	Genbank accession number	Primer	Primer sequence (5'-3')	Position	PCR product size (bp)
Hcrtr2	NM_013074.1	S	GGCTTATCTCCAAATATTCCG	782–802	68
		A	CTCTGAACCACAGAAGAAGT	831–849	
AR	NM_012502.1	S	ATCATCTCTGTGCAAGTGC	3,634–3,654	162
		A	CCCATGCCAGAGAAGTAGTG	3,776–3,795	
ER $\alpha$	NM_012689.1	S	ATGATGGGCTTATTGACCAAC	1,248–1,268	137
		A	AGGATCTCAACCAGGCACA	1,365–1,384	
ER $\beta$	NM_012754.1	S	TCTGTGTGAAGGGCATGATC	1,474–1,493	237
		A	GCAGATGTTCCATGCCCTTG	1,691–1,710	
ERR $\alpha$	NM_001008511.2	S	CTCTCTACCCAAACGCCCTCT	334–353	234
		A	CGCACACCCCTCCTTGAGCAT	548–567	
Mc $\alpha$ 2r	NM_001100491.1	S	GGACAAGGGGGAGGCAGA	110–118	201
		A	TGGCACAACTACATCAGGAC	281–300	
Hmgcr	NM_013134.2	S	GACGCAACCTCTACATCCG	1,966–1,984	142
		A	TAGTTACCAACTGACCGCCAGA	2,087–2,107	
B2m	NM_012512.2	S	CTTGCAGAGTTAACACGTCA	316–336	70
		A	CTTGATTACATGTCCTGGTC	366–385	
PBGD	NM_013168.2	S	GAAAGACCTGGAAACCTT	522–540	148
		A	AGCTCATCCAGCTCCGTA	651–669	

Gene symbol, gene names, Genbank accession numbers, oligonucleotide sequences for sense and antisense primers, their position, and product size are shown.

of DNA Sequencing and Oligonucleotide Synthesis, Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw.

## Statistical Analysis

The applied statistical analyses of gene expression are parts of the softwares used. The RT-QPCR data, on the other hand, are expressed as the mean  $\pm$  SE, and the statistical significance of the differences between the compared groups was estimated using the Student's *t*-test.

## RESULTS

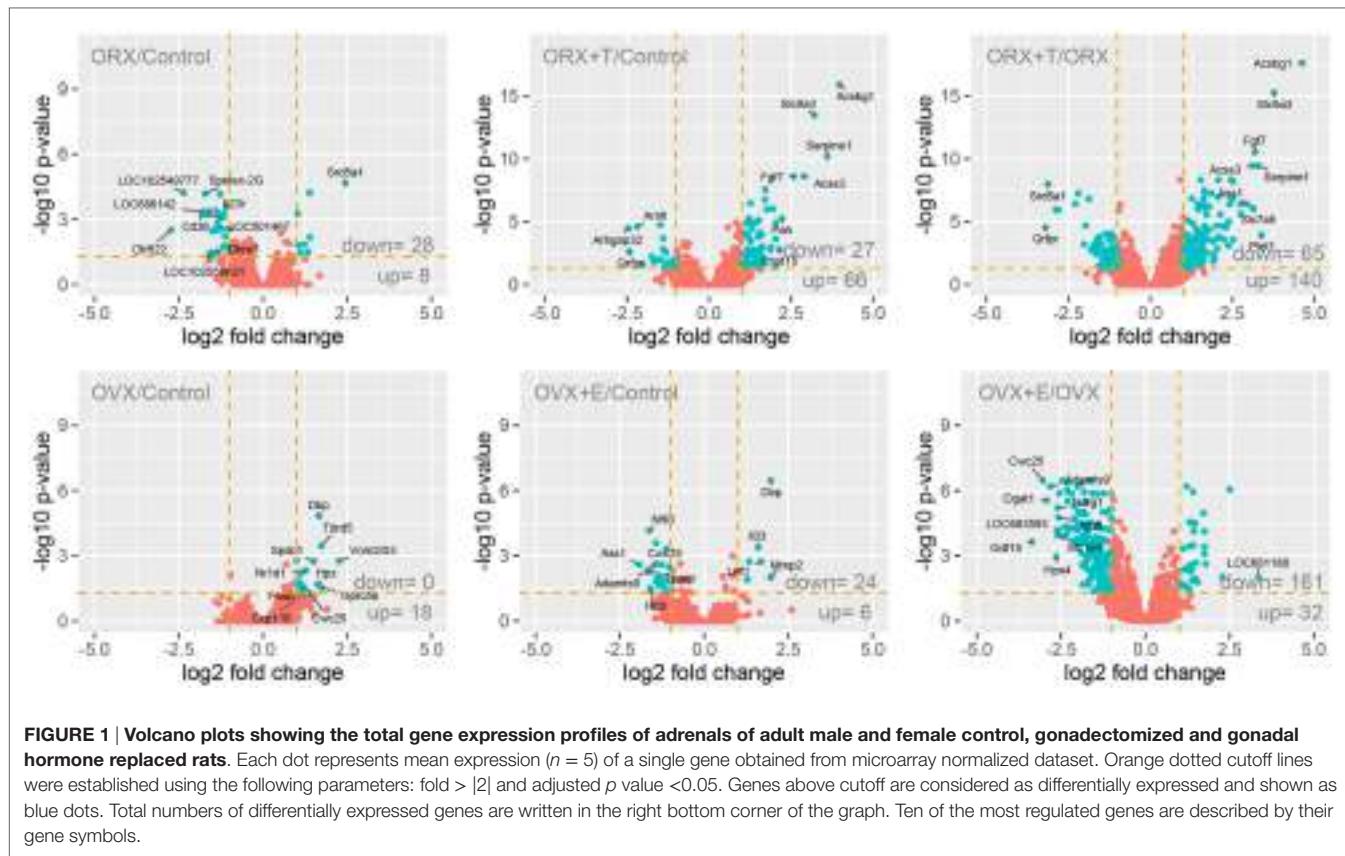
To study the modulatory effects of sex hormones on adrenal gland transcriptome profile, adult female and male rats were gonadectomized and replaced with estradiol or testosterone. The efficacy of the treatments has been demonstrated by seminal vesicles and uterine weights. As expected, weight of the examined organs decreased significantly after removal of gonads, whereas the weight of seminal vesicles and uteri were increased in the groups replaced with sex hormone [uterine weight (g): sham operated  $0.42 \pm 0.03$ , OVX  $0.06 \pm 0.01$ , OVX + estradiol replaced  $0.37 \pm 0.02$ ; seminal vesicle weights: sham operated  $0.41 \pm 0.03$ , ORX  $0.09 \pm 0.01$ , ORX + testosterone replaced  $0.85 \pm 0.03$ ; in each group  $n = 6$ ; mean  $\pm$  SE].

In these experimental conditions, ORX increases serum corticosterone concentrations, an effect reversed by testosterone replacement (Figure S1 in Supplementary Material). In the female, on the other hand, ovariectomy reduces serum corticosterone levels, while estradiol replacement restores corticosterone levels to the control values. In ORX rats, serum levels of the total cholesterol, LDL, and HDL were elevated, an effect reversed by testosterone replacement (Figure S2 in Supplementary Material). In OVX rats, serum levels of the total cholesterol, LDL, and HDL

were higher and of triglyceride lower than in sham-operated animals. Estradiol supplementation restores these changes to the normal values.

Adrenals obtained from five rats from each group were used for whole transcriptome analysis using Affymetrix microarray method, where expression of approximately 30,000 genes was examined. General gene expression profiles resulting from the comparison of male and female controls with appropriate experimental groups are shown as volcano plots (Figure 1). The selection criteria of a significantly changed gene expression level was based on expression fold difference higher than abs. 2 and adjusted *p* value  $<0.05$ . Considering these assumptions, ORX vs. control male adrenal transcription profile comparisons revealed that 36 genes were expressed differentially (28 down- and 8 upregulated genes). In the next compared groups: ORX + T vs. control males, we revealed 93 differentially expressed genes (27 down- and 66 upregulated). The last comparison of ORX + T vs. ORX groups revealed 205 differentially expressed genes (65 down- and 140 upregulated). Similar comparisons performed between OVX vs. control females, OVX + E vs. control females and OVX + E vs. OVX revealed that 18 (0 down- and 18 upregulated), 30 (24 down- and 6 upregulated), and 193 (161 down- and 32 upregulated) genes are differentially expressed, respectively.

Correctness of presented study has been verified by detailed analysis of steroid-5-alpha-reductase alpha polypeptide 1 (Srd5a1) and hypocretin (orexin) receptor 2 (Hcrtr2) gene expression. Both, studied enzyme and receptor genes were earlier described as regulated by sex hormones. Profile of Srd5a1 gene expression was extracted from normalized microarray data set and presented on Figure 2A. Expression of Srd5a1 was significantly increased after gonadectomy (fold = 5.46, *p* = 2.23E-05 for male, and fold = 2.17, *p* = 0.049 for female, in relation to appropriate control groups) and reversed by sex hormone replacement (fold = -1.6, *p* = 0.605 for male and fold = -2.28, *p* = 0.063 for

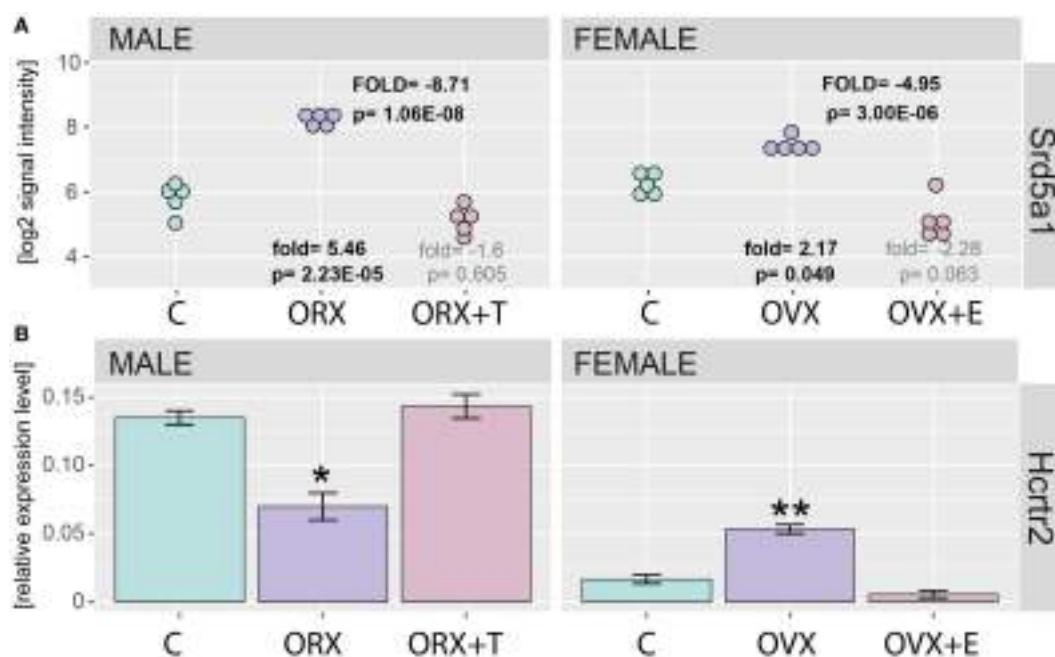


female, in relation to control groups). As revealed by QPCR, also expression of Hcrtr2 is notably decreased in ORX and reversed by testosterone. On the contrary, in OVX rats expression of Hcrtr2 was elevated and again this effect was reversed by estradiol replacement (Figure 2B).

Differentially expressed gene sets were subsequently used for screening of their overrepresentation in some particular gene ontological groups. For this reason, gene symbols from all previously mentioned comparisons were loaded to DAVID searching system. Due to the small number of differentially expressed genes in ORX vs. control, OVX vs. control, and OVX + E vs. control, we did not obtain statistically significant GO groups in the abovementioned comparisons. Comparison between ORX + T vs. control males revealed two GO terms formed by differentially expressed genes: “small molecule biosynthetic process” and “lipid metabolic process” (Figure 3A). Genes from these GO terms were upregulated in ORX + T group in relation to male control. In the next comparison, 35 GO terms were formed from genes differentially expressed in ORX + T vs. ORX groups. Some of obtained GO groups have a general meaning, e.g., “response to organic substance,” “response to chemical stimulus” but other seems to be more related to adrenal physiology, i.e., “response to steroid hormone” or “lipid metabolic process.” All of presented GO terms were upregulated in ORX + T group in relation to ORX (Figure 3B). The last presented GO term graph demonstrates OVX + E vs. OVX groups comparisons, which delivered 24 GO

terms. It is worth to notice that genes formed presented GO terms are downregulated in OVX + E vs. OVX group (Figure 3C). In this comparison, “lipid metabolic process” term was also statistically significant, and for this reason, genes belonging to this process in ORX + T vs. ORX as well as OVX + E vs. OVX were selected for hierarchical clusterization and presented as heatmap on Figure 4. To obtain the maps, 22 genes were selected from the “lipid metabolic process” in both male and female comparisons. All genes from male adrenals belonging to the “lipid metabolic process” are characterized by the highest expression levels in ORX + T group (green color) and the lowest in ORX group (red color). Expression of female genes has opposite profile. The highest gene expression levels are in OVX (green color) group, while the lowest in OVX + E (red color).

The above presented results were confirmed by another powerful bioinformatics tool that is the Gene Set Enrichment Analysis (GSEA). These analyzes were performed for: OXR/OXR + T and OVX/OVX + E experimental groups. After uploading of normalized expression level data from microarray to the software, we obtained a list of significantly represented terms from Hallmark database software. Ten of the most enrichment terms from each of the comparisons are presented on Figure 5. In accordance with DAVID and heatmap results, presented terms were enriched, it means that expressions of genes were higher in ORX + T (ORX + T/ORX) and OVX (OVX + E/OVX). The strongest enriched term in the comparison between ORX + T/ORX refers



**FIGURE 2 | Expression of Srd5a1 and Hcrt2 genes in applied experimental model.** (A) Expression values were extracted from microarray normalized data set and presented as log2 signal intensity scale. Fold and *p* values were calculated in relation to appropriate control groups (showed on bottom) or between gonadectomized [orchectomy (ORX) or ovariectomy (OVX)] and gonadectomized + sex hormone replaced (ORX + T, OVX + E) groups (showed on the top “FOLD” in capitals). In each group, *n* = 5. *p* Value obtained from moderated *t* test with FDR correction (incorporated in the Limma package of the software). (B) QPCR assay of Hcrt2 genes in adrenals of adult male and female control, gonadectomized and gonadal hormone replaced rats. Bars represent means  $\pm$  SEM (*n* = 5). HPRT gene was used as reference for data normalization. Statistically significant differences in relation to control group (Student’s *t*-test): \**p* < 0.05; \*\**p* < 0.02; \*\*\**p* < 0.01; \*\*\*\**p* < 0.001.

to “androgen response” while “TNFA signaling via NFKB” term was the strongest enriched in GSEA between OVX + E/OVX. Detailed results of those two terms are presented in Figures 6 and 7. List of the top 10 genes that strongly affect ES with their rank position are also shown.

One of the top enrichment term concerns the process strictly connected with adrenocortical steroidogenesis named “cholesterol homeostasis.” Results of GSEA are presented in Figure 8. Genes belonging to “cholesterol homeostasis” hallmark term are highly enriched in ORX + T group in relation to ORX group. Opposite results were obtained in OVX/OVX + E comparison, where gene belonging to “cholesterol homeostasis” are enriched in OVX group, it means that the expressions are higher in OVX in relation to OVX + E. Obtained results from GSEA are in commitment with previously presented DAVID GO analysis. List of the top 10 genes with their rank position are also shown in these figures.

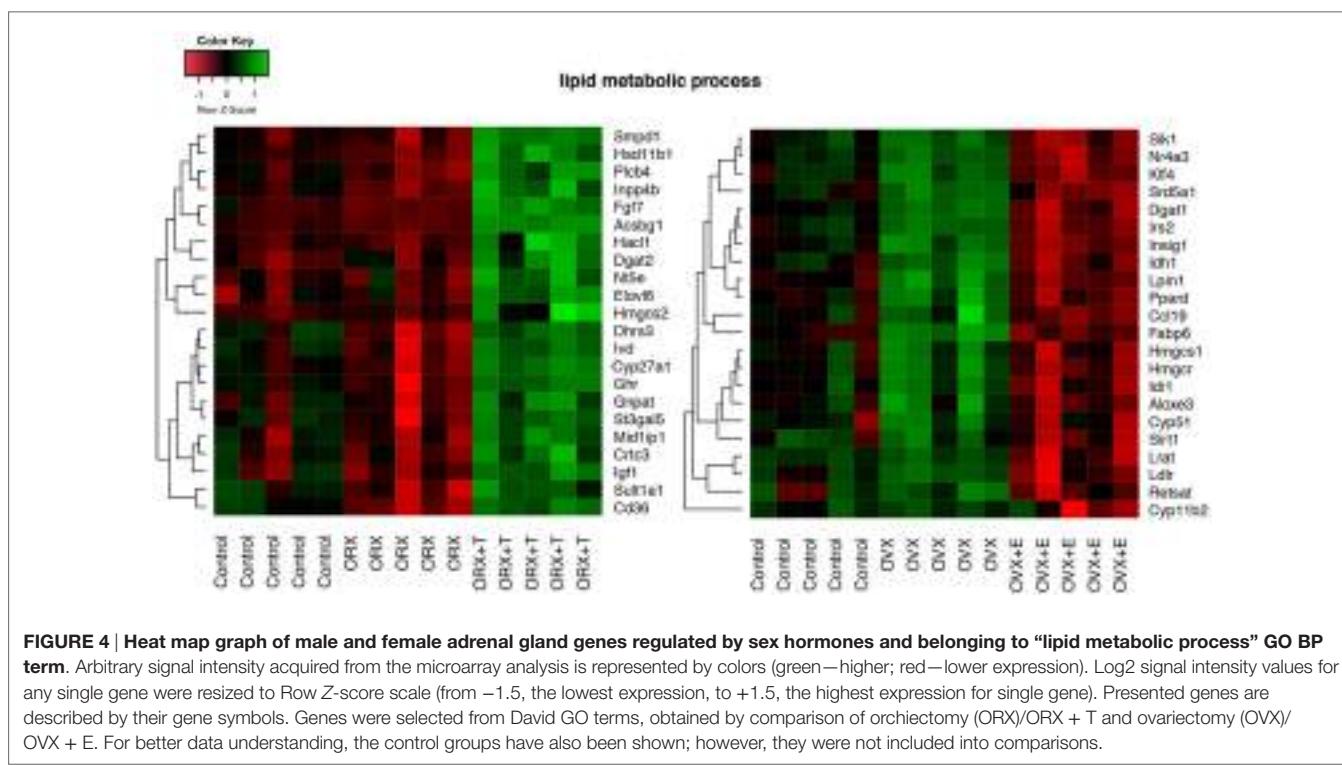
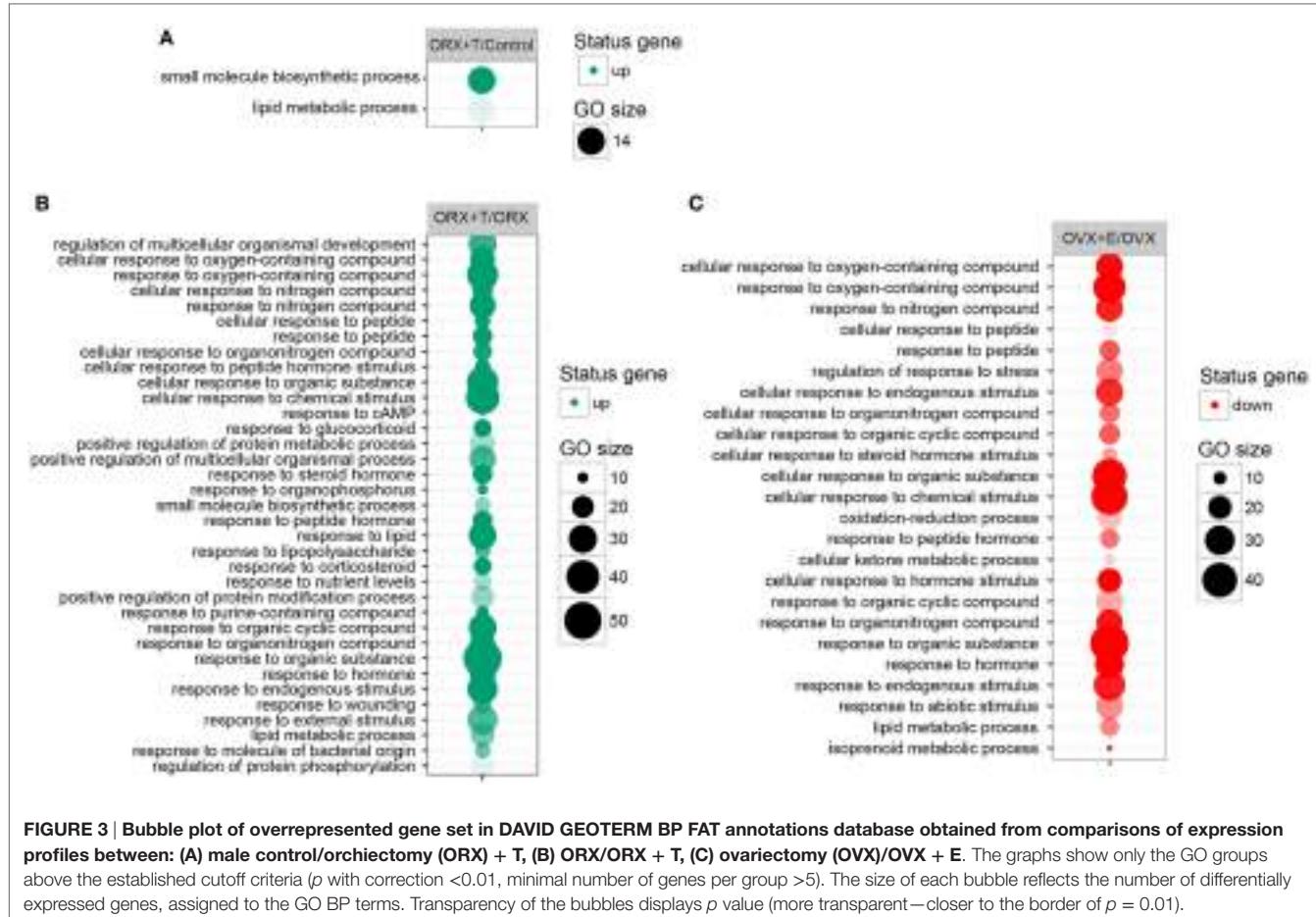
Regardless of experimental group, AR mRNA (androgen receptor) levels in rat adrenal are over 40 times higher than levels of estrogen receptors (Figure 9). As demonstrated by means of QPCR, expression levels of AR mRNA were not changed after OVX and OVX + E in relation to adrenals of control female rats. However, expression of AR was decreased after ORX and restored to control value after testosterone replacement. In both sexes, gonadectomy and sex hormone replacement did not change Esr1 and Esr2 gene expression. Expression of Esrra gene, on the

other hand, was significantly elevated after OVX and restored to control level after estrogen replacement. Expression of Esrra in male adrenals was not changed in any experimental conditions. Expression of Mc2r was not significantly regulated in any experimental conditions in female adrenals, whereas it was lowered in gonadectomized males and elevated in testosterone-replaced animals. It is interesting to note that Hmgcr gene expression in the adrenal gland of male rats is significantly higher than in females. We also revealed that expression levels of this gene were affected neither by ORX nor testosterone replacement. On the other hand, ovariectomy resulted in a notable increase in Hmgcr gene expression levels, an effect reversed by estradiol replacement (Figure 10).

## DISCUSSION

For many years, it is known that the multidirectional actions of androgen and estrogen lead to obvious sex differences in the structure and function of the adrenal cortex. As a result, in the adult female rat, adrenal cortex is larger and secretes more corticosterone than that of males (references in Section “Introduction”). It is commonly assumed that this effect depends on the stimulatory effect of estrogen on the HPA axis, while androgens have the opposite effect.

As revealed by global gene profiling method, distinct sex differences in the rat adrenal gland are accompanied by notable



GSEA result: ORX+T/ORX, enriched in ORX+T						
HALLMARK GO TERM	SIZE	ES	NES	NOM.p.val	FDR.q.val	FWER.p.val
ANDROGEN RESPONSE	98	-0.58	-2.14	0.000	0.000	0.000
FATTY ACID METABOLISM	146	-0.50	-1.97	0.000	0.001	0.002
ADIPOGENESIS	193	-0.45	-1.83	0.000	0.006	0.015
XENOBIOTIC METABOLISM	192	-0.45	-1.82	0.000	0.004	0.015
ANGIOGENESIS	36	-0.59	-1.81	0.002	0.004	0.017
ESTROGEN RESPONSE EARLY	195	-0.44	-1.78	0.000	0.004	0.020
BILE ACID METABOLISM	112	-0.47	-1.78	0.002	0.003	0.020
UV RESPONSE DN	142	-0.44	-1.74	0.000	0.005	0.030
PROTEIN SECRETION	95	-0.46	-1.68	0.000	0.008	0.053
CHOLESTEROL HOMEOSTASIS	72	-0.47	-1.68	0.008	0.007	0.054

GSEA result: OVX+E/OVX, enriched in OVX						
HALLMARK GO TERM	SIZE	ES	NES	NOM.p.val	FDR.q.val	FWER.p.val
TNFA SIGNALING VIA NFKB	200	0.72	3.00	0	0	0
CHOLESTEROL HOMEOSTASIS	72	0.76	2.72	0	0	0
MTORC1 SIGNALING	196	0.63	2.64	0	0	0
HYPOXIA	194	0.62	2.62	0	0	0
ANDROGEN RESPONSE	98	0.62	2.36	0	0	0
P53 PATHWAY	194	0.55	2.31	0	0	0
REACTIVE OXYGEN SPECIES PATHWAY	45	0.67	2.23	0	0	0
EPITHELIAL MESENCHYMAL TRANSITION	194	0.53	2.22	0	0	0
ESTROGEN RESPONSE LATE	191	0.53	2.21	0	0	0
UNFOLDED PROTEIN RESPONSE	112	0.57	2.20	0	0	0

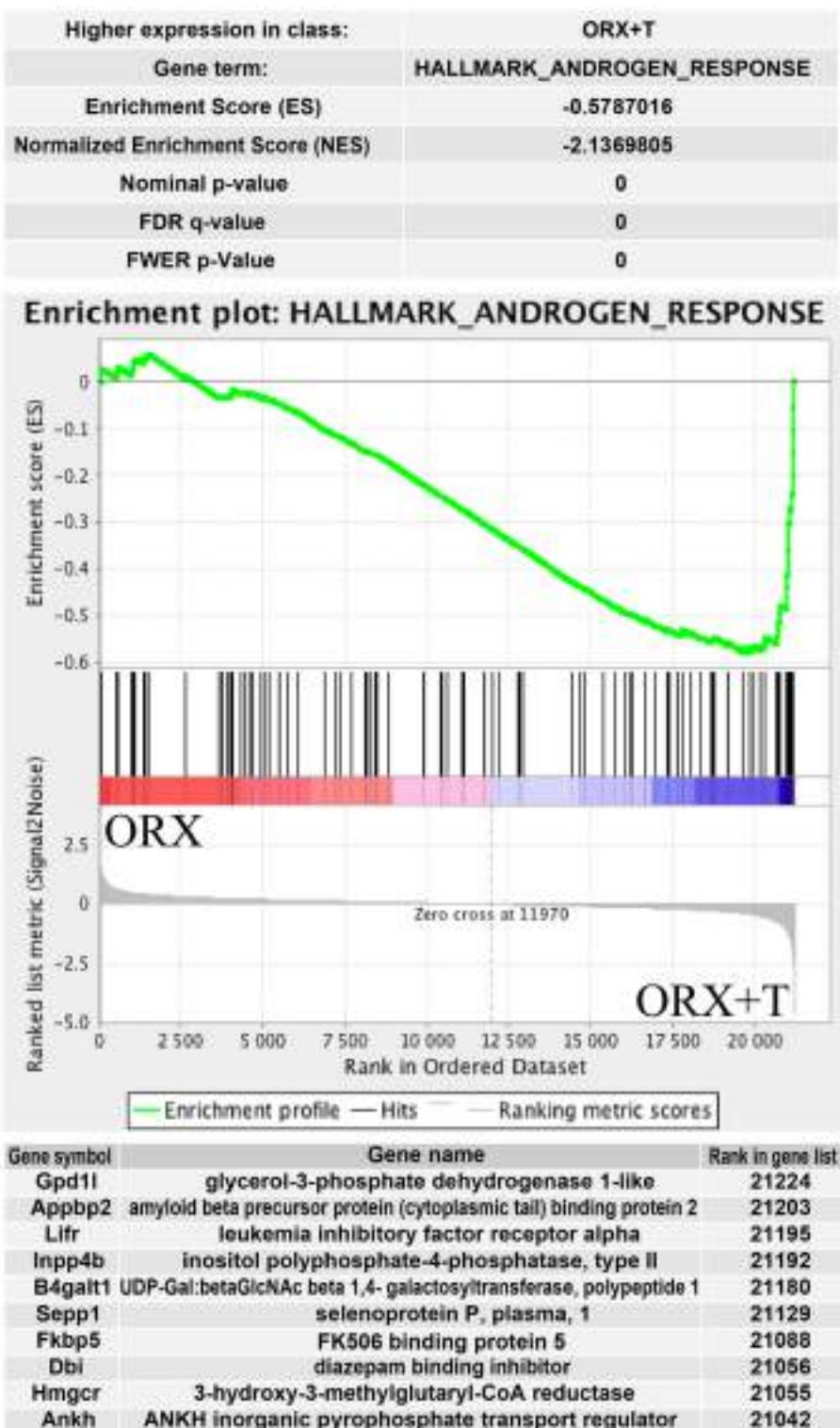
**FIGURE 5 | Ten of the highest enriched HALLMARK GO TERMS obtained from gene set enrichment analysis (GSEA) between orchectomy (ORX)/ORX + T and ovariectomy (OVX)/OVX + E groups.** Size of the category (number of genes in the “TERM”), enrichment score (ES), normalized enrichment score (NES), p value (NOM.p.val), q value of FDR correction (FDR.q.val), and p corrected by FDR (FWER.p.val) are shown.

differences in expression levels of various groups of genes (19). We demonstrated that expression levels of genes involved in corticosteroid synthesis are higher and those regulating basal cell functions lower in adrenals of adult female rats. However, the control of adrenocortical gene expression by sex hormones is totally unknown. Regarding this, only one study presents data on global gene profiling in the adrenals of female intact and testosterone-administered mice (20). However, this experimental model is not a physiological one. To get information about the regulation by testosterone and estradiol of adrenal genes in male and female rats, we used the classic “remove and replace” experiments. As evidenced by changes in uterine and seminal vesicles weights, expected, gonadectomy-induced drop in the weight of these tissues was reversed by replacement with appropriate sex hormone.

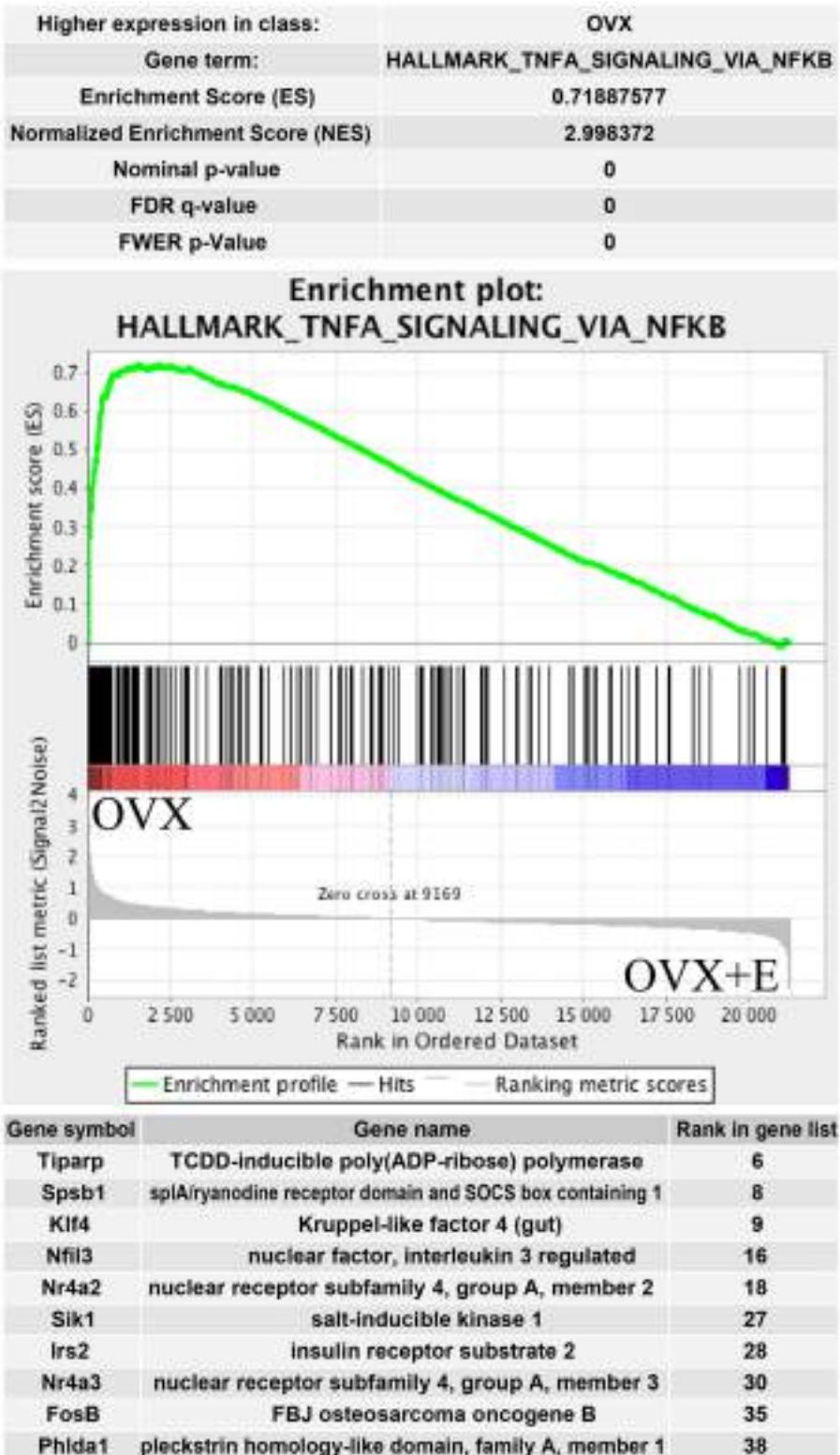
In light of the above presented discussion on sex differences in adrenals of rats, one would expect the stimulatory effect of estradiol and the opposite effect of testosterone on the glands. To our surprise, global gene profiling analyses performed on adrenals of studied rats delivered rather unexpected results. It appeared that testosterone replacement mainly upregulates expression of

adrenal genes while estradiol effect is opposite, large group of genes was downregulated. Regarding these results, the correctness of our experiments we tried to confirm by the more detailed studies of genes whose expression in a rat adrenal gland is known as dependent on testosterone or estradiol.

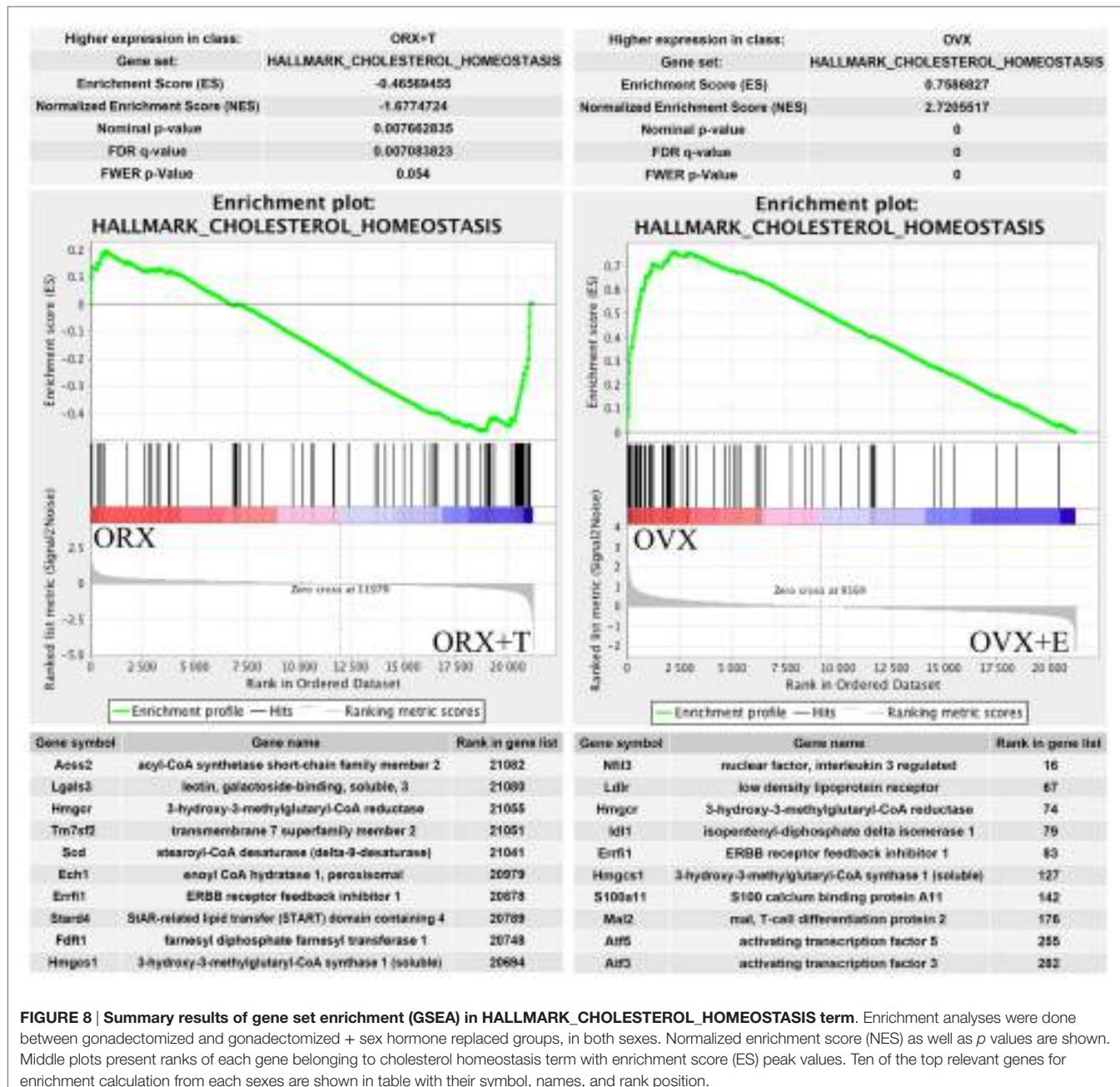
One of them is steroid-5-alpha-reductase alpha polypeptide 1 (Srd5a1). Expression of this gene and activity of the coded enzyme are notably elevated in ORX and OVX rats, changes prevented by testosterone or estradiol replacement, respectively (5, 38–43). Our microarray data are consistent with these findings. The second studied gene is orexin receptor 2 (Hcrtr2). Higher expression of Hcrtr2 in adrenals of the male rat in relation to female one as well as stimulatory effects of testosterone and inhibitory action of estradiol on this gene are well documented (19, 44–46). And again, our QPCR data are consistent with the earlier reports. Thus, obtained in the experiments performed, data on expression of specific genes in adrenals of rats of both sexes are consistent with earlier reports. In view of these, results still remains open while the global profile of gene expression regulated by sex hormones in the rat adrenal gland suggests prevalent inhibitory effect of estradiol and stimulatory effect of testosterone.



**FIGURE 6 | Summary results of gene set enrichment (GSEA) in HALLMARK\_ANDROGEN\_RESPONSE term.** Enrichment analysis was performed between orchectomy (ORX) and ORX + T groups of male adrenal glands. Normalized enrichment score (NES) as well as *p* values are shown. Middle plot presents rank of each gene belonging to androgen response term with enrichment score (ES) peak values. Ten of the top relevant genes for enrichment calculation are shown in table with their symbol, names, and rank in gene list.



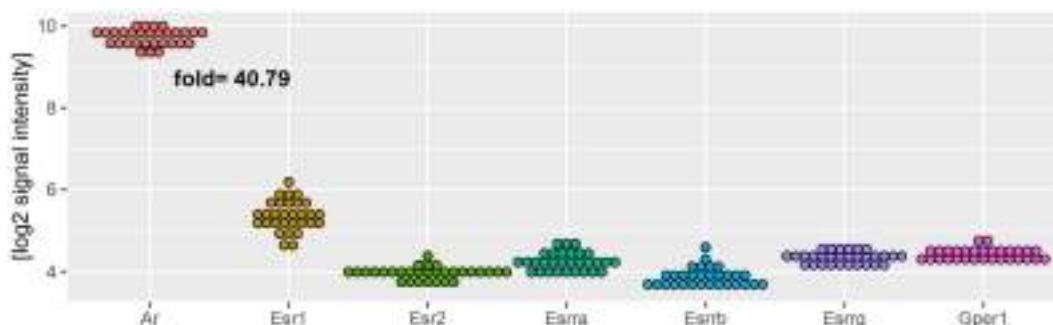
**FIGURE 7 | Summary results of gene set enrichment (GSEA) in HALLMARK\_TNFA\_SIGNALING\_VIA\_NFKB term.** Enrichment analysis was performed between ovariectomy (OVX) and OVX + E groups of female adrenal glands. Normalized enrichment score (NES) as well as *p* values are shown. Middle plot presents rank of each gene belonging to Tnfa signaling via NFKB term with enrichment score (ES) peak values. Ten of the top relevant genes for enrichment calculation are shown in table with their symbol, names, and rank in gene list.



To gain an insight into this problem, we performed numerous additional analyses of data delivered by Affymetrix microarray system. As demonstrated by Volcano plots, in adrenals of experimental rats, only small number of differentially expressed genes was found. Differentially expressed genes were further analyzed in order to get their representation in GO. This was accomplished by DAVID web-based bioinformatics tools. The highest numbers of GO terms were obtained in comparisons of ORX + T vs. ORX experimental groups (35 GO terms). In females, on the other hand, analogical comparison (OVX + E vs. OVX) delivered 24 GO terms. It is noteworthy to underline that testosterone replaced rat genes in obtained groups were

upregulated, while those in estradiol-replaced ovariectomized animals were downregulated. One of these GO terms—“lipid metabolic process”—seems to be related to adrenal physiology. This term occurs in both, testosterone- or estradiol-replaced groups; however, in androgen-replaced rats, expression levels of genes included in this group are elevated, while in estrogen supplemented animals their expression is lowered.

Our results obtained by the DAVID web-based bioinformatic tools were confirmed by GSEA. GSEA revealed that in ORX + T group (in relation to ORX) genes belonging to the Hallmark GO terms “androgen response,” “fatty acid metabolism,” “adipogenesis,” and “cholesterol homeostasis” are notably enriched.



**FIGURE 9 | Expression of androgen (Ar) and estrogen (Esr1, Esr2, Esrra, Esrrb, Esrrg, Gper) receptors in adrenal glands of studied rats (male and female control, gonadectomized and gonadal hormone replaced rats).** Data obtained from Affymetrix® Rat Gene 2.1 ST Array. From microarray data sets, expression values of analyzed genes were extracted. Each dot on graph corresponds to single log<sub>2</sub>-transformed value of signal intensity from individual sample. Fold expression change was calculated from mean of Ar receptor gene and mean of all estrogen receptors.

Moreover, in OVX group (in relation to OVX + E), enriched genes are present, among others, within the following terms: “TNFA signaling via NFKB,” “cholesterol homeostasis,” and “androgen response.” These data suggest that in adrenals of gonadectomized rats both, testosterone or estradiol replacement, potently affects genes involved in lipid and cholesterol metabolism, as well as intracellular pathways responsible for androgen response. Further GSEA analysis revealed that within the hallmark term “cholesterol homeostasis,” in both, orchietomized testosterone- and ovariectomized estradiol-replaced rats, among 10 genes that strongly affect ES Hmgcr is present.

There is some evidence that total lipid and cholesterol concentrations and contents in the rat adrenal gland are influenced by gonadal hormones. Lowered concentration of total lipid in adrenals of gonadectomized rats of both sexes was reported by Coleman et al. (47). Higher total lipid content, but not total lipid concentration in adrenals of female than male rats, has been reported by other group (48, 49). These studies demonstrated also higher concentrations and contents of total lipid in adrenals of orchietomized testosterone replaced rats, while an opposite effects were observed in ovariectomized rats replaced with estradiol.

Although cholesterol is not a classical lipid, its physiological role in the adrenal cortex is often described in the aspects of the role of lipids. In contrast to lipids, numerous data are available on cholesterol and cholesterol esters in adrenals of intact male and female rats and on impact on them of sex hormones.

HMG-CoA reductase is the rate-limiting enzyme in cholesterol biosynthesis (50, 51). Administration of 17 $\alpha$ -ethynodiol estradiol into aging male rats resulted in a notable stimulation of enzyme activity (52). Our results indicate that testosterone has no effect on the expression of Hmgcr gene; however, ovariectomy increases its expression, an effect reversed by estradiol replacement. Regarding these changes, it should be emphasized that HMG-CoA reductase is a highly regulated gene, among others by serum lipoprotein levels and intracellular cholesterol content (50, 53). Thus, it seems legitimate to suggest that observed in the present study changes in Hmgcr expression may be mediated by both, serum lipoprotein levels and adrenal cholesterol content.

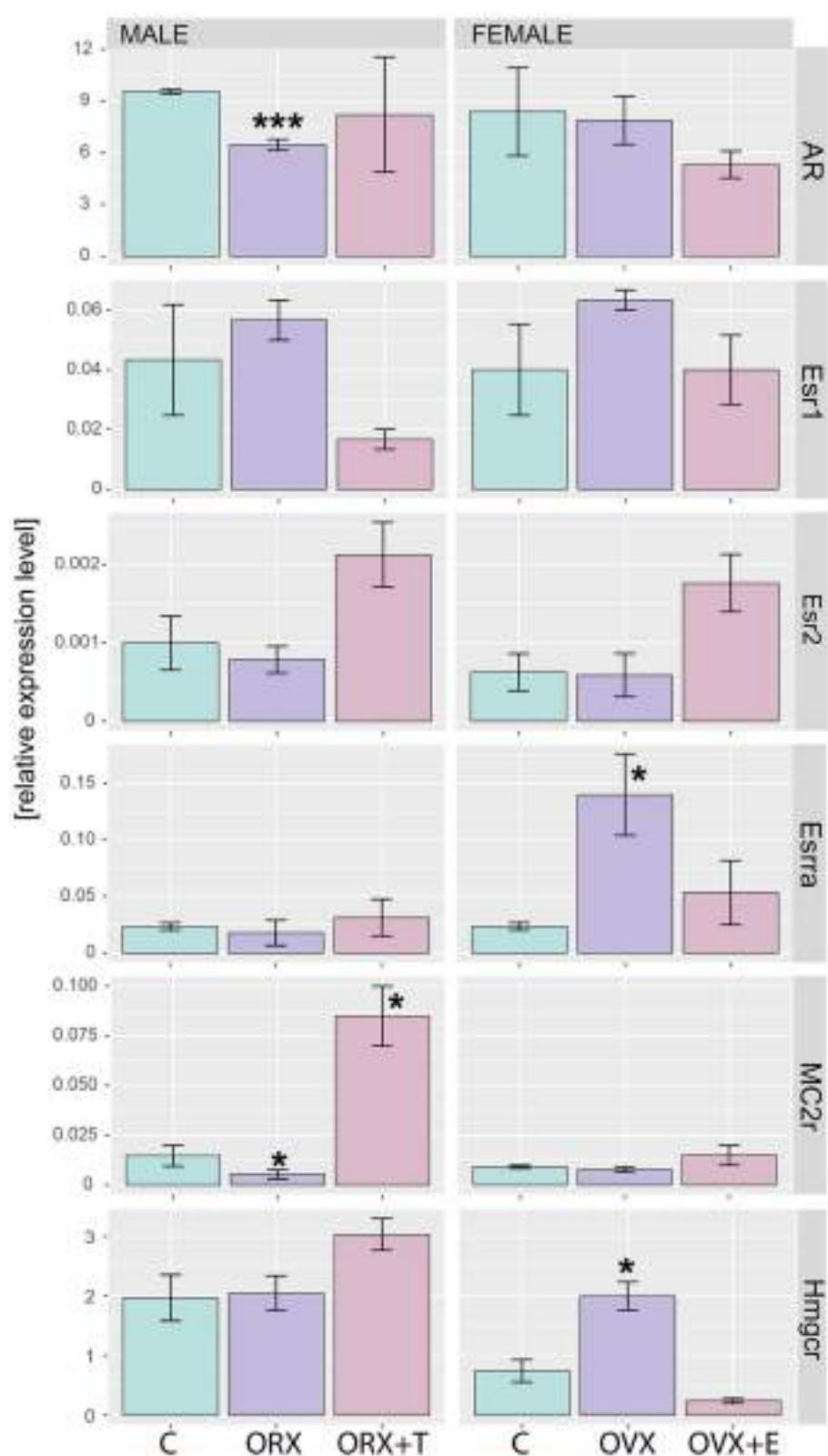
This assumption is supported by parallel changes in adrenal HMG-CoA reductase gene expression and serum HDL concentrations in studied female rats.

According to data reviewed in the earlier monographs, the majority of publications indicate a higher concentration of cholesterol (both, free or esterified) in the adrenal glands of adult female rats (5, 49). ORX increased cholesterol concentration in the gland, an effect reversed by testosterone replacement. Ovariectomy, on the other hand, either did not change or lowered the total cholesterol concentration in the rat adrenal gland. There is general agreement, on the other hand, that estrogens lower cholesterol content in adrenals of ovariectomized rats.

Free cholesterol, required for steroid hormone synthesis, is liberated from cholesterol esters by the action of ACTH-sensitive sterol ester hydrolase. The specific activity of this enzyme in adrenal homogenates is notably higher in male than female glands (54). Both, ORX and testosterone replacement had any effect on adrenal sterol ester hydrolase. Ovariectomy, on the other hand, resulted in an increase in activity of the enzyme, an effect reversed by estradiol replacement.

Thus, earlier data on the effects of testosterone and estradiol on adrenal lipids and enzymes involved in cholesterol metabolism in the rat are rather consistent with the transcriptome profile obtained in the respective experimental groups.

Also genes forming the hallmark GO term “androgen response” in rat adrenals are highly regulated by both, testosterone or estradiol replacement. In an aspect of this term in the present study, we analyzed the expression of the androgen receptor. Regardless of experimental group, AR mRNA levels in rat adrenal are over 40 times higher than levels of estrogen receptors, differences observed also by others (27, 55–59). We observed that expression of AR was decreased after ORX and restored to control value after testosterone replacement while no changes were observed after OVX and OVX + E. Our results are also consistent with the data on the sheep (60). Thus, our data did not confirm earlier reports on the effects of gonadectomy and sex hormone replacement on the levels of androgen- or estrogen-binding sites in the rat adrenals (56–58). Of estrogen receptor isoforms studied, only Esrra (estrogen



**FIGURE 10 | QPCR assay of AR, Esr1, Esr2, Esrra, MC2r, and Hmgcr genes in adrenals of adult male and female control, gonadectomized and gonadal hormone replaced rats.** Bars represent mean  $\pm$  SEM ( $n = 3$ ). All samples were amplified in triplicate, and HPRT gene was used as reference for data normalization. Statistically significant differences in relation to control group:  $*p < 0.05$ ;  $**p < 0.01$ ;  $***p < 0.001$ .

related receptor alpha, also known as NR3B1) is overexpressed in ovariectomized rats and restored to normal level by estradiol replacement.

In adrenals of female rats, hallmark GO term “TNFA signaling via NFKB” is the first term delivered by GSEA, with the size 200 (number of genes). Such a huge enrichment is observed in OVX rats, in relation to OVX estradiol-replaced rats. TNFA is a proinflammatory cytokine and TNF-induced NFKB activation induces transcription and expression of genes involved in various biological processes, including regulation of cell proliferation, differentiation, apoptosis, and immune response. The final steps of these signaling pathways are mediated through activation of nuclear transcription factors (61–63). Also other intracellular signaling pathways are affected by estradiol replacement, they predominantly downregulate respective genes. These findings may suggest inhibition of expression of numerous transcription factor genes in OVX + E female rats. Mechanism of such actions remains to be solved.

It is well documented that androgen and estrogen receptors are ubiquitously expressed in almost all tissues and organs. Therefore, *in vivo* experiments do not allow for precise identification of their action on the adrenal glands. Sex hormones exert a multidirectional effect on the function of the HPA axis, affecting primarily CRH and ACTH synthesis and/or secretion [for review, see Ref. (3–6, 64)]. Most of the experimental data based on gonadectomy and sex hormone replacement indicate that in the rat estrogens stimulate and androgens inhibit the HPA axis. However, sex hormones may also exert direct effects on the rat adrenal cortex. These effects may be mediated either via specific receptors, both nuclear and membrane (for example GPR30) or by interference with ACTH-sensitive intracellular pathways, particularly those associated with steroidogenesis (65–69). Important in the physiological control of HPA axis is also liver. This organ is critical for the regulation of CBG and lipoproteins and metabolizes circulating corticosteroids. As known, all these functions are regulated by sex hormones.

As demonstrated by means of QPCR, expression of Mc2r was not significantly regulated in any experimental conditions in female adrenals, whereas it was lowered in gonadectomized males and elevated in testosterone-replaced animals. In terms of gender differences, very contradictory data relate to gender differences in the content and secretion of ACTH by the rat pituitary gland and corticotrophin levels in blood serum are available [for review, see Malendowicz (5)]. The major part of these studies show a higher level of ACTH in the blood serum of rats treated with estrogen. Only scanty data, on the other hand, are available on expression of ACTH receptors in adrenals of male and female rats. Yoshimura et al. (70) have shown that expression levels of ACTH receptor mRNA in adrenals increased with age in the female rat, but not in the male. In these studies, ORX elevated the level of ACTH receptor, an effect reversed by testosterone replacement. In contrast, our experiments demonstrated that ORX resulted in lowering of Mc2r gene expression and testosterone replacement notable increased expression of this gene. It is difficult to explain these discrepancies, most likely they are the result of different experimental models and methods applied for determining the expression of a discussed gene [male rats aged 14 days (period

of temporal decrease of adrenal ACTH responsiveness) vs. adult animals; size and quality of applied primers]. Our results, however, are consistent with the data on the sheep. In this species, estradiol did not change adrenal Mc2r expression, while higher Mc2r expression was found in testosterone-treated rams (60).

In view of the above literature data for stimulating effects of estradiol and inhibitory of testosterone on the growth and function of the adrenal cortex of rat, obtained results of the analysis of transcriptome of sex hormone-replaced rats are surprising. Contrary to what we expected to find, it appeared that testosterone replacement resulted in a potent stimulation of expression of numerous adrenal genes, especially those involved in lipids and cholesterol metabolism. On the other hand, estradiol replacement resulted in a strong downregulation of numerous genes, especially those involved in regulation of TNFA signaling via NFKB pathways and on transcription factors synthesis. Obtained results may suggest that estradiol inhibits the expression of corepressor genes in the rat adrenal gland. It seems possible that this mechanism of action may lead to an increase in both the adrenal gland growth and secretory function. The physiological relevance of these findings await further research.

## ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the “Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes,” as stated in the Polish law “Ustawa z dnia 15 stycznia 2015 r. o ochronie zwierząt wykorzystywanych do celów naukowych lub edukacyjnych.” The protocol was approved by the Local Ethics Committee for Animal Research (Poznan, Poland) (in Polish: Lokalna Komisja Etyczna ds. Doświadczeń na zwierzętach w Poznaniu), permission number: LKE—11/2015.

## AUTHOR CONTRIBUTIONS

KJ invented and designed the experiments, performed the experiments, and performed microarrays; PC performed the experiments, QPCR; MS performed the experiments and performed QPCR; MT performed the experiments and performed QPCR; PM performed the experiments and performed QPCR; LM invented and designed the experiments, analyzed the data, and prepared the manuscript; MR invented and designed the experiments, analyzed the data, prepared graphical presentation, and prepared the manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at <http://journal.frontiersin.org/article/10.3389/fendo.2017.00026/full#supplementary-material>.

**FIGURE S1 | Serum corticosterone concentrations in adult male and female control, gonadectomized and gonadal hormone replaced rats.** Bars represent mean  $\pm$  SEM. Each circle represents an individual value. Statistically significant differences in relation to control or gonadectomized group: \* $p < 0.05$ ; \*\* $p < 0.02$ ; \*\*\* $p < 0.01$ ; \*\*\*\* $p < 0.001$ .

**FIGURE S2 | Serum total cholesterol, LDL, HDL, and triglyceride concentrations in adult male and female control, gonadectomized and**

**gonadal hormone-replaced rats.** Bars represent mean  $\pm$  SEM. Each circle represents an individual value. Statistically significant differences in relation to control or gonadectomized group: \* $p < 0.05$ ; \*\* $p < 0.02$ ; \*\*\* $p < 0.01$ ; \*\*\*\* $p < 0.001$ .

**TABLE S1 | List off all regulated genes in adrenals of adult male and female control, gonadectomized and gonadal hormone replaced rats.** Their fold change,  $p$  values, and their localization to chromosomes.

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# Circadian Clocks, Stress, and Immunity

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In mammals, molecular circadian clocks are present in most cells of the body, and this circadian network plays an important role in synchronizing physiological processes and behaviors to the appropriate time of day. The hypothalamic–pituitary–adrenal endocrine axis regulates the response to acute and chronic stress, acting through its final effectors – glucocorticoids – released from the adrenal cortex. Glucocorticoid secretion, characterized by its circadian rhythm, has an important role in synchronizing peripheral clocks and rhythms downstream of the master circadian pacemaker in the suprachiasmatic nucleus. Finally, glucocorticoids are powerfully anti-inflammatory, and recent work has implicated the circadian clock in various aspects and cells of the immune system, suggesting a tight interplay of stress and circadian systems in the regulation of immunity. This mini-review summarizes our current understanding of the role of the circadian clock network in both the HPA axis and the immune system, and discusses their interactions.

**Keywords:** circadian clock, HPA axis, immune system, glucocorticoids, stress

## INTRODUCTION

Life on Earth has evolved in the context of a rhythmic environment, characterized largely by the regular succession of night and day. This has led to the evolution of intrinsic circadian (from Latin *circa diem* – about the day) clock systems, in order to optimally time physiological and behavioral processes. Disruption of circadian timing, such as with inter-time zone travel, shift work, and mistimed eating, can have consequences for cardiovascular, metabolic, and mental health and, crucially, immune function. The hypothalamic–pituitary–adrenal (HPA) axis and the immune system show extensive crosstalk, in particular with regard to the strong anti-inflammatory effects of glucocorticoids (cortisol in humans and corticosterone in rodents). However, less well studied is the interaction of the HPA axis and immune system with regard to the circadian clock. This mini-review will summarize current knowledge regarding the role of the circadian clock in each of these systems, and the interactions that can occur in the context of disrupted circadian rhythmicity.

## THE CIRCADIAN CLOCK

Circadian rhythms are synchronized to external time by cues known as zeitgebers (German for time givers), such as light and food. In mammals, the clock system is organized in a hierarchical manner, with a master pacemaker residing in the hypothalamic suprachiasmatic nuclei (SCN), acting to synchronize peripheral clocks in all other tissues *via* endocrine and autonomic signals (1). At the cellular level, circadian clocks coordinate gene expression programs to control physiological processes over the course of the day. The primary zeitgeber for the SCN is light. From the eye, signals are relayed *via* the retinohypothalamic tract to the SCN, which in turn coordinates peripheral

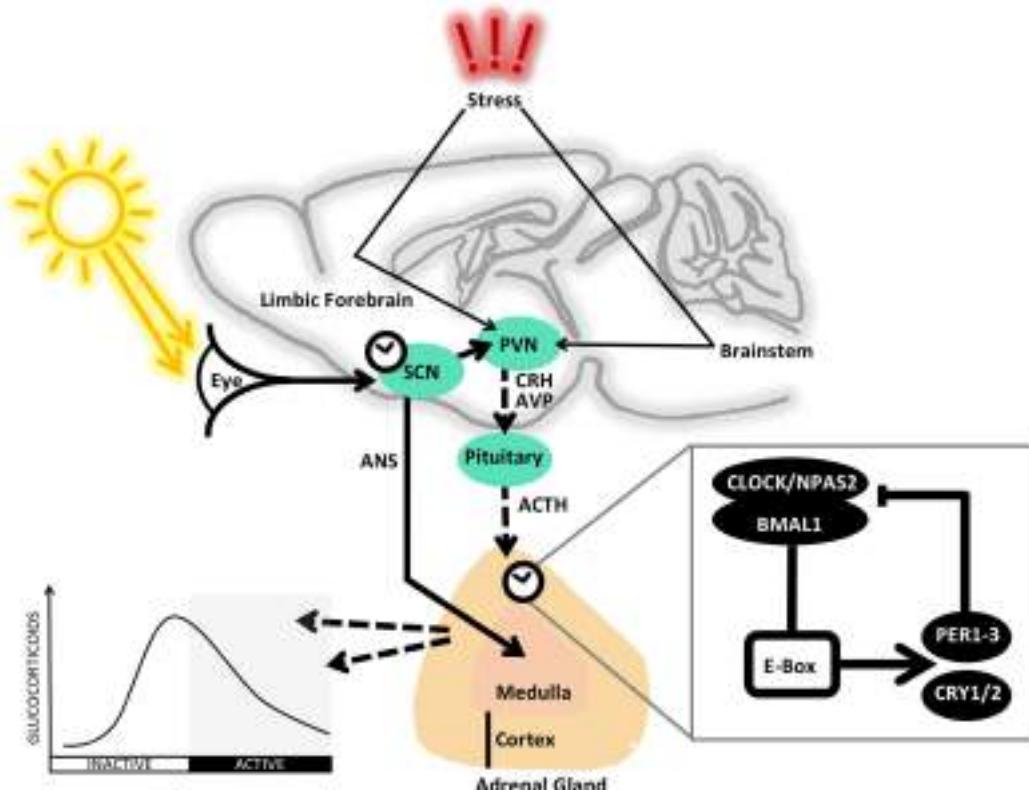
tissue clocks. Other zeitgebers can influence circadian rhythms, with mistimed feeding in particular being able to reset peripheral clocks independent from the SCN (2, 3).

The molecular circadian clock consists of interlocked transcriptional-translational feedback loops [TTLs; discussed in detail elsewhere (4, 5)]. Briefly, during the day, the transcription factors CLOCK (circadian locomotor output cycles kaput) or NPAS2 (neuronal PAS domain-containing protein 2) in complex with BMAL1 (brain and muscle aryl hydrocarbon receptor nuclear translocator-like 1) bind to *E-box* (enhancer box) promoter elements to drive expression of *Period* (*Per1-3*) and *Cryptochromes* (*Cry1/2*), along with other clock controlled genes (inset in Figure 1). PER/CRY protein complexes accumulate in the cytoplasm over the day and later relocate into the nucleus where they inhibit the activity of the CLOCK–BMAL1 (or NPAS2–BMAL1) complex. This shuts down *Per/Cry* transcription during the night. After degradation of nuclear PER/CRY complexes toward the next morning, the inhibition of CLOCK–BMAL1 is released and a new cycle begins.

## CIRCADIAN AND STRESS REGULATION OF THE HPA AXIS

The HPA axis is a key in the regulation of stress responses, with glucocorticoids mediating intermediate and chronic adaptation to stressful stimuli, complementing the rapid response of catecholamines, both secreted from the adrenal gland. The rhythmic regulation of catecholamines and other adrenal hormones is discussed elsewhere (6). Rhythmic regulation of glucocorticoid release (Figure 1) allows for anticipation of daily timing of energy-demanding situations. In addition, glucocorticoid rhythms play a key role in the systemic coordination of circadian rhythms by resetting cellular clocks downstream of the SCN.

During stress, the brainstem and limbic forebrain stimulate corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP) secretion from neurosecretory neurons of the paraventricular nucleus of the hypothalamus (PVN) (7). Via the hypophyseal portal system, these reach anterior pituitary corticotrophs, which secrete adrenocorticotrophic hormone (ACTH).



**FIGURE 1 | The rhythmic control of the HPA axis is regulated at several levels.** The master clock residing in the suprachiasmatic nucleus (SCN) is synchronized by light information received via the retinohypothalamic tract from the eye in order to exert autonomic (ANS) and hormonal influence on the clocks and rhythms of downstream tissues of the body. In addition to the direct innervation of the adrenal, the SCN influences the paraventricular nucleus (PVN) to secrete corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP), which reach the pituitary via the blood portal system to stimulate secretion of adrenocorticotrophic hormone (ACTH), which activates production and release of glucocorticoids. In addition, local adrenal clocks are thought to regulate responsiveness to ACTH in a circadian fashion. The baseline circadian rhythm of circulating glucocorticoids peaks just before the beginning of the active phase (day in humans and night in rodents). Stress-induced stimulation of the HPA axis acts via afferent signals from the limbic forebrain and brainstem to the PVN. Inset: the core transcriptional-translational feedback loop (TTL) that makes up the molecular circadian clockwork. In the positive arm of the clock, CLOCK or NPAS2 form a complex with BMAL1 and bind to *E-Box* elements in the gene promoters of PERs and CRYs, which make up the negative arm and act to inhibit the activity of CLOCK–BMAL1 or NPAS2–BMAL1, with a cycle of roughly 24 h. For further detail, see the main text.

ACTH then acts at melanocortin type-2 receptors (MC2R) in the adrenal cortex to stimulate production and release of glucocorticoids. Negative feedback from glucocorticoids acts at the level of CRH in the hypothalamus and ACTH in the pituitary (8, 9). In addition, PVN CRH expression is indirectly controlled by the SCN (8–11).

Glucocorticoids bind to glucocorticoid (GR) and mineralocorticoid receptors (MR) in target tissues. Unlike the MR, which is almost constantly activated by glucocorticoids, GR – widely expressed in the brain and periphery, but not in the SCN (12) – activation occurs only during glucocorticoid peak levels (13). This means that GR activation may occur during the peak of the circadian rhythm even during the ultradian trough, but ultradian peaks during the circadian nadir may not be sufficient for activation (14, 15). GR binds to glucocorticoid response elements (GRE or nGRE) to regulate transcription of target genes (16). GRE are present in the promoter region of the clock genes *Per1*, *Per2*, *Npas2*, and various clock controlled genes involved in the synchronization of peripheral circadian rhythms (17).

Glucocorticoid circadian rhythms peak slightly before the onset of the active phase, which is during the night for most rodent species and during the day for humans (18). This rhythm overlays a more dynamic ultradian pattern for both ACTH and glucocorticoid secretion (19) driven by feedback between glucocorticoids and ACTH release at the pituitary (20) and intra-adrenal feedback of glucocorticoids (21). The presence of GR in the adrenal cortex (22, 23) and the demonstrated inhibitory effects of exogenous corticosterone on the ACTH-stimulated corticosteroid synthesis could potentially play a role in local regulation of glucocorticoid secretion in the adrenal gland. Circadian glucocorticoid rhythms can persist independent of the SCN (24–26). Given the influence that circadian rhythmicity of glucocorticoids may have on peripheral clock function, it is perhaps not surprising that the HPA axis, which is acutely activated in stressful situations, is unlikely to be the main driver of the circadian rhythm of these hormones. Indeed, there are several components of the HPA axis which, although they express circadian rhythmicity, do not synchronize well enough to explain downstream endocrine rhythms (3, 10, 27). The circadian influence of the SCN on the HPA axis also occurs through autonomic innervation of the adrenal gland (28, 29), with SCN-dependent rapid induction of *Per1* expression being stimulated in the adrenal gland following a light pulse (30). In line with this, splanchnic nerve transection results in dampened circadian glucocorticoid rhythm in rats (31, 32).

## LOCAL REGULATION OF GLUCOCORTICOID RHYTHMS

A circadian rhythm of steroid release was first demonstrated in isolated Syrian hamster adrenals [*Mesocricetus auratus*, See Ref. (33)], and rhythmic glucocorticoid concentrations persist under constant peripheral CRH infusion in CRH knockout mice (34), or in hamsters with natural loss of ACTH rhythm (35). Adrenal circadian rhythms can however be altered by ACTH, which stimulates *PER1* and *BMAL1* expression *ex vivo* in human tissue (36) and

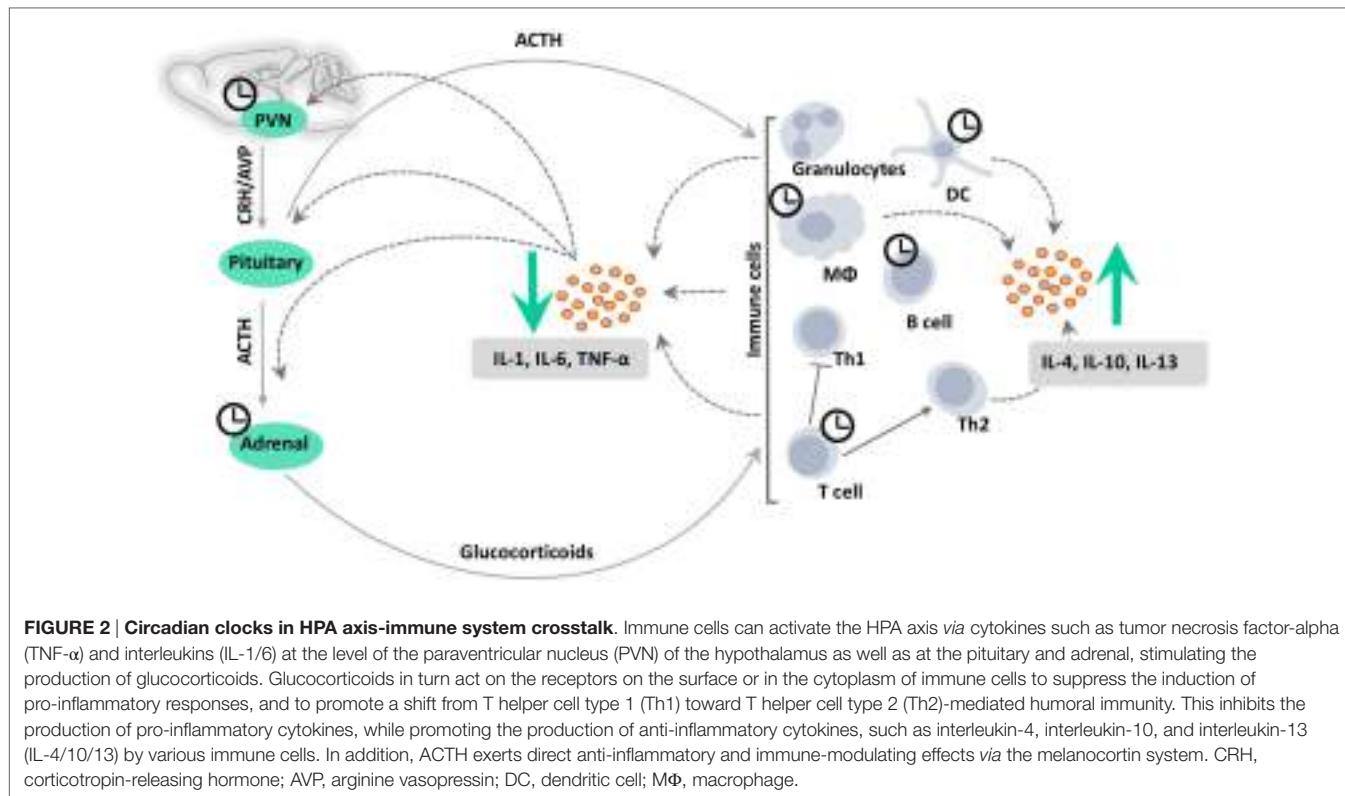
shifts clock rhythms in isolated adrenals of *Per2:LUC* reporter mice (37).

Circadian clocks within the adrenal gland also play a role in the rhythmic regulation of the HPA axis, both in glucocorticoid production and in sensitivity to ACTH. Robust clock gene expression rhythms have been demonstrated in the adrenal cortex of rodents and primates (38–43), and several steroidogenic genes show circadian expression (31, 40, 44). Work in transgenic mice has shown that those lacking genes of the positive arm of the TTL produce lower levels of corticosterone (45, 46), while those with mutations in the negative arm are chronological hypersecretors (47, 48). Evidence for the importance of adrenocortical clocks in regulating ACTH sensitivity comes from isolated adrenal tissue responses to ACTH, which differs across the day and is very low in mice deficient for *Per2* and *Cry1* (41) or *Bmal1* (46). This is further supported by evidence from primate adrenal explant studies, where knockdown of *Cry2* and subsequent downregulation of *Bmal1* lead to attenuated ACTH responses (49). Together, these studies suggest that the local adrenal clock is important for regulating the circadian glucocorticoid rhythm independent of systemic influences such as during stress, and may explain the high amplitude of glucocorticoid rhythm in the face of comparably low variations in ACTH concentrations.

## HPA AXIS INTERACTION WITH THE IMMUNE SYSTEM

A bidirectional communication exists between the HPA axis and the immune system (Figure 2). It is well understood that immune cells can activate the HPA axis *via* cytokines such as tumor necrosis factor-alpha (TNF- $\alpha$ ), interleukins (IL-1, IL-6), and the type-I interferons (IFNs) (50–53). Interestingly, some cytokines can activate the HPA axis *via* different mechanisms. Although primarily acting on the PVN to stimulate CRH release (54–56), they also have direct action at the level of the pituitary and adrenal (57, 58).

At the same time, glucocorticoids can affect viability and function of many immune cell types, including T cells, B cells, monocytes, macrophages, and granulocytes (59, 60). Glucocorticoids suppress the synthesis and release of cytokines, thereby protecting the host organism from the detrimental consequences of a long-term hyperactivity of the immune system [reviewed in Ref. (61)]. Pioneering work by Hench, Kendall, and Reichstein demonstrated the immunosuppressive actions of glucocorticoids almost 70 years ago (62). Nevertheless, glucocorticoids are still the most widely used and most effective treatment to control allergic, autoimmune, inflammatory, and hematological disorders (63). GR are found in almost all types of immune cells, and upon activation tether and trans-represses pro-inflammatory regulators such as nuclear factor kappa-light-chain-enhancer of activated B-cells (NF- $\kappa$ B) and activator protein 1 (AP-1) (61, 64) by activating anti-inflammatory molecules such as glucocorticoid-induced-leucine zipper [GILZ (65)], MAPK phosphatase-1 [MKP-1 (66)], annexin-1 (67), mitogen-inducible gene-6 [Mig-6 (68)], and SRC-like adaptor protein 1 [SLAP (69)].



In contrast to the well-described immunosuppressive effects recent studies indicate that glucocorticoids can also have permissive or even stimulatory effects on immune processes [reviewed in Ref. (70–72)]. It is thought that acute stress enhances, while chronic stress suppresses the peripheral immune response (70), but the mechanism of this dual role is not well understood. Several studies report that glucocorticoids induce expression of innate immune-related genes, including members of the toll-like receptor (TLR) family, such as TLR2 and TLR4 (73–75). Glucocorticoids also rapidly induce a central component of the inflammasome, NLRP3, in macrophages, which stimulates secretion of pro-inflammatory cytokines (76).

In addition, glucocorticoids regulate adaptive immune responses by influencing cell trafficking to the sites of inflammation and by suppressing T helper cell type 1 (Th1) and enhancing T helper cell type 2 (Th2) cytokine-driven responses (77, 78). Consequently, in contrast to the traditional view of glucocorticoids as generally immunosuppressive hormones, glucocorticoids are now more accurately regarded as immune modulators.

As an alternative to the glucocorticoid treatment of chronic inflammatory diseases such as multiple sclerosis, the use of ACTH has recently been re-employed, appearing to act not only indirectly by stimulating glucocorticoid production but also by a direct anti-inflammatory effect *via* the melanocortin system [reviewed in Ref. (79–81)]. Melanocortin receptors (MCRs) are found on lymphocytes and macrophages (82–84). Anti-inflammatory effects of ACTH are mediated primarily by MC1R and MC3R, while immune-regulatory effects rely on MC5R (80). Such glucocorticoid-independent effects of ACTH have

been demonstrated after lipo-polysaccharide (LPS)-stimulated production of IL-1 $\beta$  and TNF- $\alpha$  in human blood samples (85), in a rat gout model (86), and in TNF- $\alpha$ -induced acute kidney disease in rats (87). Furthermore, ACTH can reduce neutrophil infiltration *via* MC3R (88).

## CIRCADIAN INTERACTION OF THE HPA AXIS WITH IMMUNE FUNCTION

In mammals, the circadian clock is an important regulator of the immune system, allowing the organism to anticipate daily changes in activity and the associated risk of antigen encounter. Circadian rhythms are found in multiple aspects of immune function, such as recruitment of immune cells to tissues, antigen presentation, lymphocyte proliferation, TLR function, and cytokine gene expression (89, 90). Furthermore, several inflammatory diseases, such as bronchial asthma and rheumatoid arthritis vary in severity over the course of the day, implicating a circadian regulation of vulnerability (91, 92). Animal studies have revealed that circadian rhythm disruption by shift work or chronic jet lag leads to a dysregulation of the immune system and a higher risk for several pathologies (93, 94).

Molecular clocks have been characterized in various immune cells, including macrophages, dendritic cells, and T and B lymphocytes (95–97). In humans, under constant routine conditions, administration of exogenous glucocorticoids 10 h after awakening can entrain circadian rhythms in peripheral blood mononuclear cells (PBMCs) without changing plasma melatonin and

cortisol rhythms, thus linking HPA axis regulation to immune cell function. In line with this, oral administration of synthetic hydrocortisone shifts the expression of *BMAL1* and *PER2/3* in PBMCs by 9.5–11.5 h (98).

Stress-induced alterations of HPA axis rhythmicity can lead to wide-spread alterations in innate and adaptive immune responses and contribute to the development and progression of some types of cancer in animals (99, 100). In humans, data are less clear, with a positive association between stress and breast cancer observed in some (101), but not in other studies (102). In another context, stress-induced inflammatory priming of microglia was influenced by time of day in rats. Animals exposed to stress during the rest phase showed enhanced neuroinflammatory responses to an LPS challenge compared with animals experiencing stress during the active phase (103). Whether these effects involve circadian alterations remains to be shown. Of interest in this context, pulmonary antibacterial responses in mice appear to be gated by circadian clocks residing in the epithelial club cells lining the pulmonary airways, entrained by glucocorticoids (104).

Not only does the circadian clock regulate the immune system but immune status also feeds back on circadian rhythms. For example, administration of LPS resets activity rhythms in mice (105), and transiently suppresses *Per2* and *Dbp* expression in the SCN and liver of rats (106). LPS treatment increases AVP release from SCN explants (107), and TNF- $\alpha$  treatment downregulates SCN *Dbp* expression and causes prolonged rest periods during the active phase in rodents (108).

Recent studies reveal blunted circadian cortisol rhythms and attenuated stress responses in patients with allergic diseases, such as bronchial asthma, allergic rhinitis, atopic dermatitis, and extensive nasal polyposis (109–112). In patients with sepsis hypercortisolism persists despite low ACTH levels, suggesting that non-ACTH-mediated mechanisms are involved in the maintenance of high glucocorticoid levels. Interestingly, circadian

rhythm of both ACTH and cortisol secretion were shown to be blunted in these patients (113). Furthermore, neonatal endotoxin exposure reprograms HPA axis development in rats, leading to ACTH and corticosterone hyper-responsiveness later in life (114).

## CONCLUSION

Cellular circadian clocks in central and peripheral tissues interact to regulate the activity of the main endocrine axes. Our improved understanding about the systemic regulation of HPA axis circadian rhythms and the interplay of clock and stress functions in this context may help to optimize current treatment strategies for many immunological disorders. For example, in chronic diseases such as rheumatoid arthritis, timed administration of exogenous glucocorticoids at specific times of day may improve therapeutic effectiveness and reduce negative side effects, since lower doses are required (115). At the same time, the importance of glucocorticoids in the coordination of the circadian timing system itself has so far been largely neglected in clinical settings. Stabilizing circadian HPA axis regulation may protect against adverse external influences such as stress and infection, thus protecting the body against some of the most frequent threats of the 24/7 globalized society.

## AUTHOR CONTRIBUTIONS

RD, OM, and HO discussed the concept, compiled the literature, and wrote the paper.

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# Phase-Dependent Shifting of the Adrenal Clock by Acute Stress-Induced ACTH

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The adrenal cortex has a molecular clock that generates circadian rhythms in glucocorticoid production, yet it is unclear how the clock responds to acute stress. We hypothesized that stress-induced ACTH provides a signal that phase shifts the adrenal clock. To assess whether acute stress phase shifts the adrenal clock *in vivo* in a phase-dependent manner, mPER2:LUC mice on a 12:12-h light:dark cycle underwent restraint stress for 15 min or no stress at zeitgeber time (ZT) 2 (early subjective day) or at ZT16 (early subjective night). Adrenal explants from mice stressed at ZT2 showed mPER2:LUC rhythms that were phase-advanced by ~2 h, whereas adrenals from mice stressed at ZT16 showed rhythms that were phase-delayed by ~2 h. The biphasic response was also observed in mice injected subcutaneously either with saline or with ACTH at ZT2 or ZT16. Blockade of the ACTH response with the glucocorticoid, dexamethasone, prevented restraint stress-induced phase shifts in the mPER2:LUC rhythm both at ZT2 and at ZT16. The finding that acute stress results in a phase-dependent shift in the adrenal mPER2:LUC rhythm that can be blocked by dexamethasone indicates that stress-induced effectors, including ACTH, act to phase shift the adrenal clock rhythm.

**Keywords:** adrenal clock, acute stress, restraint stress, dexamethasone, ACTH, circadian, mPER2:LUC

## INTRODUCTION

The suprachiasmatic nucleus (SCN) is responsible for generating circadian rhythms in mammals (1). The molecular clock that underlies SCN rhythmicity is found in most mammalian cells (2), providing a peripheral clock mechanism that subserves tissue-specific functional rhythms (3). A fundamental question is how peripheral clocks are entrained to light and to other environmental signals (4). The adrenal cortex expresses a clock that can control the corticosterone rhythm by inducing rhythmic expression of the clock-controlled gene, steroidogenic acute regulatory (StAR) protein (5). Knockdown of the adrenal clock results in a corticosterone rhythm with reduced amplitude under constant dark, yet the rhythm is maintained under light-dark (LD) conditions (5). These results argue for redundancy in controlling corticosterone rhythms, with both clock-dependent and clock-independent mechanisms.

Our results using the mPER2:LUC mouse have shown that the adrenal clock can be reset *in vitro* by ACTH in a phase-dependent manner (6). The *in vitro* experiments showed that ACTH produced a phase delay when administered at a circadian time analogous to subjective night, but no phase shift at the circadian time representing subjective day. To examine whether stress is capable of phase-shifting the adrenal clock *in vivo*, we examined the adrenal clock in mPER2:LUC mice after exposure to chronic subordinate stress (7). The chronic subordinate stress model consisted of a brief period

(10 min or less) of daily physical contact between subordinate and dominant mice at early subjective day [zeitgeber time (ZT) 3] followed by cohabitation for the remainder of the 24-h period. We found that a single exposure to subordination stress produced a phase advance in the adrenal mPER2:LUC rhythm that was maintained after 14 days of chronic subordinate stress. However, it is unclear (1) whether the effect of acute stress on the adrenal clock is dependent on the phase of the circadian rhythm and (2) whether the phase shift depends on the release of ACTH. Using acute stress to activate the hypothalamic–pituitary–adrenal (HPA) axis in mPER2:LUC mice, experiments were done to determine whether stress shifts the phase of the adrenal clock in a phase-dependent fashion and whether the adrenal response occurs after blockade of ACTH release.

## MATERIALS AND METHODS

### Animals

Homozygous male mPER2:LUC mice (8) (3–6 months old) bred in-house were housed on a 12:12-h LD cycle (lights on at 0600 hours). Animals were maintained and cared for in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Experimental procedures were approved by the University of Minnesota Animal Care and Use Committee.

### Bioluminescence

Animals were killed by decapitation 3.5–4.0 h before lights out (at ZT 8–8.5). Adrenals were rapidly excised and placed in cold Hank's balanced salt solution. Cleaned and hemisected adrenals were placed on Millicell organotypic inserts (PICMORG50, 30 mm hydrophilic PTFE membrane, 0.4 µm pore size) in a 35 mm Petri dish with 1.5 ml of warmed culture media (Dulbecco's Modified Eagle media w/o Phenol Red) supplemented with luciferin and penicillin/streptomycin, as described previously (6, 8). Dishes were sealed with circular glass coverslips and silicon grease. Cultures were maintained at 36°C, and bioluminescence was measured using photomultiplier tubes in an Actimetrics Lumicycle.

## Experiments

### Experiment 1a

Male mPER2:LUC mice ( $n = 7$ /group) housed under a 12:12 L:D cycle underwent a 15-min restraint stress or no stress at ZT2. Mice were restrained by being placed in 50 ml conical tubes (Fisher Scientific, cat. # 05-539-13) with air holes drilled in tube bottoms to permit respiration. Mice were returned to their home cages following restraint and killed by decapitation at ZT8. Adrenals were processed for monitoring rhythms in bioluminescence.

### Experiment 1b

Male mPER2:LUC mice ( $n = 6$ /group) housed under a 12:12 L:D cycle underwent a 15-min restraint stress or no stress at ZT16; at ZT16 all procedures were performed under dim red light. Mice were returned to their home cages following restraint and killed by decapitation on the next day at ZT8. Adrenals were processed for monitoring rhythms in bioluminescence.

### Experiment 2a

Male mPER2:LUC mice ( $n = 5$ –6/group) housed under a 12:12 L:D cycle were injected with saline (100 µl sc; vehicle for ACTH) or ACTH (0.3 µg/100 µl sc) or underwent no stress at ZT2. Mice were returned to their home cages following injection and killed by decapitation at ZT8. Adrenals were processed for monitoring rhythms in bioluminescence.

### Experiment 2b

Male mPER2:LUC mice ( $n = 5$ /group) housed under a 12:12 L:D cycle were injected with saline (100 µl sc; vehicle for ACTH) or ACTH (0.3 µg/100 µl sc) or no stress at ZT16 under dim red light. Mice were returned to their home cages following restraint and killed by decapitation on the next day at ZT8. Adrenals were processed for monitoring rhythms in bioluminescence.

### Experiment 3

Male mPER2:LUC mice ( $n = 4$ –5/group) were pretreated with dexamethasone [Dexamethasone phosphate (Bimeda Inc.); 250 µg/kg BW sc] or needle puncture only as a control at ZT14 and underwent restraint stress for 15 min or no stress at ZT16. Mice were decapitated immediately following restraint, and trunk blood was collected for hormone assay. Plasma ACTH and corticosterone were measured by RIA, as described previously (9).

### Experiment 4a

Male mPER2:LUC mice ( $n = 4$ –5/group) were pretreated with dexamethasone (250 µg/kg BW sc) or needle puncture at ZT1 and underwent restraint stress for 15 min or no stress at ZT3. For experiment 4, the dexamethasone treatment was given at ZT1, instead of ZT0, to avoid manipulation of mice at light onset. To maintain a 2-h exposure to dexamethasone before stress, acute restraint was initiated at ZT3. Mice were returned to their home cages following restraint and killed by decapitation at ZT8. Adrenals were processed for monitoring rhythms in bioluminescence.

### Experiment 4b

Male mPER2:LUC mice ( $n = 4$ –5/group) were pretreated with dexamethasone (250 µg/kg BW sc) or needle puncture at ZT14 and underwent restraint stress for 15 min or no stress at ZT16. Mice were returned to their home cages following restraint and killed by decapitation the next day at ZT8. Adrenals were processed for monitoring rhythms in bioluminescence.

## Data Analysis

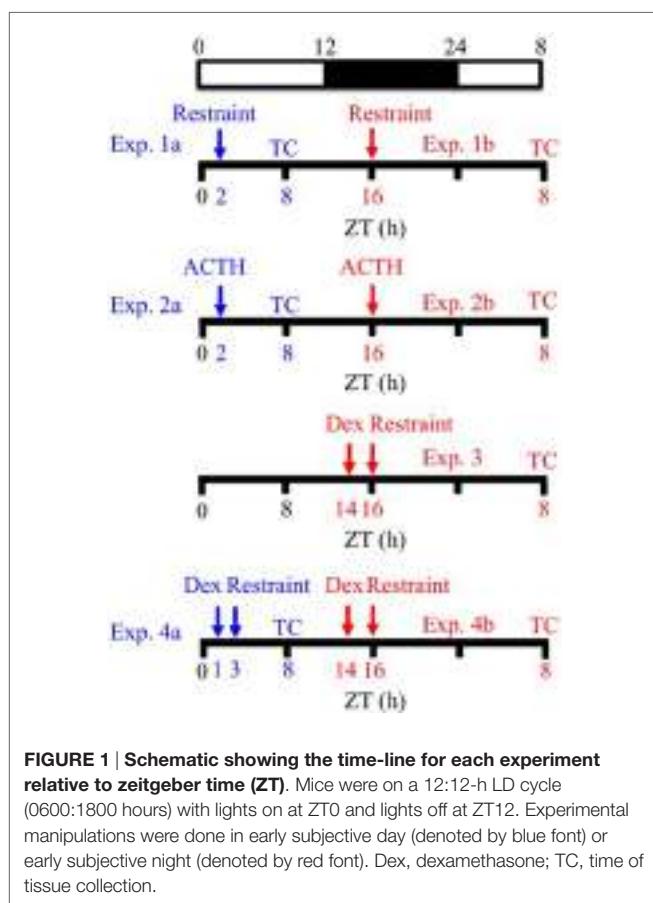
Data from the first day of recording were omitted from analysis due to transient bioluminescent activity (10). The remaining data were smoothed and detrended using a 2- and 24-h running average, baseline subtracted, and fit to a damped sine wave using Lumicycle Analysis software (Actimetrics). Only tissue showing rhythms with a goodness of fit >85% were accepted. To assess changes in the adrenal clock rhythm induced by *in vivo* manipulations, phase was determined from the peak measured on the second day of incubation, and period was calculated using data from two cycles spanning the second and third days of incubation.

## Statistical Analysis

Data are presented as means  $\pm$  SEM. Statistical differences were determined using one-way ANOVA (using Dunnett's correction for *post hoc* analysis), two-way ANOVA (using Bonferroni's correction for *post hoc* analysis), or unpaired Student's *t*-test, where appropriate, using Prism software (GraphPad). Differences were considered significant if  $p < 0.05$ .

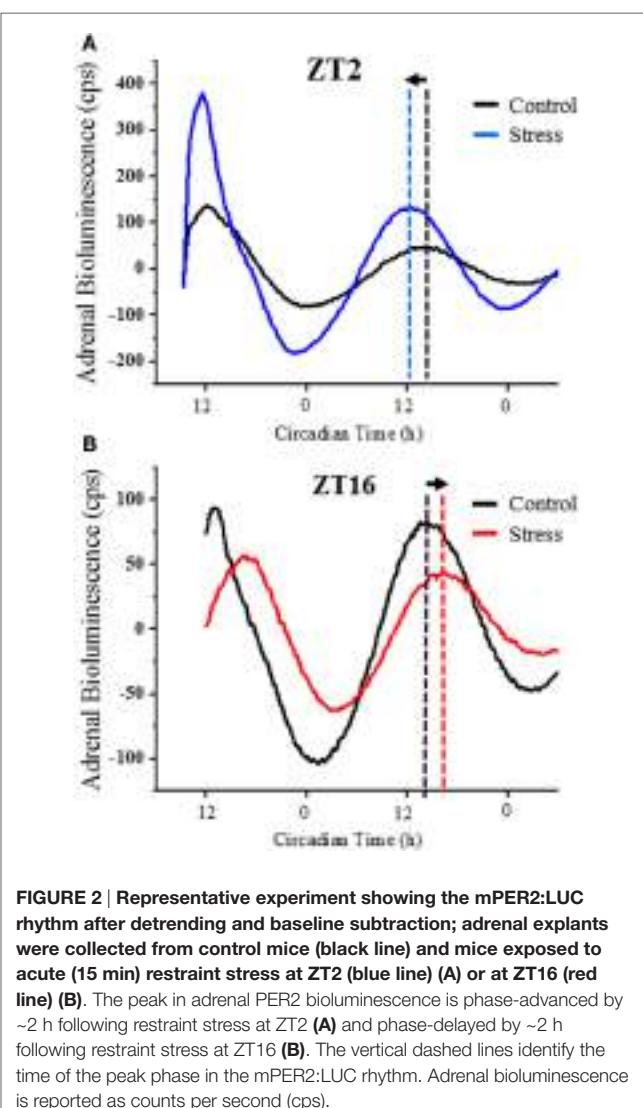
## RESULTS

A schematic showing the time line of treatments for each experiment as shown in **Figure 1**. Experiment 1 was done to determine if acute restraint stress results in a phase-dependent shift in the adrenal clock rhythm. Representative examples of mPER2:LUC bioluminescent activity are shown for adrenals collected from non-stressed mice and mice exposed to a 15-min restraint stress at ZT2 (**Figure 2A**) or at ZT16 (**Figure 2B**). Adrenals from mice that underwent a 15-min restraint stress at ZT2 showed a phase advance in the mPER2:LUC rhythm compared with adrenals from non-stressed (control) mice (**Figure 3A**), whereas adrenal rhythms from mice that were stressed at ZT16 showed a phase delay compared with controls (**Figure 3B**). Acute restraint stress had no effect on the period of the rhythm at ZT2 (CTRL:  $23.60 \pm 0.18$ ; RESTRAINT:  $23.32 \pm 0.23$ ) or at ZT16 (CTRL:  $23.37 \pm 0.08$ ; RESTRAINT:  $22.78 \pm 0.23$ ).

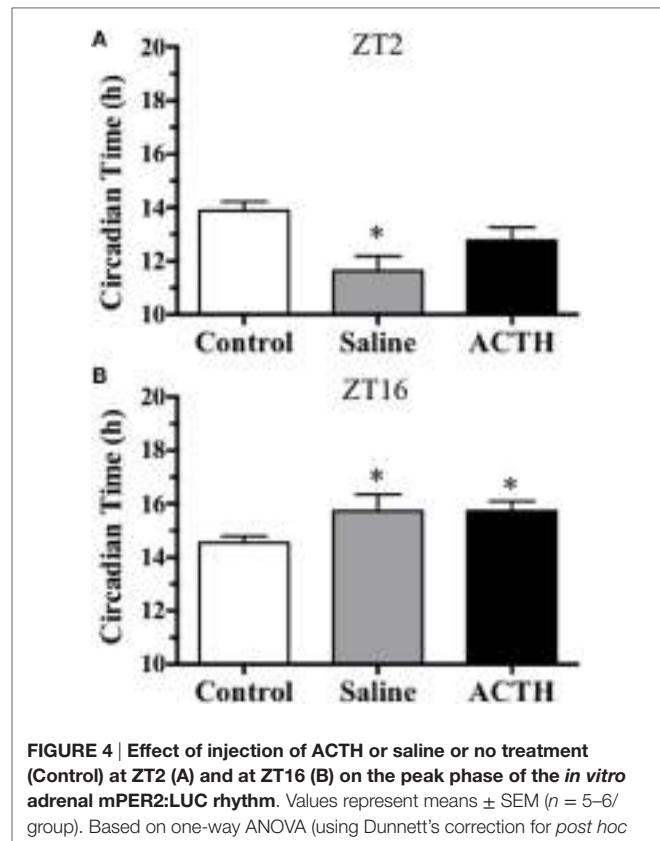
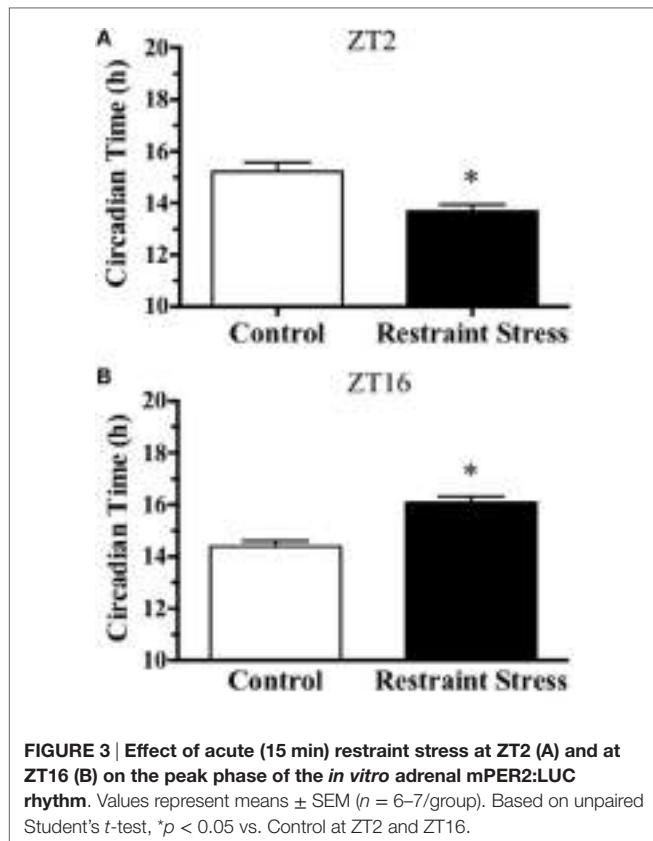


**FIGURE 1 | Schematic showing the time-line for each experiment relative to zeitgeber time (ZT).** Mice were on a 12:12-h LD cycle (0600:1800 hours) with lights on at ZT0 and lights off at ZT12. Experimental manipulations were done in early subjective day (denoted by blue font) or early subjective night (denoted by red font). Dex, dexamethasone; TC, time of tissue collection.

Two approaches were used to examine whether stress-induced ACTH contributed to stress-induced phase-dependent shifts in the adrenal mPER2:LUC rhythm. In experiment 2, mice were injected with a supramaximal dose of ACTH [100  $\mu$ l, 3.0  $\mu$ g/kg BW; Ref. (9)] and compared with mice injected with saline (100  $\mu$ l) or no injection (Control) at ZT2 or ZT16. Adrenals from mice injected with saline at ZT2 showed a phase advance in the mPER2:LUC rhythm compared with adrenals from Control mice (**Figure 4A**), whereas rhythms from adrenals from mice injected with saline or ACTH at ZT16 showed a phase delay compared with Control mice (**Figure 4B**). No differences were observed between saline and ACTH at ZT2 or ZT16, indicating that the handling and injection stress was sufficient to shift the adrenal mPER2:LUC rhythm in a phase-dependent fashion. Neither injection of saline nor ACTH had an effect on the period of the rhythm at ZT2 (CTRL:  $23.60 \pm 0.18$ ; saline:  $23.58 \pm 0.23$ ; ACTH:  $22.88 \pm 0.49$ ) or at ZT16 (CTRL:  $22.12 \pm 0.10$ ; saline:  $22.48 \pm 0.28$ ; ACTH:  $22.32 \pm 0.25$ ).



**FIGURE 2 | Representative experiment showing the mPER2:LUC rhythm after detrending and baseline subtraction; adrenal explants were collected from control mice (black line) and mice exposed to acute (15 min) restraint stress at ZT2 (blue line) (A) or at ZT16 (red line) (B).** The peak in adrenal PER2 bioluminescence is phase-advanced by  $\sim 2$  h following restraint stress at ZT2 (A) and phase-delayed by  $\sim 2$  h following restraint stress at ZT16 (B). The vertical dashed lines identify the time of the peak phase in the mPER2:LUC rhythm. Adrenal bioluminescence is reported as counts per second (cps).



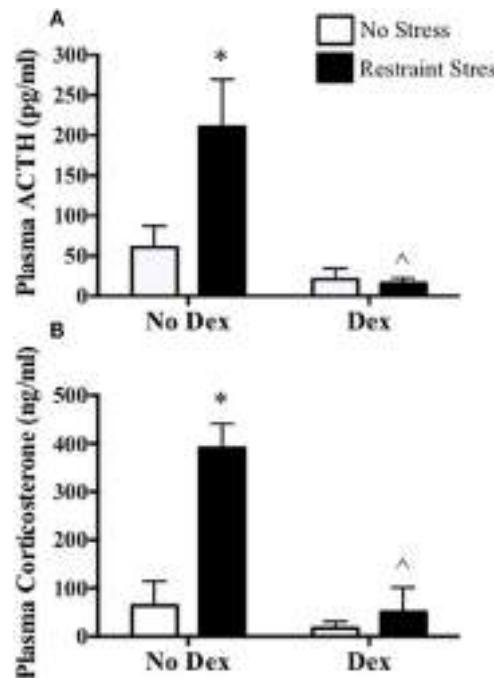
To further examine a possible role for stress-induced ACTH in the phase-dependent shift of the mPER2:LUC rhythm, dexamethasone was used to block ACTH release induced by restraint stress. In experiment 3, mice were pretreated with dexamethasone 2 h before undergoing restraint stress at ZT16. Plasma ACTH and corticosterone increased at 15 min after restraint in mice that were not treated with dexamethasone. In contrast, dexamethasone pretreatment blocked the plasma ACTH and corticosterone response to restraint (Figure 5). In experiment 4, dexamethasone was used to block ACTH 2 h prior to restraint at ZT3 or at ZT16, and adrenals were collected to measure changes in the mPER2:LUC rhythm *in vitro*. Results showed that dexamethasone prevented both the phase advance in the mPER2:LUC rhythm induced by restraint stress at ZT3 (Figure 6A) and the phase delay induced by restraint stress at ZT16 (Figure 6B), suggesting that increased ACTH induced by restraint stress contributes to the phase-dependent shift in the mPER2:LUC rhythm. Although the period of the mPER2:LUC rhythm was not affected by restraint stress in the absence of dexamethasone, the period was lengthened in adrenals from mice that underwent dexamethasone treatment followed by stress at ZT3 (Figure 7A) and ZT16 (Figure 7B).

## DISCUSSION

Experiments were done to determine whether acute stress shifts the phase of the adrenal clock in a phase-dependent manner and whether the adrenal response is dependent on stress-induced

release of ACTH. The present results show that a single 15-min restraint stress at ZT2 produced an ~2-h phase advance in the mPER2:LUC rhythm. In marked contrast, the same manipulation performed at ZT16 produced an ~2-h phase delay. To examine whether increased ACTH was responsible for the phase-dependent shift, we injected ACTH at ZT2 or at ZT16 to mimic restraint stress-induced ACTH. Injection stress was sufficient to phase shift the adrenal mPER2:LUC rhythm in a phase-dependent manner, yet responses to ACTH injection were comparable to saline injection. To assess a possible role for ACTH more clearly, mice were treated with dexamethasone to inhibit restraint stress-induced ACTH; results showed that dexamethasone blocked the phase-dependent shift in the mPER2:LUC rhythm. Taken together, these experiments show that acute stress is capable of producing a biphasic shift in the adrenal mPER2:LUC rhythm that can be blocked by dexamethasone, suggesting that ACTH is a necessary component in stress-induced alterations in adrenal clock timing.

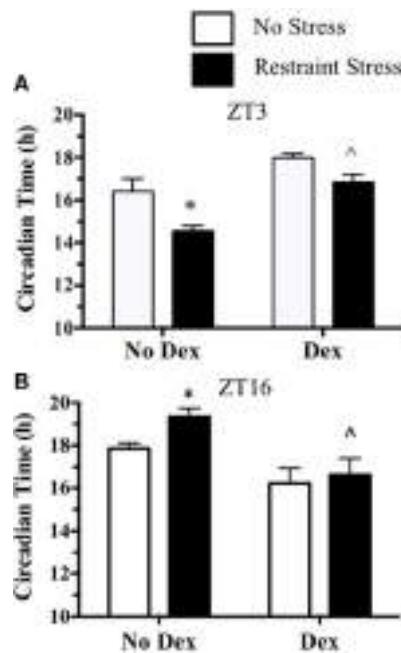
Our previous work showed that a single exposure to subordination stress at early subjective day (ZT3) followed by cohabitation for the remainder of the day produced an ~2-h phase advance in the adrenal mPER2:LUC rhythm in male mice (7). By using a single acute restraint or injection stress, the present experiments were designed to allow more precise control of the timing of stress exposure. Similar to the response to single subordination stress, acute restraint or injection stress at early subjective day (ZT2–3) phase-advanced the adrenal mPER2:LUC rhythm. In



**FIGURE 5 |** Effect of dexamethasone blockade on restraint stress-induced plasma ACTH (A) and corticosterone (B) at ZT16 in mPER2:LUC mice. Values represent means  $\pm$  SEM ( $n = 4\text{--}5/\text{group}$ ). Based on two-way ANOVA (using Bonferroni's correction for post hoc analysis), \* $p < 0.05$  vs. No Dex-No Stress and ^ $p < 0.05$  vs. No Dex-Stress.

addition, when acute stress was applied at early subjective night (ZT16), the adrenal mPER2:LUC rhythm was phase-delayed (Figures 3 and 4). Others have examined whether repeated stress results in a phase-dependent effect on the adrenal mPER2:LUC rhythm (11). A 2-h exposure to subordination stress in early subjective day for 3 weeks resulted in a phase advance in the adrenal rhythm, but repeated subordination stress in early subjective night did not produce a phase shift in the adrenal rhythm. The effect of repeated stress at early subjective day confirmed our previous work showing that the phase advance in the adrenal mPER2:LUC rhythm was maintained after chronic (14-day) subordination stress, indicating that changes in the timing of the adrenal clock did not habituate to chronic stress applied at ZT3 (7). Since exposure to a single acute stressor, but not repeated exposure to stress, results in a biphasic effect on the adrenal mPER2:LUC rhythm, it is possible that the phase delay produced by acute stress in early subjective night habituates to repeated exposure to stress. Additional experiments are required to test this possibility.

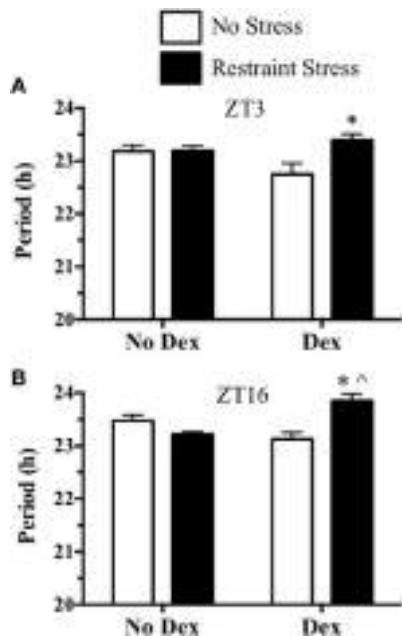
The observation that acute restraint stress shifts the phase of the adrenal mPER2:LUC rhythm led to experiments assessing whether ACTH contributed to the response. This possibility was based in part on our previous study showing that ACTH *in vitro* induced a phase shift of adrenal mPER2:LUC rhythm (6). The 2-h phase delay of the mPER2:LUC rhythm following acute restraint stress during the dark period (ZT16) parallels the phase-dependent delay produced by ACTH *in vitro*. In contrast,



**FIGURE 6 |** Effect of dexamethasone treatment before restraint stress at ZT3 (A) or at ZT16 (B) on the peak phase of the *in vitro* adrenal mPER2:LUC rhythm. Values represent means  $\pm$  SEM ( $n = 4\text{--}5/\text{group}$ ). Based on two-way ANOVA (using Bonferroni's correction for post hoc analysis), \* $p < 0.05$  vs. No Dex-No Stress and ^ $p < 0.05$  vs. No Dex-Stress at ZT3 and ZT16.

the phase advances in the mPER2:LUC rhythm observed after restraint stress during the light period (ZT2) was not mimicked by ACTH *in vivo*, experiments were done to assess the effect of ACTH directly on the phase-dependent response. Results showed that ACTH injection was comparable to saline injection in changing the phase of the adrenal mPER2:LUC rhythm, suggesting that injection stress alone was sufficient for producing a phase-dependent shift (Figure 4). To examine whether ACTH responses to acute stress were required for the phase shift, mice were treated with dexamethasone, a synthetic glucocorticoid, to block ACTH secretion. This approach takes advantage of the known effect of dexamethasone to act at the anterior pituitary as a negative feedback signal to inhibit ACTH release (12). Pretreatment with a low dose (250  $\mu\text{g}/\text{kg}$ ) of dexamethasone, similar to the dose used to block stress-induced ACTH in rats (9, 13), effectively blocked both restraint stress-induced plasma ACTH and corticosterone (Figure 5). In addition, dexamethasone pretreatment prevented both the phase advance produced by restraint stress at ZT3 and the phase delay produced by restraint stress at ZT16 (Figure 6). These findings show that acute stress shifts the phase of the adrenal clock rhythm *in vivo* and suggest that the response results at least in part from increased plasma ACTH.

Activation of glucocorticoid receptors by dexamethasone can entrain other peripheral clocks (14, 15). Thus, in addition to ACTH blockade, it is possible that the activation of glucocorticoid receptors in the adrenal (16) could be involved in



**FIGURE 7 | Effect of dexamethasone treatment before restraint stress at ZT3 (A) or at ZT16 (B) on the period of the *in vitro* adrenal mPER2:LUC rhythm.** Values represent means  $\pm$  SEM ( $n = 4\text{--}5/\text{group}$ ). Based on two-way ANOVA (using Bonferroni's correction for *post hoc* analysis), \* $p < 0.05$  vs. Dex-No Stress and \*\* $p < 0.05$  vs. No Dex-Stress at ZT3 and ZT16.

phase-dependent changes in the adrenal mPER2:LUC rhythm. Although the high doses (10 mg/kg) of dexamethasone result in phase-dependent shifts in the body temperature rhythm, lower doses like those used in the present study have no effect (17). By evaluating the effects of dexamethasone alone on the adrenal mPER2:LUC rhythm, we were unable to find differences in peak phases between non-stress groups treated with or without dexamethasone (Figure 6). However, whereas changes in the period were not observed after stress or dexamethasone alone, dexamethasone combined with stress at ZT3 and ZT16 prolonged the period of the mPER2:LUC rhythm (Figure 7). Additional experiments are necessary to determine whether the mechanism for period lengthening includes activation of adrenal glucocorticoid receptors.

In non-photic entrainment, SCN-controlled rhythms, such as locomotor activity, shift in response to acute arousal (18). Non-photic entrainment by arousal is more prevalent during subjective day (19) and is characterized by a phase advance (20). Since we observed a phase advance in the adrenal mPER2:LUC rhythm following stress at ZT2, it is possible that the adrenal response is dependent on a change in SCN activity. Although we did not assess SCN mPER2:LUC activity, others have shown that repeated subordination stress at ZT1–3 does not affect the phase of the SCN clock gene rhythm (11). Moreover, acute subordinate stress during subjective night in rats does not shift the phase of activity or temperature rhythms, reflections of SCN activity, suggesting that acute stress during the active period acts independently of

the SCN (19, 21). Since mice were housed under a 12:12-h LD cycle in the present study, it is likely that photic entrainment predominates in maintaining the phase of the SCN clock over stress-related stimuli. The biphasic stress-induced phase shifts of the adrenal clock may not result from non-photic entrainment of the SCN but instead may represent a direct phase-dependent response of the adrenal clock to stress.

The mechanism for a phase-dependent response of the adrenal clock rhythm to stress is unclear. The ability to block the phase-resetting induced by stress with dexamethasone implicates ACTH. Since the magnitude of the ACTH response to stress differs diurnally (22), exposure to different levels of ACTH may contribute to phase dependency. However, our *in vitro* data showed that the phase response was not dependent on the magnitude of the ACTH stimulus, since high concentrations of ACTH administered at early CTs were ineffective in phase-shifting the mPER2:LUC rhythm (6). It is likely that factors, in addition to ACTH, play a role. Since increases in adrenal sympathetic activity synergize with ACTH to increase adrenal steroidogenic responses (23) and also increase adrenal Per expression (24), sympathetic neural input may be involved in altering timing of the adrenal clock. The adrenal cortex includes sympathetic innervation by vasoactive intestinal peptide (VIP)-ergic fibers (25). Corticosterone rhythms are dampened in constitutive VIP knockout (26, 27) and in VIP receptor knockout mice (28, 29), supporting the possibility that stress-induced VIPergic activity could complement ACTH in phase-shifting the adrenal clock. As shown for light-induced phase shifts of the SCN clock (30), phase-shifting of the adrenal clock may result from stress-induced increases in adrenal PER that vary with the time of stress to differentially change PER negative feedback on the molecular clock. Additional studies are required to assess this possibility.

The present results show that acute stress produces a shift in the adrenal clock gene rhythm that is phase-dependent. The physiological importance of this observation stems in large part from the role of the adrenal clock in regulating glucocorticoid secretion. Rhythms in plasma glucocorticoids are paralleled by rhythms in adrenal sensitivity to ACTH (9, 22, 31), and the adrenal clock is thought to control rhythmic steroidogenesis by gating sensitivity to ACTH (32). Since the adrenal clock controls circadian rhythms in glucocorticoid production (5), it will be important to determine whether acute stress also shifts the phase of the plasma glucocorticoid rhythm in a phase-dependent manner. Since glucocorticoid rhythms entrain other peripheral clocks (14, 33), altered glucocorticoid rhythms produced by stress-induced phase shifts could have effects on metabolic, hemodynamic, and cognitive function.

## AUTHOR CONTRIBUTIONS

WE designed the experiments, assisted in performing the experiments, analyzed the results, wrote the initial draft of the manuscript, and completed the final edited version of the manuscript. JY and CK assisted in designing and performing the experiments, analyzing the results, and editing the manuscript. PK assisted in designing the experiments, performing the experiments, and editing the manuscript.

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# Stress-Related and Circadian Secretion and Target Tissue Actions of Glucocorticoids: Impact on Health

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Living organisms are highly complex systems that must maintain a dynamic equilibrium or homeostasis that requires energy to be sustained. Stress is a state in which several extrinsic or intrinsic disturbing stimuli, the stressors, threaten, or are perceived as threatening, homeostasis. To achieve homeostasis against the stressors, organisms have developed a highly sophisticated system, the stress system, which provides neuroendocrine adaptive responses, to restore homeostasis. These responses must be appropriate in terms of size and/or duration; otherwise, they may sustain life but be associated with detrimental effects on numerous physiologic functions of the organism, leading to a state of disease-causing disturbed homeostasis or cacostasis. In addition to facing a broad spectrum of external and/or internal stressors, organisms are subject to recurring environmental changes associated with the rotation of the planet around itself and its revolution around the sun. To adjust their homeostasis and to synchronize their activities to day/night cycles, organisms have developed an evolutionarily conserved biologic system, the "clock" system, which influences several physiologic functions in a circadian fashion. Accumulating evidence suggests that the stress system is intimately related to the circadian clock system, with dysfunction of the former resulting in dysregulation of the latter and vice versa. In this review, we describe the functional components of the two systems, we discuss their multilevel interactions, and we present how excessive or prolonged activity of the stress system affects the circadian rhythm of glucocorticoid secretion and target tissue effects.

**Keywords:** stress, stress system, hypothalamic–pituitary–adrenal axis, glucocorticoids, glucocorticoid receptor, circadian endocrine rhythms, clock system

## THE STRESS SYSTEM

The stress system consists of the locus caeruleus/norepinephrine autonomic nervous systems and the hypothalamic–pituitary–adrenal (HPA) axis. These two components interact with each other, as well as with other brain subsystems, such as the mesocortical and the mesolimbic dopaminergic system, which is involved in reward and motivation, the central nucleus of the amygdala, which generate fear and/or anger, and the arcuate nucleus of the hypothalamus participating in

stress system control (1–4). The activity of the stress system is influenced by several neurochemical modulators (e.g., serotonin, acetylcholine,  $\gamma$ -aminobutyric acid, glutamate and endogenous cannabinoids, and benzodiazepines) (1–4). When homeostasis is threatened or perceived by the individual as threatened by stressors, the locus caeruleus/norepinephrine/autonomic nervous systems release norepinephrine in the brain and the systemic circulation, while epinephrine is secreted by the adrenal medulla. On the other hand, the HPA axis is associated with the production and secretion of glucocorticoids by the *zona fasciculata* of the adrenal cortex (1–4). Glucocorticoids play a fundamental role in the maintenance of basal and stress-related homeostasis, regulating many physiologic functions through genomic actions mediated by their cognate intracellular receptor, the glucocorticoid receptor (GR); the latter belongs to the steroid receptor family of the nuclear receptor superfamily of transcription factors (4–8).

The human glucocorticoid receptor (hGR) is encoded by the *NR3C1* gene, which is located in the long arm of chromosome 5 and is composed of 10 exons. The alternative splicing of exon 9 generates the two main protein isoforms of the receptor, the hGR $\alpha$  and the hGR $\beta$ . Expressed in every tissue except the suprachiasmatic nucleus (SCN) of the hypothalamus, the hGR $\alpha$  is activated following binding of natural or synthetic glucocorticoids to its ligand-binding domain and binds to the regulatory regions of glucocorticoid-responsive genes through its DNA-binding domain and/or interacts with other transcription factors altering their transcriptional activities (*vide infra*) (6–10). On the other hand, the hGR $\beta$  isoform is an enigma in endocrine physiology. Exclusively localized in the nucleus of certain cell types, such as endothelial cells, the hGR $\beta$  acts as a dominant-negative inhibitor of hGR $\alpha$ -induced transcriptional activity through well-delineated molecular mechanisms (11–13). Interestingly, this receptor isoform can influence the transcription rate of several genes independently of hGR $\alpha$  (14, 15). Recent studies have demonstrated that hGR $\beta$  may be involved in insulin signaling and implicated in gluconeogenesis and inflammation in mouse liver (16, 17). New evidence suggests a pivotal role of the GR $\beta$  isoform in the molecular cascades of glioma formation and bladder cancer cells migration (18–20). Further to the alternative splicing of exon 9, Lu and Cidlowski showed that the initiation of the hGR $\alpha$  mRNA translation might occur through eight different sites giving rise to receptor isoforms with variable N-terminal domains: hGR $\alpha$ -A (classic GR $\alpha$ ), hGR $\alpha$ -B, hGR $\alpha$ -C1, hGR $\alpha$ -C2, hGR $\alpha$ -C3, hGR $\alpha$ -D1, hGR $\alpha$ -D2, and hGR $\alpha$ -D3, which have distinct properties in terms of intracellular localization and transcriptional activity (10, 21). We assume similar translation processing of the GR $\beta$  isoform.

At the cellular level, the glucocorticoid signaling pathway is initiated by ligand-induced activation of the primarily cytoplasmic hGR $\alpha$ , which dissociates from chaperon heat shock proteins and immunophillins, and translocates into the nucleus, where it binds, as homo- or heterodimer, to specific DNA sequences, the glucocorticoid response elements, within the regulatory regions of target genes, thereby influencing their transcription in a positive or negative fashion (4, 6–8, 10). In addition to direct hGR $\alpha$  binding to glucocorticoid-responsive genes, glucocorticoids can

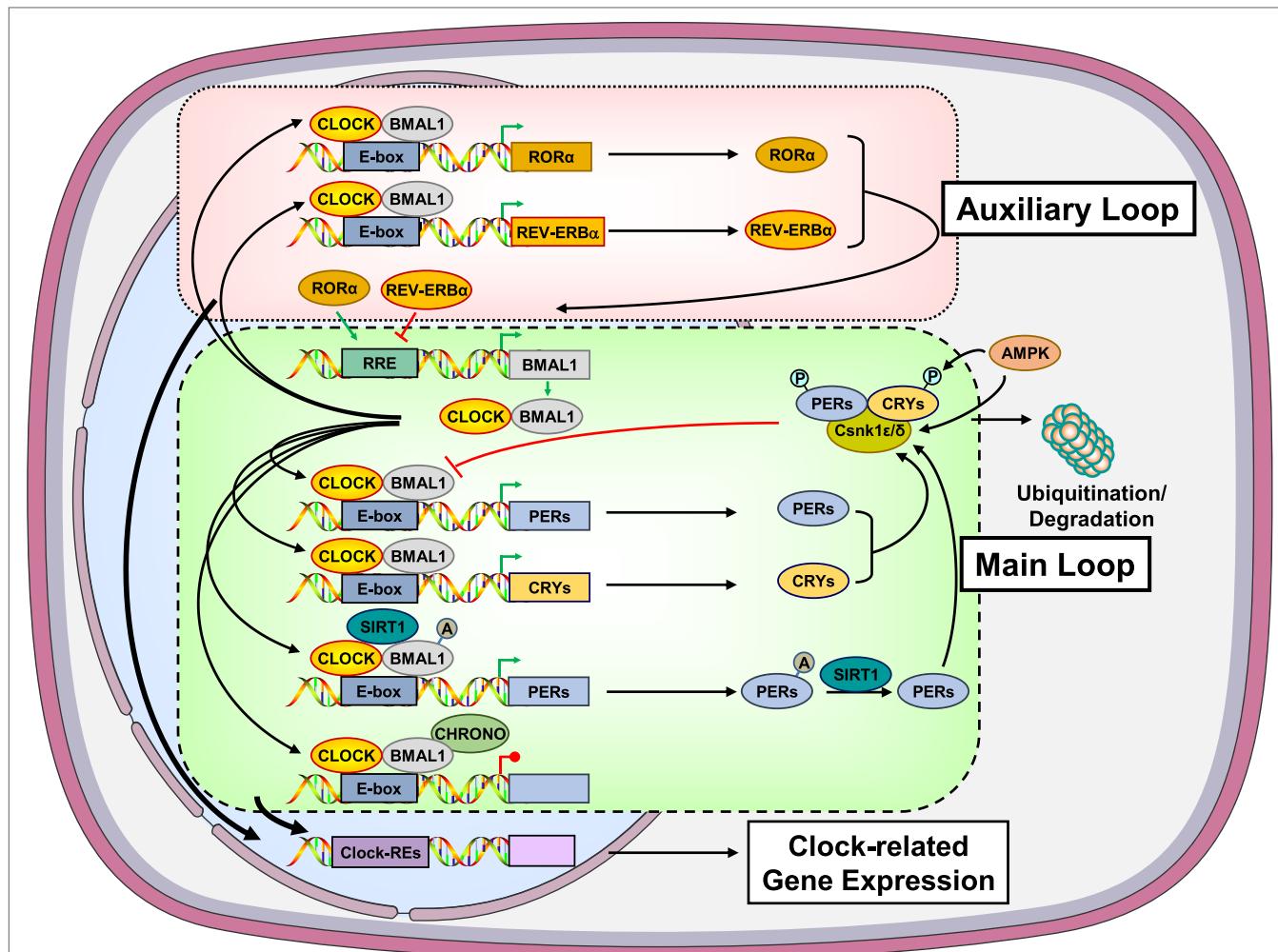
influence the transcription of several other genes independently of DNA binding. Indeed, the activated hGR $\alpha$  isoform can interact, possibly as a monomer, with other transcription factors, such as the nuclear factor- $\kappa$ B, the activator protein-1, and the signal transducers and activators of transcription, suppressing or inducing their transcriptional activity (4, 6–8, 10). In addition to the well-described genomic actions, glucocorticoids can induce some cellular effects in a very short-time frame. These effects are referred to as “non-genomic glucocorticoid actions” and are likely to be mediated by membrane-bound GRs, which may trigger the activation of kinase signaling pathways (22–24).

## THE CIRCADIAN CLOCK SYSTEM

To adjust their daily activities to light/dark changes, organisms have developed a highly conserved timekeeping system, the circadian clock system (from the Latin “circa diem” meaning “approximately a day”), which creates internal rhythmicity under the influence of day/night cycles. This regulatory system is composed of a central “master” clock located in the SCN of hypothalamus, and peripheral “slave” clocks, which are ubiquitously expressed in all tissues (25–27). Importantly, peripheral clocks are tightly synchronized to the central clock through as yet unknown possibly neural or neuroendocrine mechanisms. The central clock influences virtually all physiologic functions, such as sleep/wakefulness, feeding, thermoregulation, energy expenditure, glucose homeostasis, and the activity of the HPA axis. Similarly, peripheral clocks regulate several functions of their residing tissues, ultimately contributing to the homeostasis of living organisms (25–28).

At the cellular level, the circadian clock system is composed of transcriptional/translational loops, which create an intrinsic, self-oscillating circadian rhythm in both the central and peripheral clocks (Figure 1). At the molecular level, these feedback loops are mediated by the circadian locomotor output cycle kaput/brain-muscle-arnt-like protein 1 (CLOCK/BMAL1) heterodimer and other negative transcription factors, such as the Periods (PER1, PER2, and PER3) and Cryptochromes (CRY1 and CRY2). In the principal or core transcription loop, the activated CLOCK/BMAL1 heterodimer binds to the E-box response elements and induces the expression of *Pers* and *Crys*. These proteins associate with casein kinase (Csnk) 1 $\epsilon$  and  $\delta$  and undergo phosphorylation (29–31). The phosphorylated isoforms then translocate to the nucleus and suppress the transcriptional activity of the CLOCK/BMAL1 heterodimer. In addition, several other clock-related genes, such as retinoic acid receptor-related orphan receptor  $\alpha$  (ROR $\alpha$ ) and reverse viral erythroblastosis oncogene product (REV-ERB $\alpha$ ), are upregulated by the CLOCK/BMAL1 heterodimer, forming an auxiliary loop, which stabilizes the transcriptional activity of the core loop. The transcription factors of both principal and auxiliary loops can modulate the expression of many clock-responsive genes in various tissues (27, 29–31).

In addition to light/dark signals, the circadian clock system is strongly influenced by several metabolic inputs, particularly those associated with ingestion of food (Figure 1) (33–39). Indeed, the



**FIGURE 1 | Molecular components of the main and auxiliary transcriptional/translational loops of the circadian clock system.** In the main transcription loop, the heterodimer CLOCK/BMAL1 causes upregulation of *Per*s and *Cry*s. *PER*s and *Cry*s undergo phosphorylation by the Csnk1 $\epsilon/\delta$  and translocate to the nucleus suppressing the transcriptional activity of the CLOCK/BMAL1. Moreover, CLOCK/BMAL1 influences the transcription rate of several other clock-related genes, such as ROR $\alpha$  and REV-ERB $\alpha$ , giving rise to an auxiliary transcription loop. AMPK participates in the main transcription loop by phosphorylating *Cry*s, *PER*s, and Csnk1 $\epsilon$ . SIRT1 functions as a counter-regulatory mechanism for the acetyltransferase activity of the CLOCK by deacetylating BMAL1, PER2, and histone H3. CHRONO, a recently identified BMAL target was found to interact with BMAL1, repressing the main transcription loop via recruitment of histone deacetylase 1. A, acetyl residue on the acetylated molecules; AMPK, adenosine monophosphate-activated protein kinase; BMAL1, brain-muscle-ant-like protein 1; CHRONO, ChIP-derived repressor of network oscillator; CLOCK, circadian locomotor output cycle kaput; *Cry*s, cryptochromes; Csnk1 $\epsilon/\delta$ , casein kinase 1 $\epsilon/\delta$ ; P, phosphate residue on the phosphorylated molecules; *PER*s, periods; ROR $\alpha$ , retinoic acid receptor-related orphan nuclear receptor  $\alpha$ ; SIRT1, sirtuin 1. Modified from Ref. (32).

adenosine monophosphate-activated protein kinase (AMPK), a tissue sensor and master regulator of energy balance, seems to influence the activity of the clock system through energy-dependent signals. AMPK does so by phosphorylating *Cry*s and *PER*s leading to their degradation. AMPK can also cause destabilization of *PER*s indirectly by increasing the activity of Csnk1 $\epsilon$  through phosphorylation, resulting in Csnk1 $\epsilon$ -mediated degradation of *PER*s (Figure 1) (33–36). In addition to AMPK, the longevity and metabolism-associated sirtuin 1 (SIRT1) was demonstrated to deacetylate BMAL1, PER2, and histone H3 depending on the NAD $^{+}$  cellular levels, possibly functioning as a counter-regulatory mechanism for the histone acetyltransferase activity of the CLOCK (Figure 1) (35–39).

## MOLECULAR INTERRELATIONS BETWEEN THE HPA AXIS AND THE CIRCADIAN CLOCK SYSTEM: THE PIVOTAL ROLE OF GLUCOCORTICOIDS

### Influence of the Circadian Clock System on the HPA Axis

A growing body of evidence suggests that the stress-responsive HPA axis and the circadian clock system interact with each other at multiple levels (27, 32, 40–45). Indeed, the central clock in the SCN projects neurons in the paraventricular nucleus (PVN) of the hypothalamus providing the basis for the diurnal oscillation

of circulating glucocorticoid concentrations, which are higher during the day for diurnal species and at night for nocturnal species (28). In addition, the central clock influences the sensitivity of the adrenal cortex to adrenocorticotrophic hormone (ACTH) concentrations through a multisynaptic neuronal pathway (46, 47). On the other hand, in peripheral tissues, the CLOCK/BMAL1 heterodimer represses the hGR $\alpha$ -induced transcriptional activity through CLOCK-mediated acetylation of multiple lysine residues located in the hinge region of the receptor (48–51). In humans, the acetylation status of hGR $\alpha$  is higher in the morning than in the evening, and mirrors the circadian oscillation of cortisol concentrations; therefore, the target tissue glucocorticoid sensitivity reaches a zenith during the evening hours (52). Moreover, CRY1 and CRY2 interact with hGR $\alpha$  leading to reduced DNA binding of the receptor (53).

Recent *in vitro* and *in vivo* studies have identified a novel circadian CLOCK component and BMAL target gene, the *Gm129*, later termed as “*Chrono*” (“ChIP-derived repressor of network oscillator”) (Figure 1) (54, 55). *Chrono* mRNA was found to oscillate in a circadian fashion, which was antiphasic to that of *Bmal1* mRNA, in the mouse SCN, as well as in many peripheral tissues. *Chrono* was shown to encode a 45-kDa protein, called “CHRONO,” which displayed robust circadian oscillation with the opposite phase of BMAL1. CHRONO interacted with BMAL1, CRY2, and DEC2 and functioned as a repressor of the principal transcriptional loop through recruitment of histone deacetylase 1 (55, 56). Moreover, interesting findings from these studies indicated that CHRONO might be a potential link between the circadian clock system and the HPA axis, since this protein interacted with the GR, and *Chrono* knockout mice had increased circulating serum corticosterone concentrations, compared to wild-type mice (56, 57).

## Influence of the Circadian Clock System on Glucocorticoid Secretion

Glucocorticoids, the end products of the HPA axis, play a pivotal role in mediating the stress response and contribute to the tight synchronization of peripheral clocks. These steroid hormones are secreted into the systemic circulation in an ultradian, circadian, and stress-related fashion (58). The ultradian or pulsatile pattern of glucocorticoid release is characterized by a time period of 80–110 min in humans, and its activity is independent of SCN input (59). The circadian release of glucocorticoids is influenced by three factors: (i) the SCN-activated HPA axis, (ii) the SCN-derived autonomic innervation of the adrenal glands through the splanchnic nerve, and (iii) local adrenal clocks (60). As mentioned earlier, SCN neurons send projections into the area of PVN of hypothalamus, thereby creating the diurnal fluctuation of corticotropin-releasing hormone (CRH), arginine vasopressin (AVP), ACTH, and glucocorticoids (28). Furthermore, SCN neurons transmit light information to the adrenal glands *via* splanchnic nerve innervation and increase glucocorticoid release in an HPA-independent fashion (46, 47). The transmission of the light signal to the adrenal cortex is likely to be mediated by catecholamines and/or neuropeptides produced by the adrenal medulla (61). In addition to the photic transmission, the SCN-guided autonomic

innervation also alters the sensitivity of the adrenal cortex to ACTH concentrations (46, 47). As already known in the 1960s and as recently confirmed, isolated adrenal gland tissues and cells display robust circadian secretion of steroids, suggesting that the SCN input is not the only prerequisite for diurnal oscillation of the adrenal transcriptome (62). Indeed, adrenal glands harbor their own circadian clocks influencing the rhythmic expression of approximately 10% of the adrenal genome (63). Among genes regulated by adrenal circadian clocks, the steroidogenic acute regulatory protein, a rate-limiting gene encoding a cholesterol transporter into the mitochondria, is rhythmically expressed under the transcriptional control of the adrenal circadian BMAL1 (64).

## Influence of the HPA Axis/Glucocorticoids on the Circadian Clock System

Glucocorticoids, through binding to the hGR $\alpha$ , can effectively reset the activity of peripheral clocks, adding another level of interaction between the HPA axis and the circadian clock system (65). Glucocorticoids alter circadian oscillations of several clock-related genes, including *Pers*, by phase shifting their expression in peripheral organs (66). Therefore, *Per1* and *Per2* genes are upregulated, causing a phase delay of the peripheral clocks, but not the SCN master clock (67). Moreover, the activated hGR $\alpha$  transrepresses the *Rev-ERB $\alpha$*  and *ROR $\alpha$*  genes, influencing directly the activity of the auxiliary transcriptional/translational loop and indirectly that of the main loop (68). At the tissue level, glucocorticoids regulate several local oscillators in different brain areas, as well as in peripheral tissues, as demonstrated by rodent studies. Chronic administration of prednisolone or adrenalectomy strongly influences the expression of clock-related genes [reviewed in Ref. (69)]. In the PVN, adrenalectomy causes a reduction in the amplitude of the expression of *Per1*, whereas acute stress results in the increased expression of the same gene (70, 71). In several limbic areas, many clock genes are suppressed by adrenalectomy, such as the *Per2*. The expression of the latter was shown to be restored when adrenalectomized rats were given corticosterone placed in their drinking water (72). In the hippocampus, the expression of *Per1* was influenced by glucocorticoids (73). In peripheral tissues, glucocorticoids modulate local clocks (74–78). In kidney, adrenalectomy causes phase delay in *Per1* oscillations (74). In white adipose tissue, dexamethasone increases the amplitude of oscillations of clock genes in preadipocytes and attenuates them in differentiated adipocytes (75). In bronchial epithelial cells of the lung, the administration of dexamethasone resulted in upregulation of the *Per1* (76). In cardiac muscle tissue, dexamethasone phase shifts the expression of local clock genes (77). In cornea, adrenalectomy causes a phase delay in *Per1* (74). Finally, the bone local clock can be reset by dexamethasone (78).

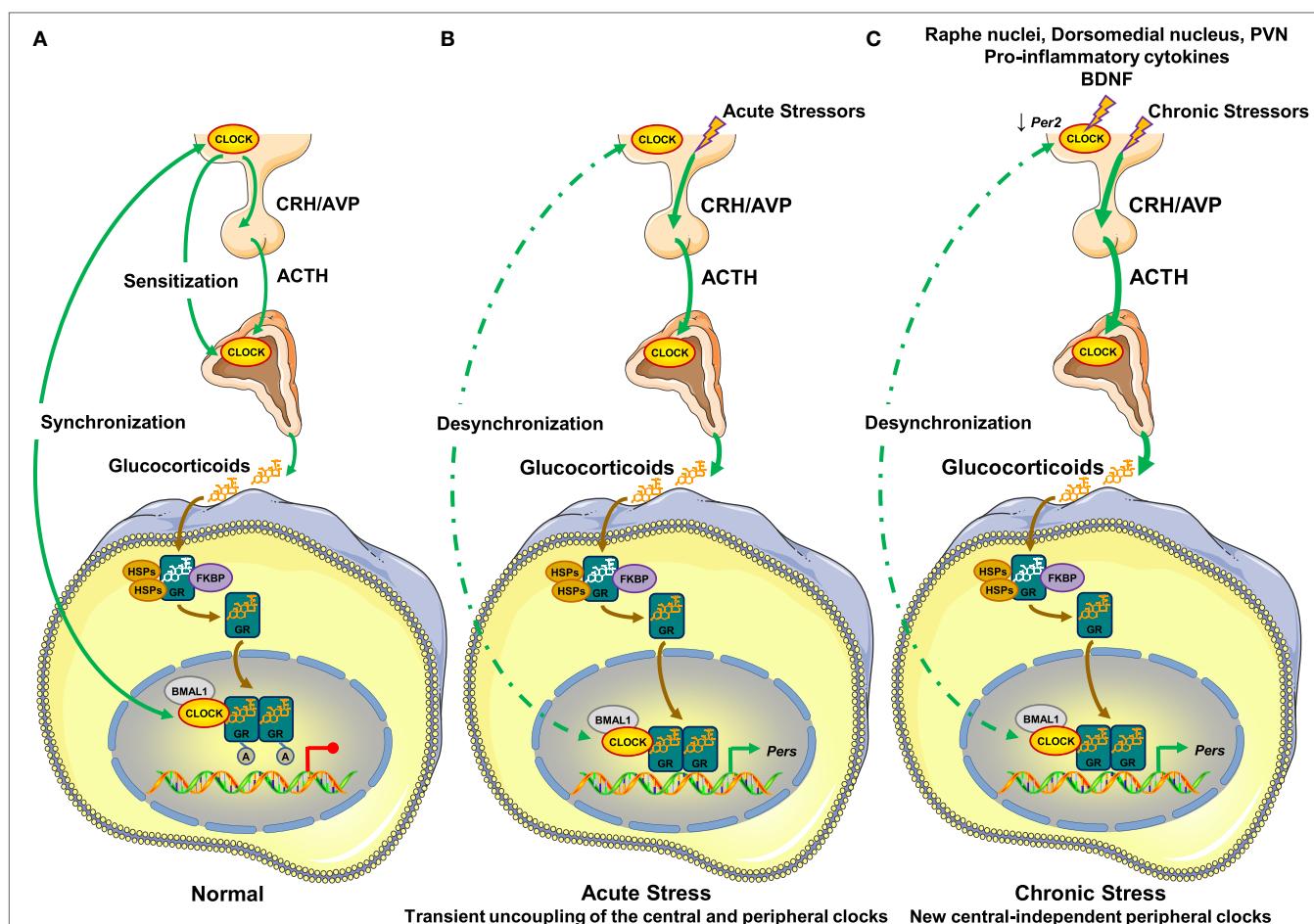
## Interplay between Stress and Circadian Clock Systems on the HPA Axis/Glucocorticoids

In the absence of stressors, the central clock regulates the HPA axis activity and influences the sensitivity of the adrenal cortex to

ACTH (28, 46, 47). These two mechanisms are responsible for the diurnal fluctuation of circulating glucocorticoid concentrations (28, 46, 47). The peripheral clocks are synchronized to the activity of the central clock through as yet unknown, perhaps neural or endocrine mechanisms (79, 80). In addition, the peripheral clocks suppress the hGR $\alpha$ -induced transcriptional activity through acetylation of the receptor by the CLOCK/BMAL1 heterodimer, possibly functioning as a counter-regulatory mechanism against the circulating glucocorticoid concentrations (48) (Figure 2).

Under stressful conditions, acute stressors induce HPA axis activity, thereby increasing the synthesis and secretion of

glucocorticoids by the adrenal cortex. Upon binding to the GR $\alpha$ , secreted glucocorticoids phase shift the expression of several clock-related genes, such as *Per1* and *Per2*, and reset peripheral clocks but not the central clock, granted that GR $\alpha$  is not expressed in SCN neurons (65, 66, 68, 81, 82) (Figure 2). Therefore, the circadian rhythm in peripheral clocks phase shifts from that of central clock under the regulation of the HPA axis, leading to transient uncoupling of the central and peripheral clocks (48). Following termination of the acute stress, the central clock can reset peripheral clocks to their initial phase within a few days (65, 83). hGR $\alpha$ -induced transcriptional activity may be influenced by



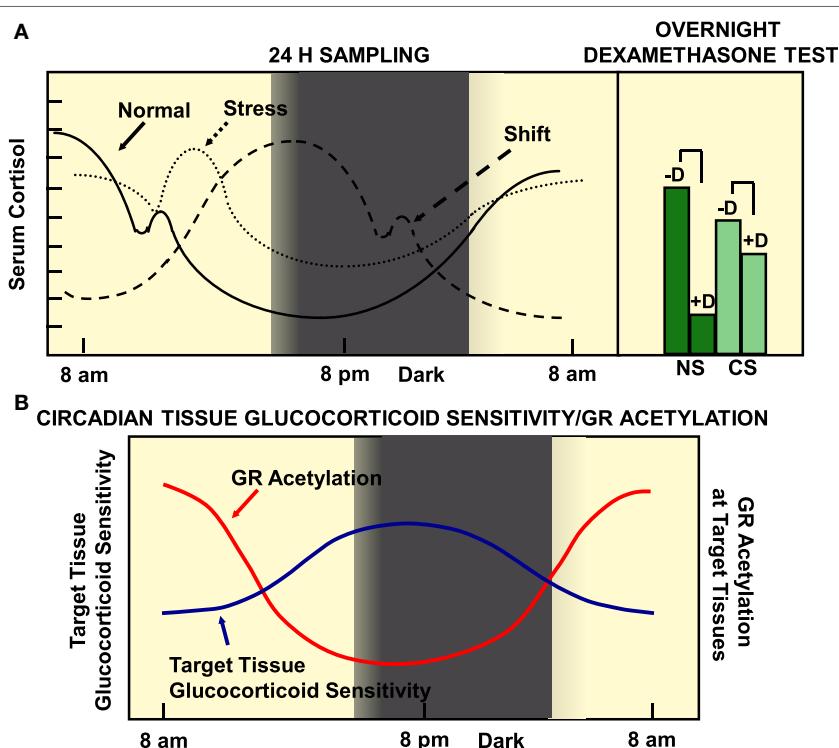
**FIGURE 2 | Molecular interactions between the hypothalamic–pituitary–adrenal (HPA) axis and the circadian clock system. (A)** In the absence of stressors, **(B)** under acute stressors, and **(C)** under chronic stressors. **(A)** In normal conditions, the central suprachiasmatic nucleus (SCN) clock creates the diurnal fluctuation of glucocorticoid concentrations by regulating the activity of the HPA axis through neuronal projections and by influencing the adrenal cortex sensitivity to ACTH through the splanchnic nerves. The peripheral clocks are synchronized to the circadian activity of the central clock through unknown mechanisms and suppress the transcriptional activity of the hGR $\alpha$  by CLOCK-mediated acetylation of the receptor. **(B)** Under stressful conditions, acute stressors activate the HPA axis leading to increased glucocorticoid concentrations independently of the central clock-mediated circadian regulation of the HPA axis. In peripheral tissues, glucocorticoids phase shift and reset peripheral clocks leading to uncoupling of the latter from the central clock, granted that the GR $\alpha$  protein is not expressed in the SCN. In addition, the transcriptional activity of the hGR $\alpha$  may be influenced by the phase-shifted peripheral clocks by unknown mechanisms. Following termination of the acute stress, the central clock can reset peripheral clocks to their initial phase. **(C)** In the presence of chronic stressors, the SCN receives indirect glucocorticoid feedback from raphe nuclei, the hypothalamic dorsomedial nucleus, and the paraventricular nucleus. Its activity is also influenced by pro-inflammatory cytokines and BDNF. Chronic stressors trigger the release of glucocorticoids by the adrenal cortex independently of the central clock-mediated diurnal regulation of the HPA axis. This stress-induced glucocorticoid secretion phase shifts and resets peripheral clocks leading to uncoupling of the latter from the central clock. A, acetyl residue on the acetylated molecules; ACTH, adrenocorticotrophic hormone; AVP, arginine vasopressin; BDNF, brain-derived neurotrophic factor; BMAL1, brain–muscle–arnt-like protein 1; CLOCK, circadian locomotor output cycle kaput; CRH, corticotropin-releasing hormone; FKBP, FK506-binding protein; GR, glucocorticoid receptor; HSPs, heat shock proteins; PERs, periods.

phase-shifted peripheral clocks in local tissues, but the specific mechanisms remain to be elucidated (**Figure 2**).

In the presence of chronic or repeated stressors, accumulating evidence suggests that the non-expressing GR $\alpha$  SCN receives indirect glucocorticoid feedback from peripheral tissues expressing GR $\alpha$ , such as the raphe nuclei, the hypothalamic dorsomedial nucleus, and the PVN (84, 85) (**Figure 2**). Indeed, chronic stressors reduce the expression of *Per2* in the SCN, suggesting an impact of chronic stress on SCN function (86, 87). In addition to glucocorticoids, several other molecules in the periphery (e.g., pro-inflammatory cytokines, brain-derived neurotrophic factor, etc.) provide feedback to the central SCN clock during chronic stress (88, 89) (**Figure 2**). As chronic or repeated stressors trigger the secretion of glucocorticoids into the systemic circulation independently of the central clock-mediated diurnal regulation of the HPA axis, stress-related glucocorticoid secretion phase shifts and resets peripheral clocks leading to uncoupling of the latter and the central clock (48) (**Figure 2**). We speculate that a prolonged and/or excessive or deficient adaptive response may not allow the proper rhythmicity of peripheral clocks under the control of the central clock, ultimately leading to several pathologic conditions.

## CLINICAL IMPLICATIONS

Alterations in circadian release of glucocorticoids have been found in several pathologic conditions, such as mood, metabolic and inflammatory disorders, as well as in cancers (60). On the other hand, chronically stressed (CS) humans, rotating shift workers and subjects frequently exposed to jet lag because of trans-timezone traveling have been demonstrated to be at increased risk for cardiometabolic disorders and their sequelae myocardial infarction and stroke (69). Compared to normal subjects, CS individuals might display an uncoupling between the circadian clock and the HPA axis, a decreased variance between evening nadir and morning zenith cortisol concentrations, as well as an inadequate response to a low-dose dexamethasone suppression test as a result of chronic hyperactivation of PVN CRH and vasopressin secretion (90) (**Figure 3**). We hypothesize that shift workers or trans-timezone travelers might show a phase-altered curve of cortisol concentrations (**Figure 3**). Individuals with alterations in circadian secretion of glucocorticoids might develop psychiatric diseases (e.g., anxiety and depression) and autoimmune/inflammatory conditions, with



**FIGURE 3 | (A)** Circadian pattern of cortisol secretion in normal humans, CS subjects and rotating shift workers (left panel), and the responses of normal and stressed subjects to overnight dexamethasone suppression test. **(B)** The target tissue sensitivity is lower in the morning and higher at night, mirroring the status of GR acetylation. **(A)** A population of 284 51-year-old men were examined by obtaining a detailed medical history, by performing anthropometry, and by measuring a series of diurnal salivary cortisol concentrations. Participants were asked to fill in a questionnaire about self-perceived stress and underwent a low-dose overnight dexamethasone suppression test. Normal participants were characterized by increased variance, distant zeniths in the morning and nadirs in the evening, and an appropriate suppression in the morning salivary cortisol concentrations following a low-dose dexamethasone suppression test. On the other hand, CS participants showed a decreased variance, evening nadir elevations and morning zenith decreases of cortisol concentrations, as well as an inadequate response to a low-dose dexamethasone suppression test. We speculate that rotating shift workers might be characterized by a phase-delayed curve of salivary cortisol concentrations, compared to that of normal participants. CS, chronically stressed individuals; D, midnight dexamethasone administration; NS, non-stressed individuals. Modified from Ref. (35, 90).

rheumatoid arthritis and asthma as representative examples (91). Furthermore, chronic stress with loss of a proper cortisol circadian rhythm result in glucocorticoid excess-related increased appetite, splachnic obesity, and metabolic disturbances, such as hyperglycemia, insulin resistance, dyslipidemia, osteopenia/osteoporosis, and hypertension (27). All the above pieces of evidence strongly suggest that any dysfunction of the stress system may cause dysregulation of the circadian clock system and *vice versa*.

Several studies in animals and humans have shown that diurnal fluctuations of cortisol concentrations are flattened in obesity. Indeed, genetically obese rats, *db/db* mice, and obese adults display damped glucocorticoid circadian rhythms (92, 93). In addition, childhood overweight and obesity are associated with a reduction in the amplitude of ultradian glucocorticoid secretion (94). Moreover, subjects carrying specific *Clock* polymorphisms are more susceptible to develop obesity and metabolic syndrome (95, 96). Not only in obesity but also in diabetes mellitus there is a flattened 24-h pattern of cortisol concentrations. In diabetic adults, salivary cortisol was low in the morning and high in the afternoon and evening (97). Finally, mice deficient in the *Cry* gene had a defective suppression of HPA axis, ultimately leading to glucose intolerance and metabolic syndrome (98).

Disruption of circadian rhythms, as often observed in shift workers, may cause mood disorders (99). Mice exposed to a short 7-h light/dark change have higher concentrations of corticosterone and exhibit depressive symptoms (100). On the other hand, subjects suffering from major depression have flattened diurnal glucocorticoid rhythms, probably due to altered secretion of CRH and AVP in the brain (101). They also display defective clock-related gene expression in the peripheral blood cells, as well as in brain tissues (102, 103). Indeed, the rhythmic expression of core clock transcription factor genes, such as *Bmal1*, *Per1-3*, *Dec1/2*, and *Rev-erba* is attenuated in brain regions influencing mood, compared to healthy controls. In addition to depression, several other psychiatric conditions, including bipolar disorder, post-traumatic stress disorder, attention deficit hyperactivity disorder, schizophrenia, and chronic alcoholism, are examples in which the interconnection between the stress system and the circadian clock system is dysregulated (104).

Chronic inflammatory disorders, such as rheumatoid arthritis and asthma, are characterized by worsening symptoms in early morning hours (60). This phenomenon has been attributed to the circadian fluctuation of circulating cytokines, such as interleukin (IL)-1 $\alpha$ , IL-6, and tumor necrosis factor- $\alpha$ , which strongly participate in the pathogenesis of these diseases (105, 106). These inflammatory cytokines reach their peak concentrations earlier than cortisol; however, the target tissue glucocorticoid sensitivity is low during that time because of CLOCK-mediated suppressed hGR $\alpha$ -transcriptional and transpressive activities (48). Furthermore, night-shift workers have increased risk for common infections and multiple sclerosis, indicating that dysregulation of the circadian clock system contributes undoubtedly to the development of inflammatory diseases (107, 108).

Subjects with prolonged night-shift work are also more susceptible to develop several cancers (109). Previous studies have shown that SCN damage, chronic jet lag, as well as *Per2* deficiency

contribute to cancer initiation and progression (110–112). On the other hand, patients with breast, ovarian, lung, and kidney cancers have flattened or antiphase diurnal oscillations of cortisol, compared to normal subjects (60). Future studies are still needed to clarify the molecular mechanisms underlying the association between stress, circadian rhythms, and carcinogenesis.

In addition to metabolic, psychiatric, autoimmune/inflammatory, and malignant disorders, sleep disturbances and disorders have been associated with alterations in circadian secretion of glucocorticoids (69). Indeed, a prolonged or excessive activation of the HPA axis results in insufficient sleep, which, in turn, may cause an elevation of glucocorticoid concentrations and cytokines in the early evening, forming a vicious cycle (69). Moreover, sleep deprivation has been associated with an increase in HPA axis activity in the evening hours, thereby altering the ability of the latter to properly autoregulate itself (113–115). Idiopathic chronic insomnia has been associated with evening hypercortisolism and hypercytokinemia influencing substantially the transcription rate of numerous genes in the brain (116, 117). On the other hand, a dysfunctional HPA axis could promote insomnia (69) or be affected by obstructive sleep apnea (118, 119). Not only chronic insomnia but also chronic fatigue syndrome, fibromyalgia and posttraumatic stress disorder have been linked to consistent alterations in HPA axis activity (120, 121).

## CONCLUDING REMARKS AND FUTURE DIRECTIONS: SYNTHETIC GLUCOCORTICOIDS IN THE ERA OF CHRONOTHERAPY

Synthetic glucocorticoids have been widely used in the treatment of several inflammatory disorders and hematologic malignancies (122). Since target tissue glucocorticoid sensitivity is lower in the morning and higher in the evening, glucocorticoid analogs should be administered in a time-of-day dependent fashion to achieve a beneficial therapeutic outcome and to avoid their detrimental side effects, such as osteoporosis, weight gain, glucose intolerance, and psychiatric symptoms. Therefore, patients with autoimmune disorders are treated with prednisolone or other synthetic glucocorticoids in the evening, given that the hGR $\alpha$  is less acetylated during that time (106). In addition to inflammatory disorders, chronic administration of glucocorticoids is frequently used as substitution treatment of hypocortisolemic disorders, such as adrenal insufficiency, regardless of its etiology. Approximately two-thirds of hydrocortisone dose is usually given in the morning, while the remainder one-third is administered in two doses (in the mid-day and in the early evening) (123). Recent advances in the therapeutic manipulation of adrenal insufficiency have shown that a dual-release hydrocortisone formulation, which resembles circadian cortisol secretion, results in improved quality of life, decreased body weight and blood pressure, and improved glucose tolerance (124, 125).

Although we have gained important insight in the molecular communication between the stress system and the circadian clock system, there are many physiologic and pathophysiological aspects of their interrelation that still elude us. The molecular

mechanisms underlying resetting of peripheral clocks by glucocorticoids are under intense investigation both in normal and pathologic conditions. Moreover, the tight synchronization of peripheral clocks with the central clock remains poorly understood. Furthermore, our understanding on the function and significance of local adrenal clocks is still increasing. Future *in vitro* and *in vivo* studies will shed light on the functional significance of the cross talk between the stress system and the circadian clock system of living organisms to increase survival chance. Importantly, in parallel with the tremendous progress of molecular, cellular, and structural biology, significant advances in the field of mathematical and computer biosciences will undoubtedly help us have a deeper understanding of system interrelations. Interestingly, accumulating evidence suggests that stress-related

“static” signaling pathways can be effectively transformed into functionally predictive computerized kinetic models (126–129). Such efforts will be useful for accurate predictions of a system response to acute or chronic stress, as well as to pharmacotherapy with novel medications.

## AUTHOR CONTRIBUTIONS

All authors contributed equally to the conception of the work, drafted the manuscript or revisited it critically for important intellectual content, finally approved the version to be published, and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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# ACTH Antagonists

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Adrenocorticotropin (ACTH) acts via a highly selective receptor that is a member of the melanocortin receptor subfamily of type 1 G protein-coupled receptors. The ACTH receptor, also known as the melanocortin 2 receptor (MC2R), is unusual in that it is absolutely dependent on a small accessory protein, melanocortin receptor accessory protein (MRAP) for cell surface expression and function. ACTH is the only known naturally occurring agonist for this receptor. This lack of redundancy and high degree of ligand specificity suggests that antagonism of this receptor could provide a useful therapeutic aid and a potential investigational tool. Clinical situations in which this could be useful include (1) Cushing's disease and ectopic ACTH syndrome – especially while preparing for definitive treatment of a causative tumor, or in refractory cases, or (2) congenital adrenal hyperplasia – as an adjunct to glucocorticoid replacement. A case for antagonism in other clinical situations in which there is ACTH excess can also be made. In this article, we will explore the scientific and clinical case for an ACTH antagonist, and will review the evidence for existing and recently described peptides and modified peptides in this role.

**Keywords:** adrenocorticotropin hormone, receptor antagonism, Cushing's syndrome, congenital adrenal hyperplasia, high throughput screening, G protein-coupled receptor, receptor modelling, peptide hormone antagonists

## INTRODUCTION

The impact of receptor antagonism on modern medicine cannot be understated. Classical examples include the β-blockers in the treatment of hypertension and cardiovascular disease (1) and histamine H2 antagonism in the treatment of gastric hyperacidity (2). Even in the field of endocrinology, receptor antagonism of steroid hormones [e.g., tamoxifen (3), eplerenone (4), and flutamide (5)] and some peptide hormones [e.g., pegvisomant (6) and conivaptan (7)] has had major life-changing impact. The pituitary–adrenal axis is one endocrine axis that when disrupted can be associated with a wide range of pathologies, and yet, despite the fact that it comprises several unique and thus highly targetable components, receptor antagonism has received little attention as a therapeutic approach.

In this article, we will examine the possible benefits of development of an effective antagonist to a key component of this axis, the peptide hormone adrenocorticotropin (ACTH). The disorders in which clinical benefit might be attained will be considered. We will then consider the nature of the target – ACTH and the ACTH receptor complex, and certain unique features before discussing the history of ACTH antagonist research, ending with a description of the current state-of-the art. Initially, a brief description of the pituitary–adrenal axis and its key components is necessary.

## THE PITUITARY-ADRENAL AXIS

The corticotroph cells of the anterior pituitary gland are responsible for synthesis and secretion of the 39 residue peptide, ACTH (8). ACTH is derived from a larger precursor protein, pro-opiomelanocortin (POMC), by the action of a specific pro-hormone convertase enzyme (PC1 or PCSK1) (9). In other tissues – for example, the hypothalamus – this precursor is processed differently to produce  $\alpha$ -MSH instead of ACTH (10). ACTH is synthesized and secreted by the pituitary in response to tonic control from the hypothalamus – principally in the form of two peptide hormones – corticotrophin-releasing hormone (CRH) and vasopressin (AVP), which in turn are regulated by multiple higher factors including stress (11).

Adrenocorticotropin has a short half-life in the circulation (12) and acts on a highly specific G protein-coupled receptor expressed almost uniquely in the adrenal cortex (13). This receptor, the MC2R is one of five members of the melanocortin receptor family – see **Table 1**. ACTH can activate all five of these receptors, although at physiological circulating levels, the sensitivity of the other receptors is such that they are not activated. Importantly, the naturally occurring agonists for these other receptors –  $\alpha$ -MSH,  $\gamma$ -MSH, and possibly  $\beta$ -MSH – have no affinity for the MC2R (14, 15). Thus the MC2R is a highly sensitive and highly specific receptor for ACTH with a major, essential function of stimulating the fasciculata cells of the adrenal cortex to synthesize and secrete glucocorticoid. In addition, ACTH can stimulate zona glomerulosa cells to secrete mineralocorticoid and zona reticularis cells to secrete adrenal androgens.

Glucocorticoid (cortisol in man and most other species, corticosterone in rodents), secreted by the adrenal gland exert a plethora of physiological actions on virtually every cell in the organism. These actions are the result of interaction with the widely expressed glucocorticoid receptor – a nuclear hormone receptor. Glucocorticoid may also activate a second related receptor – the mineralocorticoid receptor – which is less widely expressed. However, the action of the 11  $\beta$ -hydroxysteroid dehydrogenase type 2 enzyme inactivates glucocorticoid in mineralocorticoid receptor expressing tissues under normal circumstances leaving these receptors responsive to aldosterone (16). From an endocrine perspective, a key role of glucocorticoid is to feedback negatively on the pituitary and hypothalamus to inhibit ACTH secretion (17).

From this brief description, it can be seen that in theory, the MC2R should provide a perfect substrate for receptor targeting. This is a receptor with, effectively, a single function, expressed

in a highly tissue-restricted way and activated by a single, highly specific agonist. The question is – if it were possible to design the perfect antagonist – what clinical role might it play?

## DISORDERS OF THE PITUITARY-ADRENAL AXIS

Disorders of this axis are, fortunately, uncommon and can be subdivided into disorders of hormone deficiency and excess. Glucocorticoid deficiency seems unlikely to benefit from MC2R antagonism, but in certain specific circumstances, there could be a valuable role for this therapeutic option as discussed later.

### Glucocorticoid Excess

Glucocorticoid excess may result from primary adrenal disease – typically an adrenal adenoma or carcinoma – and is independent of ACTH. Indeed ACTH is normally suppressed by the actions of the negative feedback loop. More often, cortisol excess or Cushing's syndrome is the result of a pituitary adenoma secreting excess ACTH – known as Cushing's Disease – or less commonly a non-pituitary tumor that "ectopically" secretes ACTH. This group of disorders might theoretically provide a suitable target for an MC2R antagonist.

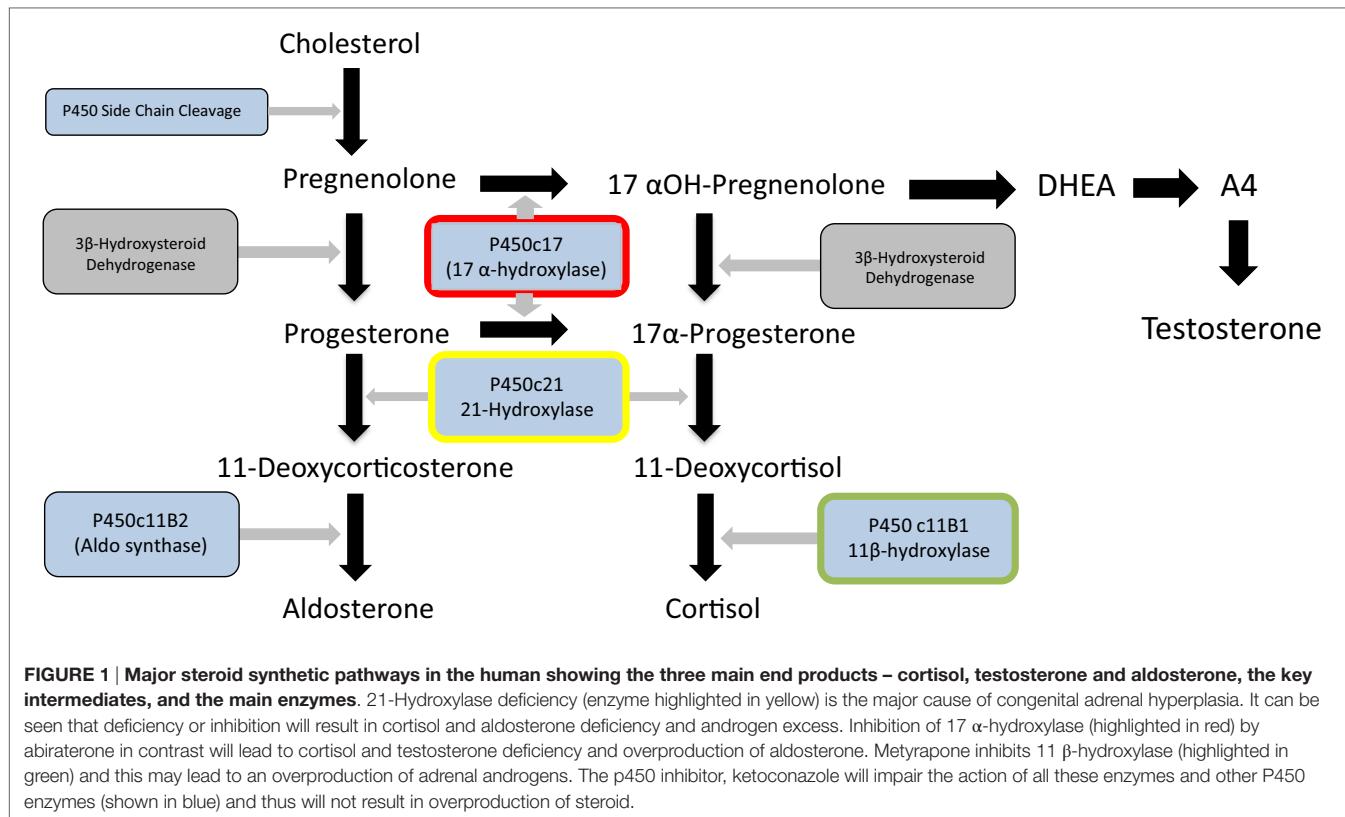
### Cushing's Disease

Corticotroph adenomas are small, usually slow growing, benign tumors that normally come to clinical attention as a result of the effects of glucocorticoid excess, rather than because of the physical effects of an expanding tumor. Typically, Cushing's syndrome may take many years to develop. Consequently the diagnosis of the disorder and exclusion of other causes of Cushing's syndrome is a significant challenge. Once a diagnosis is conclusively made, the optimal treatment is surgical removal of the tumor – ideally preserving the remaining pituitary function. Surgery for Cushing's disease requires extensive experience and skill and is normally undertaken in specialist centers (18).

In some patients, the metabolic consequences of their untreated glucocorticoid excess are so significant that there would be risks in immediately proceeding to complex or prolonged surgery. The glucocorticoid synthesis blockers metyrapone and/or ketoconazole are frequently used in this situation to reduce steroid production (see **Figure 1**), and most patients tolerate and respond to this treatment reasonably well (19–21). However, an MC2R antagonist could be equally effective in this situation.

**TABLE 1 | A summary of the main features of each of the melanocortin receptors in the human.**

Major sites of expression	Ligand preference	Function	Effect of deletion	Comments
MC1R	Melanocytes	$\alpha$ -MSH > ACTH > $\gamma$ -MSH	Pigmentation of hair and skin	Red hair, pale skin
MC2R	Adrenal cortex	ACTH	Steroidogenesis adrenal growth	Adrenal failure
MC3R	Brain, spinal cord	$\gamma$ -MSH > $\alpha$ -MSH = ACTH	Complex, inhibits POMC neurones	Obesity
MC4R	Brain, spinal cord	$\alpha$ -MSH > ACTH > $\gamma$ -MSH	Appetite regulation	Obesity
MC5R	Multiple tissues	$\alpha$ -MSH > ACTH > $\gamma$ -MSH	Exocrine gland function	Defective water repulsion Enhanced action with MRAP2 AGRP is natural antagonist



Following surgery, the glucocorticoid excess will come under rapid control in a minority of patients. More frequently, there will be a reduction in steroid over-secretion that may tail off over several weeks. In other cases, it may be necessary to re-explore the pituitary surgically, and this may result in pituitary clearance with a loss of other pituitary hormones (18). Control of glucocorticoid excess during this interim period will often necessitate the use of metyrapone and/or ketoconazole.

If further surgical measures are unsuccessful, endocrinologists may turn to the somatostatin receptor 5 agonist, pasireotide, which directly targets the corticotroph adenoma and provides partial or complete control of Cushing's in a proportion of cases (22). Other second-line options in this situation include pituitary radiotherapy or adrenalectomy. The former may take several years to normalize the glucocorticoid excess, necessitating metyrapone and/or ketoconazole during this time. An MC2R antagonist could be a possible alternative.

While glucocorticoid synthesis inhibitors are usually effective and reasonably well tolerated in these situations, there are some potential disadvantages to their prolonged use. Metyrapone blocks the 11 $\beta$ -hydroxylase enzyme, required for the last step of cortisol synthesis from 11-deoxycortisol. Consequently, as shown in **Figure 1**, steroid precursors are channeled through the androgen pathways resulting in increased secretion of adrenal androgens. In women, this may induce hirsutism and in pre-pubertal children both virilization and early puberty (23, 24). Ketoconazole, a cytochrome P450 inhibitor, inhibits several

steps of steroidogenesis and does not usually cause androgen excess. However, complications include potential rare but serious hepatotoxicity and multiple drug interactions. Consequently, although these treatments are effective and inexpensive, there could be a place for a highly specific MC2R antagonist in the management of Cushing's disease.

### Ectopic ACTH Syndrome

The mechanisms of ectopic ACTH syndrome are essentially the same as those of Cushing's disease except that the underlying tumor is outside the pituitary gland. These rare tumors are often small carcinoid tumors that may occur anywhere in the lungs and gastrointestinal tract. Improvements in imaging over the last 10–20 years together with intravenous sampling for ACTH have made identification of the primary source far simpler. Once identified, and if surgical removal of the underlying tumor is possible, then this can be curative. As with Cushing's disease, it may be helpful to use a cortisol synthesis blocking drug while waiting for definitive treatment (18) and in cases where definitive treatment is not possible. Hence, for the same reasons described above, an MC2R antagonist may have a place in the clinical management of such conditions.

Small cell lung cancer makes up about 20% of all lung cancer and is a highly malignant neuroendocrine tumor of poor prognosis. This tumor is frequently associated with ectopic ACTH secretion, and the development of Cushing's syndrome in this disease may obviously worsen prognosis. Thus there may also be value in using an ACTH antagonist in this clinical situation.

## Glucocorticoid Deficiency

The second group of disorders in which there may be an place for antagonizing ACTH action are those in which the physiological negative feedback of cortisol is reduced or lost, leading to a compensatory increase in ACTH secretion. In this situation, it is the stimulation of non-glucocorticoid adrenal steroidogenesis by ACTH, which requires control.

## Congenital Adrenal Hyperplasia

The most prominent example of such a situation is that of congenital adrenal hyperplasia, caused in the majority of cases by mutations in both alleles of the *CYP21* gene encoding the 21-hydroxylase enzyme necessary for the penultimate step of cortisol synthesis (**Figure 1**) (25, 26). This is one of the commonest human autosomal recessive disorders occurring in about 1 in 15,000 live births. Reduced glucocorticoid feedback results in ACTH stimulation of adrenal androgen production. As a consequence, affected female children are likely to be virilized or have ambiguous genitalia at presentation. Life-long treatment with glucocorticoids will restore any cortisol deficiency and suppress ACTH secretion and subsequent androgen production (27). However, achieving the optimal dose and timings of hydrocortisone replacement to avoid the adverse effects of excessive glucocorticoid on growth and metabolism while maintaining adequate androgen suppression is challenging, resulting in poor health outcomes (28). Availability of an easily administered ACTH antagonist would be likely to facilitate treatment, allowing a “block and replace” approach in which the physician could focus on treatment of glucocorticoid replacement alone rather than androgen suppression.

## Prostate Cancer Treatment

The use of the drug Abiratarone in the treatment of prostate cancer induces a form of acquired adrenal hyperplasia. Abiratarone is a potent inhibitor of the 17  $\alpha$ -hydroxylase and 17,20 lyase enzymes in the adrenal and is used to very effectively reduce the production of adrenal androgens in castration-resistant prostate cancer, with valuable benefits to prostate cancer treatment (29). Examination of adrenal steroid synthetic pathways (**Figure 1**) demonstrates that this inhibition is likely to channel steroid synthesis toward deoxycorticosterone synthesis and aldosterone production, leading to mineralocorticoid excess and glucocorticoid deficiency. The latter requires glucocorticoid replacement, but the fluid overload, hypertension, and hypokalemia resulting from aldosterone excess require treatment with a mineralocorticoid antagonist such as eplerenone (30). Use of an ACTH antagonist together with a replacement dose of hydrocortisone may be a preferred approach in this situation.

## Investigation of Endocrine Disease

A further potential use of an ACTH antagonist is in the investigation of adrenal disorders. One of the key questions in the investigation of Cushing’s syndrome is whether the cortisol excess is ACTH dependent. A number of tests have been used to determine this including the dexamethasone suppression test and the CRH stimulation test, combined with measurements of plasma ACTH and imaging studies. Hypoglycemic stress tests and metyrapone tests may also be required in complex cases (31). It is conceivable

that the use of a single dose ACTH antagonist test could provide a simple and clear solution to this question, although it is more likely that its use in combination with other investigations would be required in most cases.

## THE TARGET

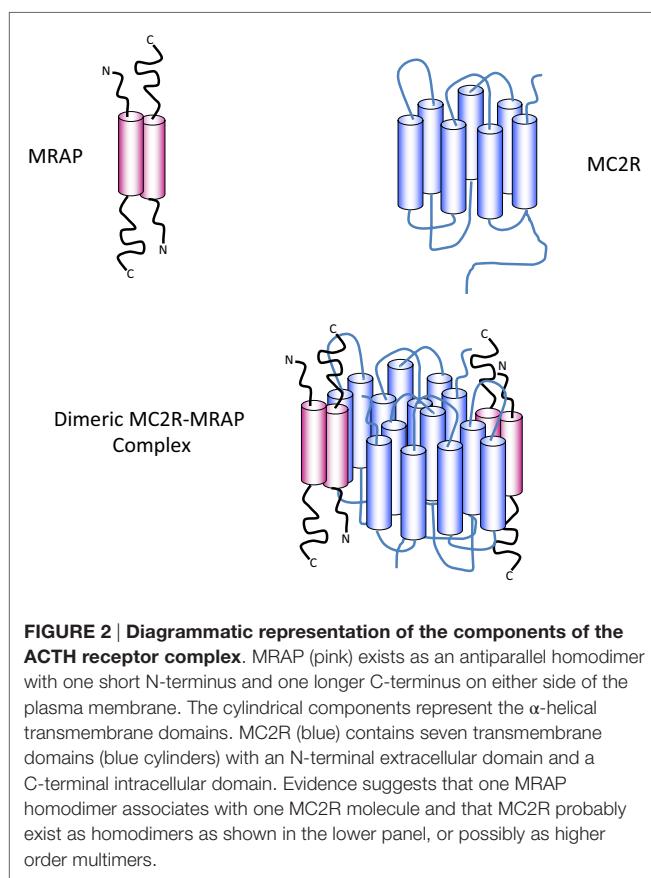
As discussed in the Section “Introduction,” the receptor for ACTH presents a remarkably attractive target for pharmacological manipulation. It is highly specific for a single peptide agonist – ACTH [1–39], and has no affinity or response to any other naturally occurring agonist. It is expressed in functional quantities only in the adrenal cortex, and thus the possibility of unwanted off-target effects of an antagonist is unlikely.

The key component of the ACTH receptor complex is the seven transmembrane domain MC2R – perhaps surprisingly, the smallest of all the G protein-coupled receptor (GPCR) family at only 289 residues in length (32). The MC2R cannot function alone as an ACTH receptor, which led to many difficulties in its characterization after initial cloning (33). The discovery that deficiency of a small, single transmembrane domain protein caused a clinical syndrome essentially identical to that caused by MC2R deficiency led to the identification of the melanocortin 2 receptor accessory protein (MRAP) as the MC2R co-receptor (34, 35).

Melanocortin receptor accessory protein is a highly unusual protein in that it naturally exists as an antiparallel homodimer and seems to be necessary for trafficking and cell surface expression of the MC2R, as well as binding of ACTH and hence signal transduction (36, 37). In common, with many other GPCRs, MC2R has the potential to homodimerise and the evidence suggests that it exists as a homodimer with two MRAP molecules, in an antiparallel homodimer formation, associated with each MC2R component (38) (**Figure 2**).

The nature of the ligand, ACTH is important in understanding receptor function (**Figure 3A**). The strongly conserved N-terminal 24 residues of ACTH are almost as efficient as the 39 residue naturally occurring peptide in activating this receptor. Further truncation of ACTH from the C-terminus is associated with gradual loss of activity until removal of the four basic residues (Lys-Lys-Arg-Arg) in positions 15–18, which inactivates this peptide at the ACTH receptor (39, 40). The first 13 residues are however active at all the other melanocortin receptors and thus it seems that this “tetrabasic” region acts as a “key” to unlock the MC2R-MRAP complex.

The evidence suggests that once the receptor is “unlocked,” the N-terminal region is an effective agonist for the receptor. As with all the melanocortin receptors, the His-Phe-Arg-Trp sequence (or HFRW sequence using the single letter amino acid code) at positions 6–9 and to some extent those residues flanking this induce the conformational changes required to activate the receptor. This HFRW sequence is fundamental to activation of all the melanocortin receptors and can be considered the “message” region of the peptide (40, 43, 44). Interestingly, a naturally occurring human mutation of Arg 8 in the HFRW sequence results in biologically inactive ACTH (45). The most N-terminal region (Ser-Tyr-Ser) has been reported to potentiate the action of the HFRW sequence (46).



Great strides have been made in recent years in understanding the three dimensional nature of GPCRs, based around a growing number of receptor crystal structures. No melanocortin receptor crystal structure has yet been reported, but increasingly sophisticated modeling exercises combined with receptor mutagenesis and substitution studies are providing information on how ligands interact with their receptor.

Pogozheva et al. studied the MC4R-binding site for NDP-MSH (a highly potent analog of  $\alpha$ -MSH) and two small molecule agonists using a combination of alanine scanning mutagenesis of the receptor followed by functional analysis and *in silico* modeling. They concluded that the HFRW sequence of NDP-MSH was required to form a  $\beta$ -hairpin-like structure so that the phenyl ring of Phe 7 interacts with the indole ring of Trp 9 (**Figure 3B**). This allows interaction between His 6 of NDP-MSH and Glu 100 in transmembrane domain 2 (TM2) of MC4R, and Arg 8 of NDP-MSH and Asp 115 and 119 in TM3 of MC4R. The interacting aromatic residues of this sequence, Phe 7 and Trp 9 interact with the aromatic Phe at positions 261 and His 264 of MC4R in TM6 (41).

A relatively similar picture of NDP-MSH binding to the MC5R was constructed by Yang and colleagues using site-directed mutagenesis and structural modeling. They also suggested that Asp 115 and 119 in TMD 3 interact with Arg 8 of the HFRW sequence and that Phe 195 (in TMD 5) and Phe 254 (TMD 6) interact with Phe 7 and Trp 9 of HFRW. All of these residues are conserved in the MC2R (and other melanocortin

receptors), and it seems highly likely that these interactions are critical in determining the HFRW binding and activation of this receptor (42). Indeed, naturally occurring homozygous mutations of Asp 103 and 107 in MC2R, the equivalent conserved Asp residues in this receptor, lead to ACTH resistance/Familial Glucocorticoid Deficiency (47). This arrangement is shown in **Figure 3C**.

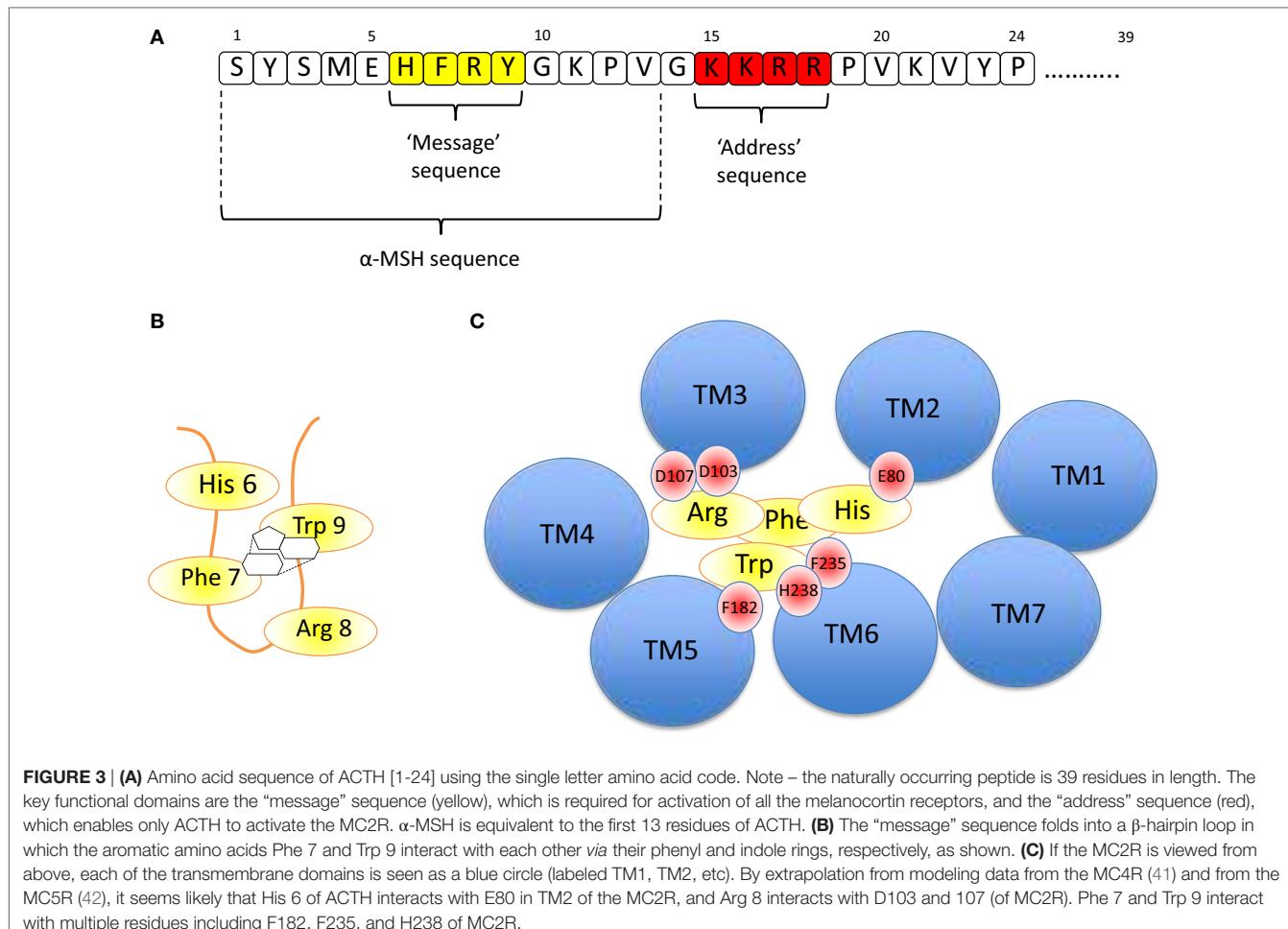
Using a receptor chimera approach in which regions of the MC4R were substituted into the MC2R, Fridmanis et al. suggested that one of the MRAP molecules binds to MC2R in the region of transmembrane domains 4 and 5 to create a binding pocket for the tetrabasic “address” sequence in ACTH. Following this interaction, a conformational shift in the receptor transmembrane domains takes place, which permits the formation of the HFRW-binding pocket (14). Although this remains speculative, it is an attractive hypothesis. It is notable that Malik et al. have shown that it is the N-terminal region of the MRAP molecule that is required on the extracellular surface of the cell for ACTH binding (48). Clearly complete understanding of this complex area will ultimately require determination of a crystal structure of the MC2R-MRAP-ACTH complex.

## APPROACHES TO ANTAGONIZING ACTH

Given the extensive knowledge of the interaction of ACTH with its receptor gained over about 50 years one might anticipate that it would be a relatively straightforward matter to design an ACTH-like peptide with antagonist properties. The first attempts to do this resulted in peptides that retained the tetrabasic address region, but lacked the HFRW message sequence. This led to the development of ACTH [11–24] (49, 50) as a potential receptor antagonist. Li et al. isolated a naturally occurring peptide, ACTH [8–39], from human pituitary, which they showed to have ACTH antagonistic effects *in vitro*, and they called this corticotrophin-inhibiting peptide (CIP) (51). However, the data with each of these potential antagonists has been confusing with discrepant results for steroidogenesis and cAMP generation in some cases. For example, Szalay demonstrated that ACTH [11–24] stimulated steroidogenesis in dispersed zona glomerulosa and zona fasciculata cells (52), and Goverde and Smals (53) demonstrated some steroidogenesis with this peptide.

More recently, Kovalitskaia et al. investigated the binding of a wide range of ACTH fragments derived from an ACTH [11–24] parent peptide. They reported that the ACTH [15–18] tetrabasic fragment alone was an effective competitor for ACTH [11–24] in ligand-binding assays, and that it also failed to stimulate cAMP generation in adrenocortical membranes (54). Its use in competition with ACTH in cAMP generation or steroidogenesis has not been reported.

The consensus from most researchers seems to be that ACTH [11–24] is not an effective ACTH antagonist. This may be because the interaction between the tetrabasic region of ACTH converts the MC2R into a “primed receptor with an unoccupied HFRW-binding site, which may then be activated by the natural agonist.” Hoffman therefore used a different approach and developed an analog in which the Trp residue at position 9 of the HFRW message sequence was substituted with Phe or *N*-methyl Trp,



and showed inhibition of ACTH stimulated cAMP generation on bovine adrenal membranes (55).

Liang et al. has described a number of peptide analogs of ACTH based on alanine and histidine substitutions around the HFRW region and in the spacing between this and the tetrabasic region (56). In this work, they described the marked reduction in MC2R activation observed with some of these peptides and, in a US patent filed the preceeding year, they reported potent ACTH antagonism with an ACTH [15-24] decapeptide (57).

In all of the above studies, the actions on adrenal tissues, slices, cells, or membranes have been studied, but little or no data on the selectivity of these peptide antagonists for the MC2R, or even melanocortin receptors in general have been obtained. It is usually highly desirable that any receptor active drug used therapeutically is selective for its target receptor and lacks off-target effects. The functions of the other melanocortin receptors and the effects of antagonizing or deleting them are summarized in Table 1, and it can be seen that a non-selective agent could induce a number of unwanted effects.

Bouw and colleagues reported an approach in which ACTH peptides that retain the intact tetrabasic region were substituted at various positions in the HFRW sequence and in some cases were cyclized in order to enhance stability. HEK293 cells stably expressing human MC2R and MRAP were used, and cAMP

production was measured with a luminescence assay. Several peptides exhibited significant antagonist actions among which GPS1573 – a variant of ACTH [7-18] with an N-terminal nor leucine – proline sequence and d-Phe and dd-Trp (in place of the L-Phe and L-Trp) in the HFRW sequence, and a cyclized variant of this – GPS1574 were most potent ( $IC_{50}$ s of  $66 \pm 23$  nM and  $260 \pm 1$  nM, respectively). These peptides retain some antagonist effect on the MC3R, MC4R and MC5R at approximately an order of magnitude less than that on the MC2R (58).

In work published in this issue, Nensey et al. report the actions of these same analogs on rat adrenal cells and show inhibitory effects in which the dose responses to ACTH [1-39] were shifted to the right by one log order or more. They also conducted *in vivo* experiments in young rats but were unable to show inhibition of the ACTH response even at 400-fold molar excess of antagonist in the case of GPS1573. GPS1574 was partially inhibitory at 30 min after ACTH injection (59).

In a recent study, presented in abstract form, researchers from Ipsen Bioscience Inc. reported the development of an ACTH-related peptide, IRC-274. This peptide was shown to inhibit ACTH binding to the human MC2R and MRAP expressed in HEK 293 cells with an  $IC_{50}$  of 3 nM (60). cAMP generation in response to ACTH in this same model is inhibited with an  $IC_{50}$  of 38 nM. Using an *in vivo* hypophysectomized rat model in which ACTH is

infused by osmotic minipump, significant inhibition of corticosterone production was observed. Using a second model in which mouse AtT20 pituitary corticotroph tumor cells were implanted into athymic nude mice, inhibition of corticosterone was again observed until the implanted tumors outgrew the inhibitory action of IRC-274. Interestingly, this antagonist exhibits a high degree of selectivity for the MC2R and has no significant actions on other melanocortin receptors. The sequence and structure of this peptide have not been revealed.

## ALTERNATIVE APPROACHES

### Small Molecules

From the above, it seems that after a rather long and chequered history some progress is now being made in developing a peptide ACTH antagonist that might ultimately be developed for use *in vivo*. The problems associated with peptide-based medication are well-known and include a short half-life in the circulation, the need to administer them by injection and the risk of inducing immunogenicity. In certain circumstances, the benefits of a peptide outweigh these potential disadvantages, and advances are being made in deriving preparations of peptides that may be taken orally or intranasally. However in many cases, there will be a need for a reliable long-term therapy as discussed earlier. Under these circumstances, it would be desirable to have an orally active agent, which would most likely be a small (non-peptide) molecule. Substantial efforts have been made to develop small molecules as agonists of the MC4 receptor with some limited success (61). With this objective in mind, we have undertaken a high throughput screen of about 200,000 small molecules using a cell-line expressing the human MC2R and MRAP, and this may provide a promising approach if a molecule with sufficient potency and selectivity can be identified.

### Antibody-Based Approaches

Humanized monoclonal antibodies directed against key signaling molecules have proven to provide effective therapeutic solutions in inflammatory diseases and cancer. This approach has been used to target ACTH and the pituitary–adrenal axis by a number of investigators and one pharmaceutical company aims to begin human studies in the near future (62). While such antibodies may not necessarily provide a long-term therapeutic solution, they seem likely to have potential in shorter-term therapeutic situations, such as around the time of pituitary surgery for Cushing's disease.

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### Corticostatins

Solomon and colleagues identified a novel lung and neutrophil peptide belonging to the defensin class of highly cationic antimicrobial peptides. They showed this peptide exhibited a number of functions including inhibition of ACTH binding and corticosterone secretion and named this corticostatin (63). It is also known as defensin  $\alpha$ -4. These functions appear to be relatively non-specific, and little work has been published on this in recent years. We are not aware that this action of corticostatin has been explored for therapeutic purposes.

## SUMMARY

We have reviewed the case for the development of an ACTH antagonist for therapeutic purposes. The conditions in which there is a potential clinical indication are relatively uncommon, and alternative therapies are well described in each case. However as a refinement to existing therapies or for the treatment of particularly difficult or complex cases, there would be a real clinical benefit. We have not considered a number of more common conditions, such as depressive illness or septic shock in which there might ultimately be a role for an ACTH antagonist, although these have been considered elsewhere (64).

The evidence suggests that progress is being made on more than one front in developing an antagonist. This has been delayed for many years by the absence of “clean” systems in which to test candidate peptides and compounds, owing to the problems in expressing the MC2R. This should no longer be a problem as a result of the identification of MRAP that enables cell surface expression of the MC2R. As a result there does seem to be a growing interest in this area and the next decade may witness exciting developments.

## AUTHOR CONTRIBUTIONS

This work is part of the result of a 5-year project. Over this time, all authors have contributed information, ideas, and data to the work. The manuscript was primarily written by AC, with comment and editing from the other authors.

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# From Bioinactive ACTH to ACTH Antagonist: The Clinical Perspective

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The adrenocorticotrophic hormone (ACTH) is a pituitary hormone derived from a larger peptide, the proopiomelanocortin (POMC), as are the MSHs ( $\alpha$ -MSH,  $\beta$ -MSH, and  $\gamma$ -MSH) and the  $\beta$ -LPH-related polypeptides (Figure 1A). ACTH drives adrenal steroidogenesis and growth of the adrenal gland. ACTH is a 39 amino acid polypeptide that binds and activates its cognate receptor [melanocortin receptor 2 (MC2R)] through the two regions H<sub>6</sub>F<sub>7</sub>R<sub>8</sub>W<sub>9</sub> and K<sub>15</sub>K<sub>16</sub>R<sub>17</sub>R<sub>18</sub>P<sub>19</sub>. Most POMC-derived polypeptides contain the H<sub>6</sub>F<sub>7</sub>R<sub>8</sub>W<sub>9</sub> sequence that is conserved through evolution. This explains the difficulties in developing selective agonists or antagonists to the MCRs. In this review, we will discuss the clinical aspects of the role of ACTH in physiology and disease, and potential clinical use of selective ACTH antagonists.

**Keywords:** stress, cortisol, adrenals, ACTH, antagonist

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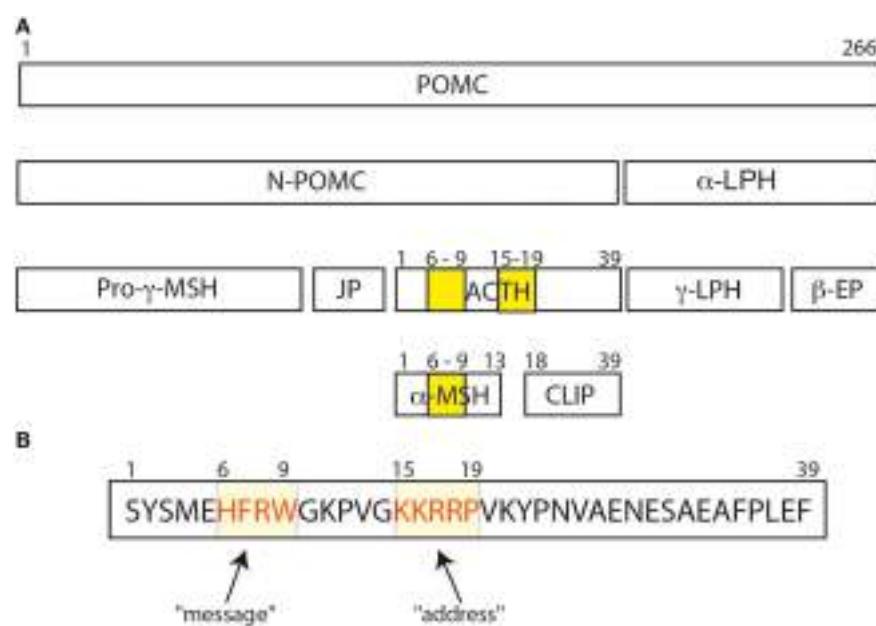
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## FROM THE CONCEPT OF “STRESS” TO THE DISCOVERY OF ADRENOCORTICOTROPIC HORMONE (ACTH)

Adrenocorticotrophic hormone and cortisol are nowadays associated with the physiological response to stress. Historically, the concept of stress was first introduced in 1936 by Hans Selye (1). It was first called the “general adaptation syndrome” and later renamed by Selye as “stress response.” Stress results from the balance between the stressor, an agent that produces stress at any time, and the body’s adaptive response to it. His experiments on rats in 1936 have shown that a stressor often alters the adrenal cortex, the immune system, and the gut. Indeed, rats exposed to various nocuous chemical or physical stimuli had a hypertrophy of the adrenals, an involution of the lymphatic nodes and developed gastric erosions (2).

In the 1930s–1940s, Selye performed extensive structure–function studies, resulting in the first classification of steroid hormones, e.g., corticoids, testoids/androgens, and folliculoids/estrogens (3, 4). During those years, he recognized the respective anti- and pro-inflammatory actions of gluco- and mineralocorticoids (named by Selye) in animal models, several years before demonstration of anti-rheumatic actions of cortisone and ACTHs in patients. In 1935 and 21 years after having isolated thyroxine in crystalline form, Kendall isolated and identified the structure of cortisone, and in 1936, Reichstein identified the structure of cortisol. In 1948, Hench and Kendall demonstrated that cortisone and ACTH exert a profound anti-inflammatory effect in patients with rheumatoid arthritis. In 1950, Hench, Kendall, and Reichstein were awarded the Nobel Prize in Physiology and Medicine for these discoveries. In the same year, Harris established that ACTH

**Abbreviations:** ACTH, adrenocorticotropin; PBMAH, primary bilateral macronodular adrenal hyperplasia; AGRP, agouti-related protein; ASIP, agouti signaling protein; CAH, congenital adrenal hyperplasia; FGD, family glucocorticoid deficiency; MSH, melanocyte-stimulating hormone; MCR, melanocortin receptor; MRAP, melanocortin receptor-associated protein; POMC, proopiomelanocortin.



**FIGURE 1 | (A)** The POMC protein and its various peptide cleavage products: the yellow bands correspond to the amino acid sequences His<sup>6</sup>Phe<sup>7</sup>Arg<sup>8</sup>Trp<sup>9</sup> and Lys<sup>15</sup>Lys<sup>16</sup>Arg<sup>17</sup>Arg<sup>18</sup>Pro<sup>19</sup>. His<sup>6</sup>Phe<sup>7</sup>Arg<sup>8</sup>Trp<sup>9</sup> is important for binding and signal transduction of α-MSH. **(B)** His<sup>6</sup>Phe<sup>7</sup>Arg<sup>8</sup>Trp<sup>9</sup> sequence is important for adrenocorticotrophic hormone (ACTH) signal transduction and for ACTH binding to melanocortin receptor 2 (MC2R) (26), and was called the “message” sequence (13). The amino acids Lys<sup>15</sup>Lys<sup>16</sup>Arg<sup>17</sup>Arg<sup>18</sup>Pro<sup>19</sup> was initially defined as the “address” sequence allowing specific ACTH binding to MC2R (27).

secretion involves “neuronal control *via* the hypothalamus and the hypophyseal portal vessels of the pituitary stalk” (5). ACTH was first isolated in 1943 and then synthesized in the 1960s and 1970s by different groups (6–9). Next, the amino acid sequences of ACTH from four mammalian species were elucidated between 1954 and 1961 by three groups of Bell, Li, and Lerner (6, 10, 11). In 1955, Guillemin (a former student of Selye’s) and Rosenberg demonstrated in rats the existence of CRF, the hypothalamic factor that allows ACTH release (12).

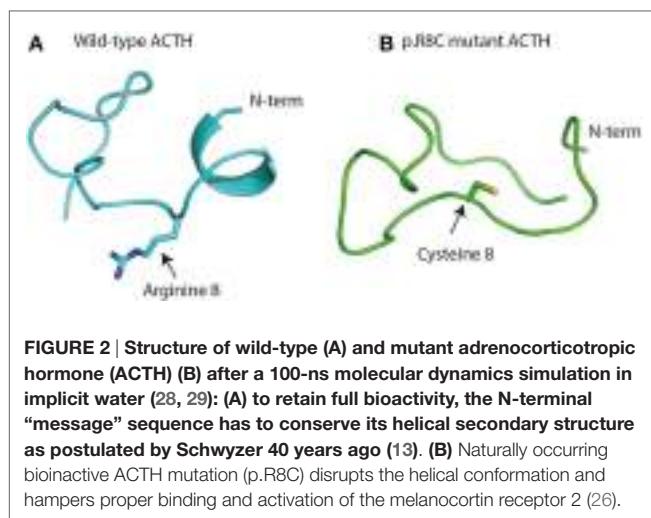
## ACTH STRUCTURE AND FUNCTION

Adrenocorticotrophic hormone, adrenocorticotropin, corticotropin, or ACTH, transmits information from the anterior lobe of the pituitary to the adrenal cortex. ACTH is a linear non-atriacontapeptide with species differences in the COOH terminal two-third of the molecule (13). Initially, an analysis of the ACTH crystal structure was still lacking. In the early 1970s, different polypeptides were isolated from different lobes of the vertebrate pituitary (14). Lowry showed that ACTH and β-lipotropin (β-LPH) shared the same amino acid motif H<sub>6</sub>F<sub>7</sub>R<sub>8</sub>W<sub>9</sub>, even if they have different functions (14, 15) (Figure 1). α-MSH and β-MSH were found to be embedded within the primary sequences of ACTH and β-LPH, respectively, in the melanocorticotrophic cells (in the intermediate pituitary) (16). A few years later, the prediction that ACTH, the MSHs, and the β-LPH-related polypeptides were derived from the same precursor (17) was confirmed by the cloning of the proopiomelanocortin gene (POMC) mRNA (18). Initially, Schwyzer and Sieber suggested that ACTH is a linear polypeptide (19). Later, Schiller showed that residues 10–21 have

a flexible conformation (20). Squire and Bewley had already suggested that a small region close to N-terminus had a tendency to form a helical structure (21). Low et al. have confirmed that a helical structure is produced in a solvent, the trifluoroethanol (22). It was suggested that this conformational change could occur when ACTH binds its receptor, to ensure a kinetically and a thermodynamically optimal hormone–receptor contact (23, 24). Later, Hruby et al. showed that the N terminal region of ACTH, between residues 4 and 10, adopts a β-turn conformation (25). Molecular modeling supports this hypothesis, given the presence of an N-terminal helical structure in the wild-type ACTH model (Figure 2A). This helical secondary structure is disrupted by validated inactivating mutations, which suggests that it is essential for ACTH bioactivity (Figure 2B).

Due to different bioassay systems, several research teams screened the biological activity of C terminally truncated analogs of ACTH (1–39) (30). ACTH (1–24) was as potent as ACTH (1–39) to induce glucocorticoid production by adrenal cells in culture (31), while ACTH (1–16) was unable to do so. It was concluded that the critical portion of ACTH for activating adrenal cells resided between residues 17 and 24 (31). However, the analog ACTH (11–24) was unable to induce glucocorticoid production and was even an antagonist at high concentrations (31). Fauchère et al. showed that dimers of ACTH (11–24) and ACTH (7–24) were potent antagonists, whereas ACTH (1–24) dimers remained agonists but were 70 times less potent when compared to its monomeric ACTH (1–24) (32–34).

Altogether, Schwyzer concluded that residues 17–24 were important for recognition of ACTH by its adrenal receptor, and he coined the term “address sequence” to indicate that



these residues allowed the ligand to find the cognate receptor (**Figure 1B**) (13, 27). As Costa et al. suggested, the position 20 should be included in the address site (35). To activate the adrenal receptor, a “message” region is needed. Several studies suggested that the H<sub>6</sub>F<sub>7</sub>R<sub>8</sub>W<sub>9</sub> motif should be considered as the “message sequence” (13) (**Figure 1B**). Indeed, both address and message sequences are necessary to activate the adrenal receptor: the address sequence interacts with the adrenal receptor and then the message sequence enters in contact with the receptor to induce the conformation change in the receptor that activates the adrenal cell (27). This could explain why α-MSH could not activate adrenal cells, even if it has the H<sub>6</sub>F<sub>7</sub>R<sub>8</sub>W<sub>9</sub> motif. On the other hand, Schwyzer considered also the effect of analogs of ACTH (1–39) on the activation of melanocytes. All analogs of ACTH (N-terminally as well as C terminally truncated forms) were able to activate melanocytes as long as the H<sub>6</sub>F<sub>7</sub>R<sub>8</sub>W<sub>9</sub> motif was intact (13, 27), and the H<sub>6</sub>F<sub>7</sub>R<sub>8</sub>W<sub>9</sub> motif is probably both the message and the address sequence in these cells.

In the 1990s, five melanocortin receptor (MCR) genes were identified in the genome of mammals, tetrapods, birds, bony fish, and cartilaginous fish (36–39). MC1R mediates pigmentation, MC2R activates glucocorticoid biosynthesis in the adrenal cortex, MC3R and MC4R influence metabolic homeostasis in the central nervous system and periphery (40), and MC5R regulates sebaceous gland secretions (41). The MCRs belong to the rhodopsin family of G protein-coupled receptors (GPCRs) (37, 42). They induce intracellular cyclic AMP production following activation by ligands (43, 44). ACTH (1–39) or α-MSH can activate MC1R, MC3R, MC4R, and MC5R (37), but MC2R can only be activated by ACTH and not by α-MSH or other analogs (45). The MCR genes have now been cloned and expressed in cell lines, allowing *in vitro* binding and activation assays of ACTH analogs against all MCRs. Recently, it has been shown that MC2R requires the presence of the melanocortin receptor-associated protein (MRAP) to be expressed and active at the cell surface (46–49).

Although ACTH and α-MSH both have the H<sub>6</sub>F<sub>7</sub>R<sub>8</sub>W<sub>9</sub> motif, only ACTH can activate MC2R. This raises the question

of differences between the H<sub>6</sub>F<sub>7</sub>R<sub>8</sub>W<sub>9</sub>-binding site in MC2R when compared to the other MCRs. Due to a crystal structure of the H<sub>6</sub>F<sub>7</sub>R<sub>8</sub>W<sub>9</sub>-binding site and site-directed mutagenesis analyses of human MC4R, we know that vertebrate MCRs have a common H<sub>6</sub>F<sub>7</sub>R<sub>8</sub>W<sub>9</sub>-binding site (50–53), which further confirms that the H<sub>6</sub>F<sub>7</sub>R<sub>8</sub>W<sub>9</sub> motif is both the message sequence and the address sequence for the α-MSH. Does hypothesized that, prior to stimulation, MC1, MC3, MC4, or MC5 receptors have a binding site that is in an open conformation for the ligand. When the ligand interacts with the receptor, it leads to a conformational change (a “docking event”) that activates the receptor (27). Therefore, the activation of MC2R may be a multistep process that requires (i) a conformational change after docking of the H<sub>6</sub>F<sub>7</sub>R<sub>8</sub>W<sub>9</sub> motif on its MC2R-binding site and, in the second step, (ii) docking of the K<sub>15</sub>K<sub>16</sub>R<sub>17</sub>R<sub>18</sub>P<sub>19</sub> motif of ACTH to activate the MC2R receptor (27, 54). This could explain the differential ligand selectivity of the MCRs. However, further analyses and experiments are necessary to confirm this hypothesis.

## FAMILIAL GLUCOCORTICOID DEFICIENCY (FGD) AND POMC DEFICIENCY

### Familial Glucocorticoid Deficiency

The glucocorticoid deficiency syndrome (FGD) is an autosomal recessive disorder characterized by insensitivity to ACTH action on the adrenal cortex (55), thereby resulting in glucocorticoid insufficiency with intact mineralocorticoid secretion. It may manifest during early neonatal life but can be diagnosed later during childhood. Clinical manifestations include recurrent hypoglycemia that may lead to seizures and coma, chronic fatigue, failure to thrive, recurrent infections, and skin hyperpigmentation. Typically, plasma cortisol levels are very low or undetectable without response to ACTH, while endogenous ACTH levels are very high, which clearly shows a specific resistance to the action of ACTH in these patients. The aldosterone and renin levels are usually normal and correctly match the activation of the renin–angiotensin axis. Even if congenital adrenal hyperplasia (CAH) (due to defects in the glucocorticoid synthesis pathway), congenital adrenal hypoplasia, and X-linked adrenoleukodystrophy are associated with high ACTH and low cortisol levels, they are distinct from FGD.

So far, mutations in five genes have been associated with isolated FGD and four with FGD associated with various syndromic features. In all these genetic disorders, low cortisol and a high ACTH suggest ACTH resistance. Causative genes of *isolated* FGD are the ACTH receptor (*MC2R*; also formerly defined as FGD 1) (56), the melanocortin receptor-associated protein (*MRAP*; also formerly defined as FGD 2) (46), the DAX1 transcription factor (*NR0B1*) (57), nicotinamide nucleotide transhydrogenase (*NNT*) (58–60), and mitochondrial thioredoxin reductase (*TXNRD2*) (61). Interestingly, *NNT* and thioredoxin reductase (*TXNRD2*) are crucial enzymes for the production of sufficient NADPH to maintain the redox potential in the steroid-producing cells. Four *syndromes* have been reported with FGD: (i) the AAA(A) syndrome (ACTH resistance, alacrimia, achalasia, and autonomous

system dysfunction) due to *AAAS* mutations (62); (ii) the IMAGe syndrome (intrauterine growth restriction, metaphyseal dysplasia, adrenal hypoplasia congenita, and genital anomalies) due to *CDKN1C* mutations (63); (iii) a syndrome with short stature, recurrent infection due to natural killer cell deficiency, and chromosomal fragility associated with mutation of the DNA helicase, minichromosome maintenance 4 (*MCM4*) (64); and (iv) the MIRAGE syndrome (myelodysplasia, infection, restriction of growth, adrenal hypoplasia, genital phenotypes, and enteropathy) due to mutations in *SAMD9* (65).

## POMC Deficiency

Propiomelanocortin (POMC) deficiency causes severe monogenic obesity that begins at an early age, adrenal insufficiency, red hair, and pale skin. Affected infants have a normal weight at birth, but they develop early hyperphagia that leads to excessive weight gain starting in the first year of life. During the neonatal period, patients usually present with hypoglycemic seizures, hyperbilirubinemia, and cholestasis due to secondary hypocorticism (66). Subclinical hypothyroidism was also reported. The incidence is very low, about one in 1 million. Complete POMC deficiency is an autosomal recessive disorder and is caused by homozygous or compound heterozygous loss-of-function mutations in the POMC gene on chromosome 2p23.3.

POMC is regulated by leptin and is cleaved by prohormone convertases to produce ACTH and MSH  $\alpha$ ,  $\beta$ , and  $\gamma$ . In POMC deficiency, the serum concentrations of these cleavage products are low. The red hair pigmentation, adrenal insufficiency, and obesity are caused by inability to activate the MC1, MC2, and MC4 receptors, respectively (66). In addition to complete POMC deficiency, isolated deficiency of  $\beta$ -MSH has been described. This isolated deficiency of  $\beta$ -MSH leads to severe obesity without adrenal insufficiency or red hair (67, 68).

A few years ago, we reported glucocorticoid deficiency in two unrelated patients with apparent ACTH resistance due to an unusual mutation in POMC: the p.R8C mutation in the sequence encoding ACTH and  $\alpha$ -MSH (26). The patients (a 4-year-old girl and a 4-month-old boy) presented with hypoglycemia, low cortisol, normal electrolytes, and high ACTH. Both patients had red hair, adrenal insufficiency, and developed early-onset obesity. They were initially treated with gluco- and mineralocorticoids, but after the identification of the mutation in POMC, mineralocorticoid treatment was discontinued in both patients. Whole exome sequencing revealed that the girl was compound heterozygous for POMC mutations: one previously described null allele and one novel p.R8C mutation in the sequence encoding ACTH and  $\alpha$ -MSH. The boy was homozygous for the p.R8C mutation. We demonstrated that even if ACTH-R8C was immunoreactive, it failed to bind and activate cAMP production in melanocortin-2 receptor (MC2R)-expressing cells. We demonstrated also that  $\alpha$ -MSH-R8C failed to bind and stimulate cAMP production in MC1R- and MC4R-expressing cells.

Discovery of this mutation indicates that in humans, unlike rodents, the amino acid sequence H<sub>6</sub>F<sub>7</sub>R<sub>8</sub>W<sub>9</sub> is important not only for cAMP activation but also for ACTH binding to the MC2R. Furthermore, *in silico* modeling suggested that the p.R8C mutation disrupts the secondary structure of ACTH, which implies

that this secondary structure may have a crucial role for M2R activation (Figure 2).

Diagnosis of complete POMC deficiency may be suspected on the basis of clinical manifestations and can be confirmed by identification of mutations in the *POMC* gene. To treat POMC-deficient patients, only hydrocortisone substitution is currently available and is required. An intranasal administration of the ACTH 4–10 melanocortin fragment did not lead to a reduction in body weight. Administration of thyroid hormone had no effect on obesity (69). Recently, two extremely obese POMC patients have been substituted with a MC4R agonist (setmelanotide), and a significant reduction of their weight has been achieved (more than 10% after 10 weeks of treatment) (70). This paves the way for treatment of this condition with specific MC4R agonists and should also prompt research into developing agonists to other MCRs such as MC2R.

## PERSPECTIVES: A ROLE FOR SPECIFIC ACTH ANTAGONISTS IN HUMAN DISEASE

Specific ACTH antagonists would be of great value especially to treat two conditions: CAH and primary bilateral macronodular adrenal hyperplasia (PBMAH). Moreover, MC2R antagonists could represent a valuable alternative to anticortisol drugs in clinical management of ACTH-dependent hypercortisolism, essentially Cushing's disease, when removal of the source of ACTH excess is impossible or incomplete.

## Congenital Adrenal Hyperplasia

Adrenocorticotrophic hormone drives the synthesis and secretion of cortisol as well as androgens from the adrenal gland. In CAH due to 21 hydroxylase deficiency, excess of ACTH leads to overandrogenization. CAH is due to the enzymatic defect in the cortisol synthesis pathway, with subsequent hypocortisolism, ACTH overproduction, accumulation of androgen precursors, and adrenal gland hyperplasia. Treatment with glucocorticoids at physiological doses is life saving but is not sufficient to suppress the elevated ACTH levels and androgen overproduction. Failure to suppress excess androgens results in height acceleration, advanced skeletal maturation, and eventually leads to decreased final adult height. Other effects of androgen excess include clitoromegaly and hirsutism in females, isosexual pseudoprecocious puberty in males, and acne and deepening of the voice in both sexes. Only supraphysiological doses of glucocorticoids suppress ACTH in CAH but at the cost of hypercortisolism with its adverse effects such as hyperglycemia, arterial hypertension, reduced growth, and osteoporosis. Since MC2R is expressed only in the adrenals, it could become a specific target. Therefore, an adjuvant drug able to specifically block the MC2R would obviate the need for supraphysiological dose of glucocorticoids in CAH and prevent the undesirable effects inherent to glucocorticoid overtreatment.

## MC2R Expressing PBMAH

Primary bilateral macronodular adrenal hyperplasia (also known as ACTH-independent macronodular adrenal hyperplasia) is a rare, sporadic disease affecting men and women with an almost

equal ratio. PBMAH has a bimodal age distribution: during the first year of life (minority) when it may be associated with the McCune–Albright syndrome (71) and during the fifth decade of life (majority) (72). Occurrence of familial cases of PBMAH and involvement of both adrenal glands (even in sporadic cases) strongly suggest involvement of germline genetic predisposition in PBMAH. Indeed, inactivating mutations of the armadillo repeat containing 5 (ARMC5) have been reported in 25–55% of patients with sporadic PBMAH (73–75).

The most frequent clinical manifestation of PBMAH is Cushing syndrome. In PBMAH, one previously believed that glucocorticoid secretion is ACTH independent given that, most of the time, plasma ACTH levels are undetectable, and high-dose dexamethasone administration fails to suppress cortisol secretion. This notion has been revised following the demonstration of local production of ACTH. Indeed, Cheitlin et al. studied the adrenal cells from a patient with PBMAH *in vitro*; the cells were cultured on an extracellular matrix and demonstrated rapid growth and a high rate of cortisol secretion in the absence of ACTH (76). Not only the MC2R was expressed in PBMAH tissue (77) but also ACTH was abnormally expressed, further stimulating the cortisol secretion through a paracrine effect (78). These findings represent a strong incentive to treat PBMAH-associated hypercortisolism with MC2R antagonists. This contrasts with primary pigmented nodular adrenocortical disease, sporadic large benign adenomas, and adrenocortical carcinomas, which are mostly ACTH unresponsive (79).

Even if these observations suggest that specific MC2R antagonist could help patients with PBMAH, the specific structure of the ACTH produced by PBMAH cells (78) and in ectopic Cushing's syndrome (80) might raise unexpected difficulties. Indeed, these bioactive ACTH are not detected by antibodies directed to the ACTH C terminal portion. This may result from extrapituitary sources of ACTH producing increased amounts of precursors (pre-ACTH and POMC) due to impaired POMC processing (81, 82). Reports on receptor affinity and steroidogenic potency of ACTH precursors are conflicting (81), which may complicate the design of MC2R antagonists that also block ACTH precursors.

## A Specific MC2R Antagonist: An Elusive Target

In the last four decades, extensive studies that have been performed to determine the molecular basis of the interaction of ACTH with cognate MCRs have shown inconsistent results, mainly due to technical limitations. First, the expression at the cell membrane of human MC2R in cell lines was not possible until the discovery of the melanocortin receptor-associated protein (MRAP) (46), which is required to address MC2R at the cell membrane. Second, studies on putative ACTH antagonists failed to perform a systematic analysis of ACTH antagonists' activation and binding against all cognate MCRs to assess specificity (83, 84).

Melanocortin receptors belong to the GPCRs family that has natural agonists and antagonists. The melanocortin antagonists, agouti-related protein (AGRP) and agouti signaling protein (ASIP), are the only two endogenous antagonists

of MCRs identified to date (85–87). The primary sequences of the endogenous agonists (MSHs) and antagonists (AGRP and ASIP) are different, and they interact differently with the MCRs to produce the active and inactive conformations of the ligand–receptor complex.

The design of a specific peptide remains a challenge that has not been resolved because of the difficulty of designing a peptide specific for one MCR (e.g., MC2R) (37). Indeed, all melanocortin ligands receptors shared the same H<sub>6</sub>F<sub>7</sub>R<sub>8</sub>W<sub>9</sub> motif, which is important for MCR binding and stimulation (88) and which bind to all MCR receptors. Development of selective ligands for the melanocortin system is challenging due to the conserved amino acid sequences of the MCRs and of their structural similarity in the seven-transmembrane GPCR fold (89). The limited structural variations of the endogenous melanotropin ligands further reduce options in the design of MCR ligands for achieving selectivity. If they are not selective, these peptides could be the source of severe undesirable effects due to the numerous specific roles of the five MCRs. Of note, some ACTH fragments show variable specificity across species. For example, ACTH (7–38) fragment antagonizes the human MC2 receptor but stimulates aldosterone secretion from the rat adrenal cortex through a mechanism involving the angiotensin receptor (90). Variable specificity between species is therefore another challenge for the preclinical phase of the development of new ACTH receptor agonists. A table listing MC2R agonists and antagonists is available in Table S1 in Supplemental Material.

Designing molecules that possess both functional selectivity and human melanocortin receptor (hMCR) subtype selectivity from the melanotropin core sequence H<sub>6</sub>F<sub>7</sub>R<sub>8</sub>W<sub>9</sub>, has been difficult. Many peptides are conformationally flexible in aqueous solution, but upon interacting with their biologically relevant molecule, they assume a preferred conformation. The reduction of conformational freedom may eventually lead to the receptor bound conformation, which results in the selective interaction of a ligand with a receptor. A major step is to determine which peptide conformation is required for binding to the receptor and resulting in an agonist or antagonist effect. A critical approach is to understand if changes in ACTH three-dimensional (3D) conformation are associated with antagonist properties (Figure 2). X-ray crystal structures provide 3D conformation but may be misleading in terms of function. Incorporation of these X-ray coordinates into a computer-aided examination of function into 3D space is being pursued (91). This allows one to further explore the region/site or the surrounding 3D space occupied by the key amino acids of the protein (where the potent ligand has an affinity) so as to better understand biological actions. Nevertheless, the numerous physiological functions of the five known subtypes of hMCRs continue to be a stimulus for the production of selective melanocortin agonists and antagonists (92).

## CONCLUSION

The ACTH is the pituitary hormone that allows growth and development of the adrenal cortex and glucocorticoid

synthesis. Even if the structure of ACTH has been known for decades, the exact mechanism of activation of MC2R, the specific ACTH receptor on adrenal cells, is still unknown. Understanding this activation and the development of specific MC2R antagonists would allow the treatment of some difficult-to-treat diseases such as CAH or PBMAH.

## AUTHOR CONTRIBUTIONS

CG wrote the review and revised the final version; J-MV performed bioinformatic analysis and revised the final version; and JD wrote the review and revised the final version.

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# ACTH Receptor (MC2R) Specificity: What Do We Know About Underlying Molecular Mechanisms?

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Coincidentally, the release of this Research Topic in *Frontiers in Endocrinology* takes place 25 years after the discovery of the adrenocorticotrophic hormone receptor (ACTHR) by Mountjoy and colleagues. In subsequent years, following the discovery of other types of mammalian melanocortin receptors (MCRs), ACTHR also became known as melanocortin type 2 receptor (MC2R). At present, five types of MCRs have been reported, all of which share significant sequence similarity at the amino acid level, and all of which specifically bind melanocortins (MCs)—a group of biologically active peptides generated by proteolysis of the proopiomelanocortin precursor. All MCs share an identical –H–F–R–W– pharmacophore sequence.  $\alpha$ -Melanocyte-stimulating hormone ( $\alpha$ -MSH) and adrenocorticotrophic hormone (ACTH) are the most extensively studied MCs and are derived from the same region. Essentially,  $\alpha$ -MSH is formed from the first 13 amino acid residues of ACTH. ACTHR is unique among MCRs because it binds one sole ligand—ACTH, which makes it a very attractive research object for molecular pharmacologists. However, much research has failed, and functional studies of this receptor are lagging behind other MCRs. The reason for these difficulties has already been outlined by Mountjoy and colleagues in their publication on ACTHR coding sequence discovery where the Cloudman S91 melanoma cell line was used for receptor expression because it was a “more sensitive assay system.” Subsequent work showed that ACTHR could be successfully expressed only in endogenous MCR-expressing cell lines, since in other cell lines it is retained within the endoplasmic reticulum. The resolution of this methodological problem came in 2005 with the discovery of melanocortin receptor accessory protein, which is required for the formation of functionally active ACTHR. The decade that followed this discovery was filled with exciting research that provided insight into the molecular mechanisms underlying the action of ACTHR. The purpose of this review is to summarize the advances in this fascinating research field.

**Keywords:** MC2R, ACTHR, specificity, mutation, mutagenesis, site directed

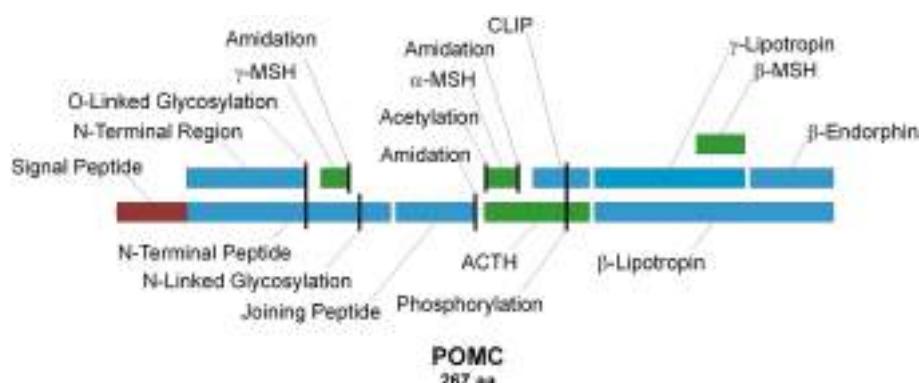
## INTRODUCTION

Adrenocorticotrophic hormone (ACTH), discovered in 1933 (1), is the primary regulator of aldosterone and corticosterone/cortisol production in mammalian adrenal glands (2–5). It is secreted into the circulating blood stream by corticotropin cells in the anterior pituitary (6) in response to short- and/or long-term stress (7). Due to its chemical structure, biological activity, and origin,

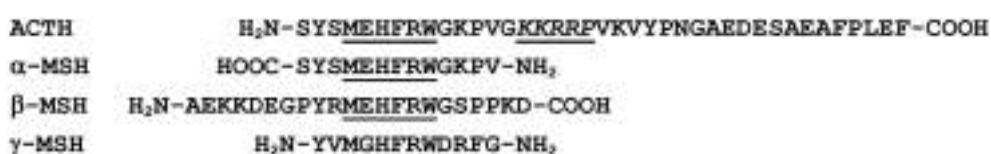
ACTH is classified as a member of the peptide hormone group named melanocortins (MCs), which in addition to ACTH also comprises  $\alpha$ -,  $\beta$ -,  $\gamma$ - and  $\delta$ -melanocyte-stimulating hormones (MSHs), of which the latter is only found within cartilaginous fish (8). All MCs are formed through tissue- and site-specific proteolysis of the propeptide proopiomelanocortin (POMC; **Figure 1**) (7, 9), and all share the  $-M-X-H-F-R-W-$  consensus sequence (**Figure 2**), which is the main determinant of their biological activity (8, 10). Experimental data suggest that this core sequence adopts a  $\beta$ -turn secondary structure in most MCs that is generally essential for ligand binding (11). The exception to this rule is  $\gamma$ -MSH, but since it is not directly relevant to the main subject of this article, the reasons underlying this difference are not discussed [for details on molecular mechanisms underlying the action of this peptide see Ref. (12)].  $\alpha$ -MSH and ACTH are overlapping peptides since the aa sequence of the former is identical to the first 13 aa of the latter, because the former is the result of proteolytic cleavage of the latter (**Figure 1**). Following their discovery and aa sequence determination (1, 13–19), there was an ongoing discussion about the melanotropic activity of ACTH and the possible adrenocorticotrophic activity of  $\alpha$ -MSH (13, 20). However, the latter of these was soon dismissed following overwhelming evidence from *ex vivo* assays that suggested ACTH-derived peptides, which lack the basic  $-K-K-R-R-$  motif (**Figure 2**), have minimal effect on steroid production [(21, 22); reviewed in Ref. (23–25)]. Detailed mutational research performed a few decades later extended this motif by including a C-terminal proline, since the replacement

of this residue with alanine or tryptophan significantly decreased the potency of the peptide (26). A similar alanine scanning mutagenesis experimental approach was also used to investigate the properties of the ACTH region, which is located between the  $-M-X-H-F-R-W-$  and  $-K-K-R-R-P-$  motifs (**Figure 2**). The results demonstrated that although replacement of one or two residues had no effect on receptor activation, substitution of all five residues ( $-G-K-P-V-G-$ ) resulted in dramatic decrease in response sensitivity. Thus, it was concluded that the secondary structure of this motif is of paramount importance because it properly orients the other two motifs in relation to each other so that they can properly fit into the receptor-binding pocket(s) (27). This highly condensed introduction to ACTH, the ligand of ACTHR that is the main subject of this review, barely skims the enormous amount of knowledge acquired for mammalian and other lineages (6, 8, 10, 28–37). However, although having a detailed understanding of the functional properties of the ligand is very important because the interaction of both elements results in the physiological effect, reviewing the ligand in detail is beyond the scope of this article. We believe the core information provided here will be sufficient for interpretation of the research on the molecular mechanisms underlying the action of the adrenocorticotrophic hormone receptor (ACTHR).

Although the main subunit of the receptor was discovered in 1992 (38) and the functional receptor was obtained in 2005 (39), research on the structure and function of the mammalian ACTHR dates back as far as 1954, when the aa sequence of ACTH was determined (14–18), or perhaps even to 1933, when



**FIGURE 1 | Schematic representation of the proopiomelanocortin precursor, the products of its proteolysis, and known posttranslational modifications.** Melanocortins are colored green, signal peptide is colored red, and other peptides are colored blue. Posttranslational modification sites are marked with vertical black lines.



**FIGURE 2 | Sequence alignment of melanocortin (MC) peptides.** All MCs share the conserved  $-M-X-H-F-R-W-$  motif, which serves as the pharmacophore for receptor binding (8, 10). The  $-K-K-R-R-P-$  motif within adrenocorticotrophic hormone (ACTH) is considered the second pharmacophore and is required for successful activation of adrenocorticotrophic hormone receptor (26).

the hormone itself was discovered (1). Although to some readers this statement might seem exaggerated, *ex vivo* research on the physiological effects of ACTH was performed on tissue samples containing endogenously expressed ACTHR from these dates. Thus, a large amount of knowledge was accumulated well before the discovery of the receptor itself, and some of these early studies revealed that the receptor was also present in murine fat cells (40–42) and in addition to increasing corticosteroid production (1, 43), activation of the ACTHR is accompanied by the activation of phosphorylases (44, 45), an increase in cyclic adenosine 3',5'-monophosphate (cAMP) concentration (46–50), and subsequently desensitization (51). Research performed in the 1980s also revealed that stimulation with ACTH increases the number of ACTH-binding sites at adrenal cell membranes (52) and promotes calcium influx (53–56). The information gained during these studies was extensive, and it provided researchers with clues on the nature of the ACTHR and accelerated research in related areas.

At the beginning of the 1990s, a significant number of G-protein coupled receptors (GPCRs) were already identified, all of which share the typical seven transmembrane (TM) domain core structure with the N-terminus located on the exterior of the cell. These receptors were shown to activate intracellular signaling pathways through coupling to the  $\alpha$ -subunit of heterotrimeric G-proteins ( $\text{G}\alpha_s$ ) that comprise also  $\beta$  and  $\gamma$  subunits. At the time, it was already known that there are several types of G-proteins that induce distinct intracellular responses (57). The most notable of these from the perspective of melanocortin receptors (MCRs) and the ACTHR were those coupling to the  $\text{G}\alpha_s$  subunit that activates adenylyl cyclase, which in turn increases the intracellular concentration of cAMP. This knowledge was consistent with the observed ACTH-induced intracellular responses, which indicated that MCRs are members of this structurally conserved GPCR family.

The coding sequences of receptors that specifically bind ACTH were first reported in 1992 by two research groups, one led by Cone, and the other led by Wikberg (38, 58). Although both groups employed similar methodology (degenerate primer PCR followed by northern blot screening of melanoma cDNA libraries), the first group identified two receptor-encoding genes, and the second group identified one such gene. This discrepancy could partially be explained by the source of the PCR template, since Cone's group used cDNAs from melanoma cells, while Wikberg's group used genomic DNA. Subsequent pharmacological characterization of receptors and post-publication sequence comparison revealed that both groups discovered the same MCR, which is highly expressed within melanocytes and specifically binds all MCs (ACTH,  $\alpha$ -,  $\beta$ - and  $\gamma$ -MSH), and was thus designated the MSH receptor (MSHR) (38, 58). In addition to MC1R, Cone's group also discovered a receptor sharing significant aa sequence identity with MSHR (~39%), yet it proved to be difficult to characterize *in vitro*. Functional expression of this protein was achieved only in an endogenous MCR-expressing Cloudman S91 melanoma cell line, and unlike MSHR, this receptor was found to be expressed in adrenal tissue and activated only by ACTH and was hence named the ACTHR (38). In the following 2 years, three additional MC-binding receptors were uncovered—two

by Yamada's group (59, 60) and one by both the Wikberg's and Yamada's groups (61, 62). Due to this simultaneous discovery, there was some ambiguity in the nomenclature of these novel MCRs, but the scientific community soon came to agreement and named them using Yamada's nomenclature as the MC type 3 receptor (MC3R), MC4R, and MC5R. Evaluation of the pharmacological properties of these novel receptors revealed that, like previously characterized homologs, they all coupled with  $\text{G}\alpha_s$  [it was later discovered that the exception to this rule is MC3R, which is also able to interact with the  $\text{G}\alpha_{q/11}$  subunit (63)]. However, from a ligand selectivity and *in vitro* expression perspective, they are more akin to MSHR since they are activated by all four MCs and successfully expressed in non-melanoma cells. Since these new receptors also shared significant aa sequence identity with previously discovered homologs (all MCRs share 39–61% sequence identity), their nomenclature was reviewed and, as a result, MSHR became synonymous with MC1R, and ACTHR with MC2R. To avoid any further confusion, in this article, we use ACTHR throughout, since this name better describes the unique functional properties, while the HGNC approved name of MC2R is a better descriptor of its evolutionary origin.

Considered together, the results on MCR expression and ligand selectivity highlighted the unique nature of each receptor because all displayed distinct pharmacological and tissue distribution profiles (Table 1). From this perspective, MC3R and ACTHR are the most specialized MCRs, because MC3R is the only receptor in this family that effectively binds  $\gamma$ -MSH and, as mentioned above, ACTHR specifically binds only ACTH (64). The molecular mechanisms underlying the ability of MC3R to effectively bind  $\gamma$ -MSH were soon uncovered (12, 65) following the expression of this receptor in a variety of mammalian cell lines. However, the mechanisms underlying the ACTHR ligand selectivity remained concealed for more than a decade due to the unusual expression selectivity of this receptor.

Although the period following the determination of the DNA sequence encoding the ACTHR was marked by very few molecular studies, this knowledge did facilitate functional and genetic research, which confirmed expression in the *zona reticularis* and *zona fasciculata* of the adrenal cortex, where its activation upregulates its own mRNA production (66). ACTHR mRNA was also found to be present in murine adipocytes (67–69) [where

**TABLE 1 | Ligand selectivity and expression profiles of melanocortin receptors.**

Receptor	Potency of ligands	Site of expression
MC1R	$\alpha$ -MSH = ACTH > $\beta$ -MSH > $\gamma$ -MSH	Melanocytes
ACTHR	ACTH	Adrenal cortex, adipocytes
MC3R	$\alpha$ -MSH = $\beta$ -MSH = $\gamma$ -MSH = ACTH	Hypothalamus, limbic system, placenta, digestive tract
MC4R	$\alpha$ -MSH = ACTH > $\beta$ -MSH > $\gamma$ -MSH	Hypothalamus, limbic system, cerebrum, brain stem
MC5R	$\alpha$ -MSH > ACTH > $\beta$ -MSH > $\gamma$ -MSH	Muscles, liver, spleen, lungs, brain, adipocytes

it affects lipolysis (67, 70) as well as leptin (71) and interleukin 6 production (69)], skin (72–74), pituitary (75), rat sympathetic ganglia (76), fetal and neonatal mouse testis (77, 78), human endometrium (79), human erythroblasts (80), and human osteoblasts (81, 82). The concept of receptor desensitization was not forgotten and was investigated in detail, and findings were somewhat contentious, but all agreed that receptor phosphorylation by various kinases (GPCR kinase and protein kinases A and C) is essential for desensitization and subsequent internalization via clathrin-coated pits (83–87).

There are several disorders associated with aberrant ACTH action. Familial glucocorticoid deficiency (FGD), which is also known as familial glucocorticoid insufficiency, hereditary adrenocortical unresponsiveness to ACTH, and familial Addison's disease, is a rare, early onset, autosomal recessive disorder characterized by low or undetectable plasma cortisol levels, normal mineralocorticoid levels, excess plasma ACTH, and hypoplasia of adrenal cortex *zona fasciculata* and *zona reticularis*. Typical physical symptoms include frequent hypoglycemia and/or infective episodes accompanied by excessive skin pigmentation. This disease was described in detail for the first time by Shepard in 1959 (88), and other studies soon followed (89–96). All reports highlighted an unusual resistance to ACTH in patients and a positive response to glucocorticosteroid treatment. Nevertheless, until 1993, when the first mutations within *ACTHR* coding sequence were discovered (97), the genetic cause of this disorder remained unknown. From this pivotal moment, the number of reports involving sequencing of *ACTHR*, which is located on the small arm of chromosome 18 (18p11.21-pter) (98), increased significantly (99–101) and facilitated accumulation of some structural information that is reviewed in a separate chapter. However, although this information did provide some clear links between genetic cause and physiological effect, surprisingly, mutations within *ACTHR* were the cause of FGD in only 25% of cases, indicating additional *ACTHR*-related factors. In the aftermath of these initial genetic discoveries, the disorder caused by a faulty *ACTHR* gene was designated as FGD type I, while disorders of unknown cause were designated FGD type II (100, 102–104).

As the incoming information from genetic studies was in a good agreement with the difficulties in expressing *ACTHR*, interest remained intense and molecular research continued. The first breakthrough was achieved in 1995 when Schimmer and colleagues revealed that ACTH-resistant Y6 and OS3 cell lines derived a decade earlier from the Y1 adrenal cell line (105) completely failed to express *ACTHR* (106). This provided for the first time a platform for sensitive and unbiased *ACTHR* characterization (107–109). An important study using one of these cell lines was performed by Noon and colleagues who expressed an *ACTHR*-green fluorescent protein (GFP) fusion protein and observed that the receptor is retained within the endoplasmic reticulum (ER) of CHO cells, but successfully reached the cell membrane in Y6 cells. Thus, they concluded that adrenal cells produce some kind of *ACTHR* accessory factor that overcomes receptor trafficking arrest (110).

The discovery of this accessory factor was reported in 2005 by Metherell and colleagues (39) who performed SNP array genotyping on an FGD type 2 family to map the causal mutation

to chromosome 21 locus 21q22.1, which spanned 30 known or predicted genes at the time. Subsequent *in silico* and mRNA expression analyses revealed that one of these genes, a predicted small single TM domain protein encoding a six exon gene named “chromosome 21 open reading frame 61 (C21orf61),” was expressed in adrenals and carried a mutation within the third intron donor splice site. *In vitro* co-expression of this protein with *ACTHR*-GFP in CHO and SK-N-SH cells confirmed that the receptor successfully reached the cell membrane and was functionally active. Based on these results, the newly identified protein was renamed the melanocortin receptor accessory protein (MRAP). Following the confirmation of its functional purpose, Matherell and colleagues performed an in-depth analysis of the gene and its expression profile. They discovered two isoforms of MRAP resulting from alternative splicing: MRAP- $\alpha$  and MRAP- $\beta$ . The 172 aa MRAP- $\alpha$  is the product of the first five exons, while the 102 aa MRAP- $\beta$  is formed from the third, fourth, and sixth exons. Since the “Start” codon is located within the third exon, both isoforms have identical N-termini and TM domains, but distinct C-termini. Analysis of protein expression patterns revealed that both proteins are differentially expressed in various tissues. Simultaneous expression was observed in the adrenals, testis, breast, ovary, adipocytes, skin, and jejunum. MRAP- $\alpha$  is also expressed in the thyroid, lymph nodes, ileum, liver, stomach, and pituitary, while MRAP- $\beta$  is only expressed in the brain (39). It was later revealed that mutations in *MRAP* account for another 20–25% of FGD cases, thus it was established that there are additional causal factors for this disease that distinguish FGD type 2 and FGD type 3, the latter referring to cases of unknown genetic cause (111).

As would be expected, the discovery of MRAP stimulated research aimed at uncovering the molecular mechanisms of *ACTHR* action. However, it also initiated an additional line of research aimed at investigating MRAP itself. These studies demonstrated that *ACTHR* is differentially affected by MRAP isoforms, with MRAP- $\alpha$  providing higher sensitivity to ACTH, whereas higher cAMP response and membrane trafficking occur during co-expression with MRAP- $\beta$  (112). Detailed investigations on the actions of the accessory proteins revealed a unique feature; in the cell, MRAP is present as a highly stable, SDS-resistant, antiparallel homodimer in which the C-terminus of one monomer is on the cytoplasmic side of the cell membrane, while the C-terminus of the other is on the extracellular side of the cell membrane (113, 114). A few years later, the same dimer topology was also observed in another membrane protein sharing 39% aa sequence identity with MRAP that was also able to facilitate *ACTHR* trafficking to the cell membrane, and this evolutionary related protein was therefore named MRAP2 (115). There was, however, one major difference between MRAP1 (the name was accordingly updated) and MRAP2; when co-expressed with MRAP2, *ACTHR* was located on the surface of the cell, but stimulation with ACTH induced the cAMP response with 1000-fold lower potency than when co-expressed with MRAP1 (116). This observation indicated that MRAP1 is more than a mere “deliverer” and is instead an essential functional component of *ACTHR* (115). Indeed, it was soon discovered that the N-terminal region comprising residues 18–21 (−L–D–Y–L−) is essential for

ACTHR ligand recognition (117). Its substitution with alanine residues resulted in an MRAP2-like protein that promoted only the trafficking of receptors to the plasma membrane. Alignment of both MRAPs revealed that this is the exact region missing from the MRAP2 sequence, and its insertion using site-directed mutagenesis allowed MRAP2 to form a functionally active ACTHR (117, 118). Further mutagenesis of various MRAP1 regions also revealed that the N-terminal side of TM region, formed from residues 31–37 (−L-K-A-N-K-H-S−), is required for MRAP1 to form an antiparallel dimer, since its removal resulted in the formation of a protein with the N-terminus always oriented on the extracellular side of the cell membrane (117). Additional aa replacement experiments showed that the TM domain of MRAP1 provides specific coupling to ACTHR, although the nature of this interaction remains unclear (117, 118). It should be noted that this review only considers MRAP1 in relation to ACTHR, but the functions of this protein extend beyond formation of functionally active ACTHR (115, 119–123).

The latest development in the field of ACTHR research regards the dimerization of the receptor itself. It has been known for a long time that various GPCRs form homo- and heterodimers and that dimerization is essential for the formation of functionally active receptors and implementation of their physiological effects (124–126). Therefore, the discovery of MCR homo- and heterodimerization was not surprising (127). However, initial difficulties with expression prevented such experiments on ACTHR, but this changed in 2011 when Cooray and colleagues performed bioluminescence resonance energy transfer analysis on functionally active ACTHR (the ACTHR and MRAP1 complex) which revealed that, just like other GPCRs, ACTHR formed dimers.

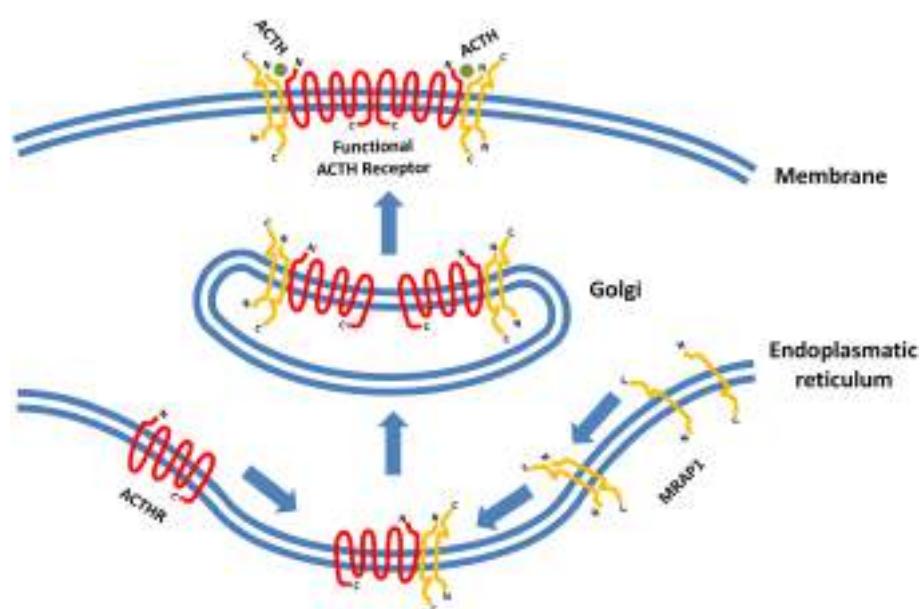
Furthermore, only when each ACTHR subunit was accompanied by an MRAP1 dimer was a functionally active ACTHR formed, hence the realization that a hetero-hexameric structure is required for full activity (**Figure 3**) (128).

Finally, we would like to highlight the important contribution made by *ACTHR* knock-out mice. Phenotypic data from these animals were in a very good agreement with observations by medical practitioners on the effects of ACTHR deficiencies in humans. *ACTHR* knock-out mice therefore provided an experimental system for investigating the complexity of ACTHR action in mammals (129–134), but these studies are too voluminous and are beyond the immediate scope of this article.

## MCs AND MCRs

More targeted research aimed at understanding the molecular mechanisms that underlie the action of MCRs was undertaken soon after their discovery. The majority of these studies focused on MC1R or MC4R, as they were either considered the best representatives, or the pharmacologically most relevant MCRs. Some research was also carried out on MC3R and MC5R, but due to expression difficulties, targeted studies on ACTHR were not performed until after 2000. Nevertheless, despite being highly specific, ACTHR is a member of the MCRs family and thus general MCR ligand-binding mechanisms are relevant and likely to apply.

Among the first to explore these mechanisms through active intervention were Frandberg and colleagues who performed PCR-based site-directed mutagenesis, a novel method at that time, to substitute selected aa residues with alanine to evaluate



**FIGURE 3 | Formation of functionally active adrenocorticotrophic hormone receptor (ACTHR).** The functional form of ACTHR is a hetero-hexameric structure formed from two molecules of ACTHR and four molecules of MRAP1. The antiparallel dimer of MRAP1 is formed within the endoplasmic reticulum, where it couples to one molecule of ACTHR. Afterward, this complex is transported to the Golgi where it “dimerizes,” thus forming the hetero-hexameric functional ACTHR (128). Symbols representing ACTHR and MRAP1 were selected to highlight the protein secondary structure. The depicted intermolecular interactions are therefore general and do not reflect the actual situation in detail.

the role of four human MC1R aa residues ( $D^{117}$ ,  $H^{260}$ ,  $F^{179}$ , and  $H^{209}$ ) in ligand recognition and receptor activation. The selection criteria for these residues were the location within the TM domains, the presence of reactive groups, and sequence conservation between known MCRs. The employment of the last two criteria was somewhat traditional, since reactive groups are necessary for molecular interactions, whereas the degree of conservation indicates structural and/or functional importance of residue. However, the first criterion was unusual because known binding pockets for the majority of receptors were on their surface. MCRs were the smallest known GPCRs, and they bind relatively large peptide ligands, thus it was predicted that their binding pocket would be located between the helices. The results of these experiments revealed that two residues of the four ( $D^{117}$  and  $H^{260}$ ) were essential for binding of  $\alpha$ -,  $\beta$ - and  $\gamma$ -MSH peptides, while their substitution had no effect on coupling with artificial ligand NDP-MSH ( $[Nle^4, D-Phe^7]-\alpha$ -MSH). This demonstrated not only the importance of these residues but also the principle that different ligands may interact with different regions of the binding pocket (135).

Researchers employed a more sophisticated approach in one subsequent study; instead of creating a large number of receptors by mutating conserved residues, they generated a three-dimensional molecular model of human MC1R based on the cryo-electron microscopy structure of bacteriorhodopsin and the electron density footprint of bovine rhodopsin and docked several natural and synthetic ligands. The results suggested the cavity of MC1R consists of two distinct acidic- and aromatic-binding pockets. According to this model, the acidic pocket is formed from  $E^{94}$  (TM2),  $D^{117}$  (TM3), and  $D^{121}$  (TM3), while the aromatic pocket is formed from  $F^{175}$  (TM4),  $F^{179}$  (TM4),  $F^{195}$  (TM5),  $F^{196}$  (TM5),  $F^{257}$  (TM6),  $Y^{182}$  (TM4), and/or  $Y^{183}$  (TM4). Being acidic, the first pocket interacts with basic R residues and to some extent H residues in the MC consensus motif  $-M-X-H-F-R-W-$ , while the second pocket interacts with aromatic F and W residues of the same motif (136). In the following years, this research group rigorously tested this model with various point mutations, different native and synthetic ligands, and even through conversion to MC4R. Their results confirmed that residues in the predicted acidic pocket were of paramount importance for ligand binding and do indeed interact predominantly with R residue from the  $-M-X-H-F-R-W-$  motif. Data on the aromatic-binding pocket were harder to interpret because considerable alterations in ligand binding were achieved only when multiple mutations were introduced at the same time, suggesting each individual residue plays a small part, and the loss of one may be compensated by the others. However, this conclusion was not supported by an acidic pocket eradication experiment in which all three pocket-forming residues were replaced with alanines. The resulting receptor was functionally inactive yet able to bind  $\alpha$ -MSH with modest affinity, indicating other potentially more important ligand-binding elements within the aromatic pocket. Thus, the repertoire of mutated aromatic residues was extended, and  $W^{254}$  (TM6),  $Phe^{257}$  (TM6), and  $H^{260}$  (TM6) were recognized as crucial elements of this ligand-binding pocket (137–139). In the following years, this type of research was performed on all five MCRs, which highlighted the uniqueness of the individual receptor-binding pockets

by identifying the aa residues responsible for differential ligand recognition. However, all studies agreed that the six previously mentioned aa residues (Figure 4) form the “backbone” of the MCR-binding pocket, while the residues surrounding these act as binding pocket “modifiers” [(65, 140–143); reviewed in Ref. (144–147)].

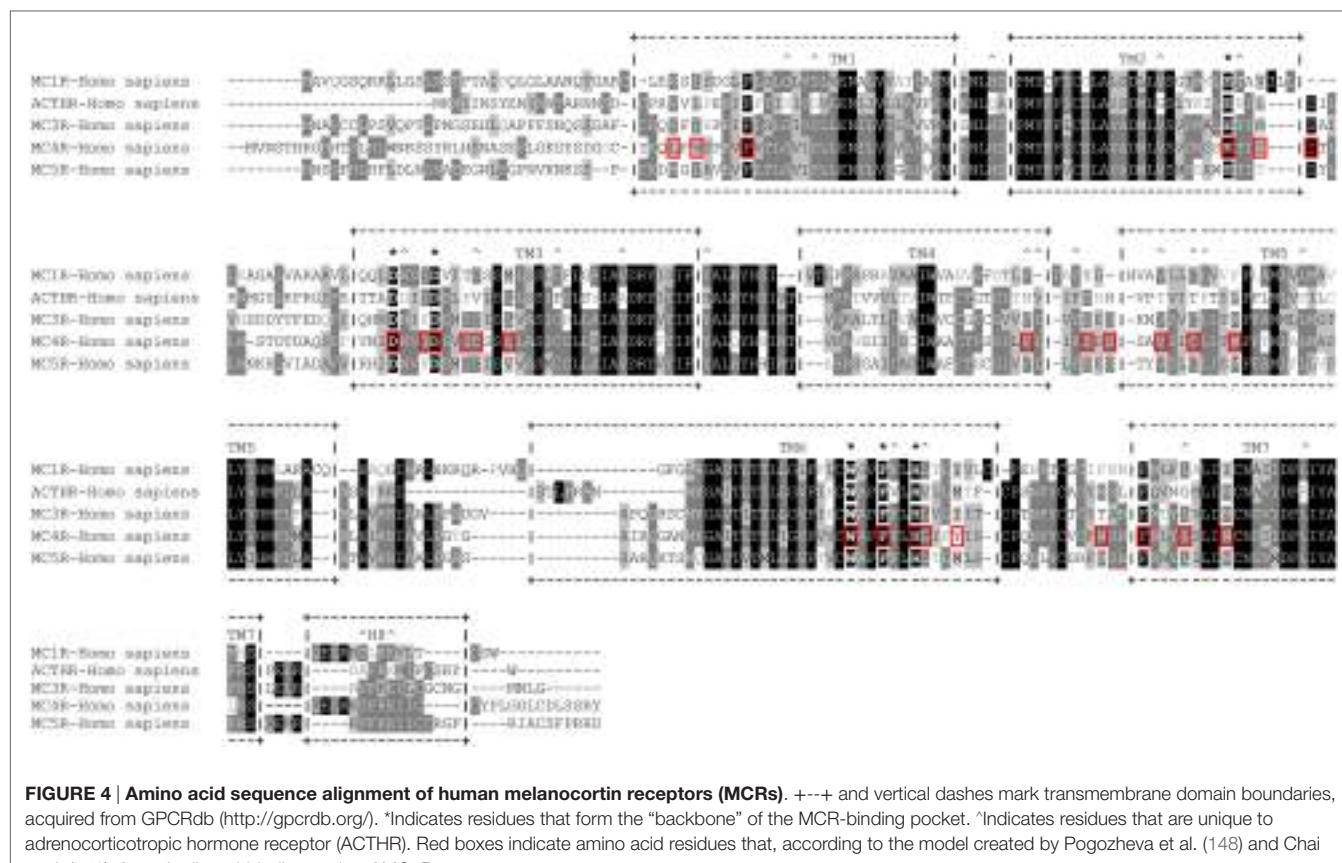
An interesting attempt to redesign the three-dimensional model of MC4R was undertaken by Pogozheva et al. (149) and Chai et al. (150). Unlike previous groups, these researchers employed data from functional analysis of receptors with point mutations and also used the crystal structure of bovine rhodopsin which had been recently determined (150). In addition, to test the validity of their model, they also performed docking of various native and artificial ligands. According to their model, the binding pocket of MC4R is a large elongated cavity formed by  $L^{44}$ ,  $V^{46}$ ,  $F^{51}$ ,  $E^{100}$ ,  $I^{103}$ ,  $I^{104}$ ,  $D^{122}$ ,  $I^{125}$ ,  $D^{126}$ ,  $I^{129}$ ,  $C^{130}$ ,  $L^{133}$ ,  $F^{184}$ ,  $S^{188}$ ,  $S^{190}$ ,  $V^{193}$ ,  $C^{196}$ ,  $M^{200}$ ,  $W^{258}$ ,  $F^{261}$ ,  $H^{264}$ ,  $L^{265}$ ,  $Y^{268}$ ,  $M^{281}$ ,  $F^{284}$ ,  $L^{288}$ , and  $M^{292}$ . This cavity contains several acidic and multiple aromatic residues located in groups on opposing sides thus forming the predicted-binding pockets with “backbone” MC-binding residues (Figures 4 and 5). Although hypothetical, this model clearly demonstrates that a significant amount of space is required to incorporate the relatively large  $\beta$ -turn structure of MCs (148), and a similar situation would be expected with all MCRs (links to models are available at <http://www.uniprot.org/uniprot/P32245>).

In the following years, a number of groups generated 3D models of various MCRs, and each explored MCR-specific themes, whether mutation-induced structural changes or differences in ligand recognition. However, the majority of these models are unavailable for download, and we were therefore unable to assess their intramolecular and intermolecular interactions in detail. For this reason, we used the model generated by Pogozheva et al. (148) and Chai et al. (149) as a reference in our following analysis.

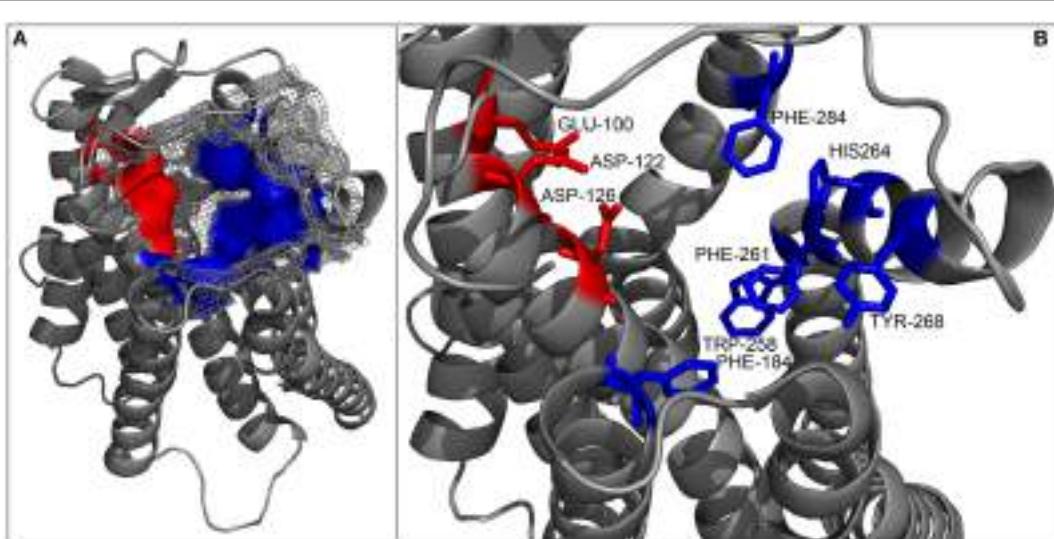
## NATURALLY OCCURRING MUTATIONS

Historically, the first source that provided information on the molecular mechanisms underlining the action of ACTHR were genetic studies on FGD—a rare autosomal recessive disorder that, as mentioned in Section “Introduction,” can be caused by defects in the *ACTHR* gene. From a genetic standpoint, all these alterations can be subdivided in three groups: mutations within regulatory sequences, mutations that result in severely truncated proteins (frameshift mutations or mutations that introduce STOP codons), and mutations that alter a single aa residue. Since the first two groups, from perspective of this article, are uninformative, they are not reviewed in detail [for reports on this subject see Ref. (107, 151–160)]. However, the third group has provided the scientific community with some valuable knowledge.

The first report on a missense mutation within FGD patients was published by Clark and colleagues soon after the discovery of the ACTHR coding sequence (97). The mutation was located within the extracellular part of the TM2 domain and resulted in substitution of  $S^{74}$  with I (Figure 6). Subsequent functional characterization of the mutated receptor confirmed that its ability to induce cAMP (107, 161, 162), bind ACTH (107), and even reach the cellular surface (despite being able to couple to



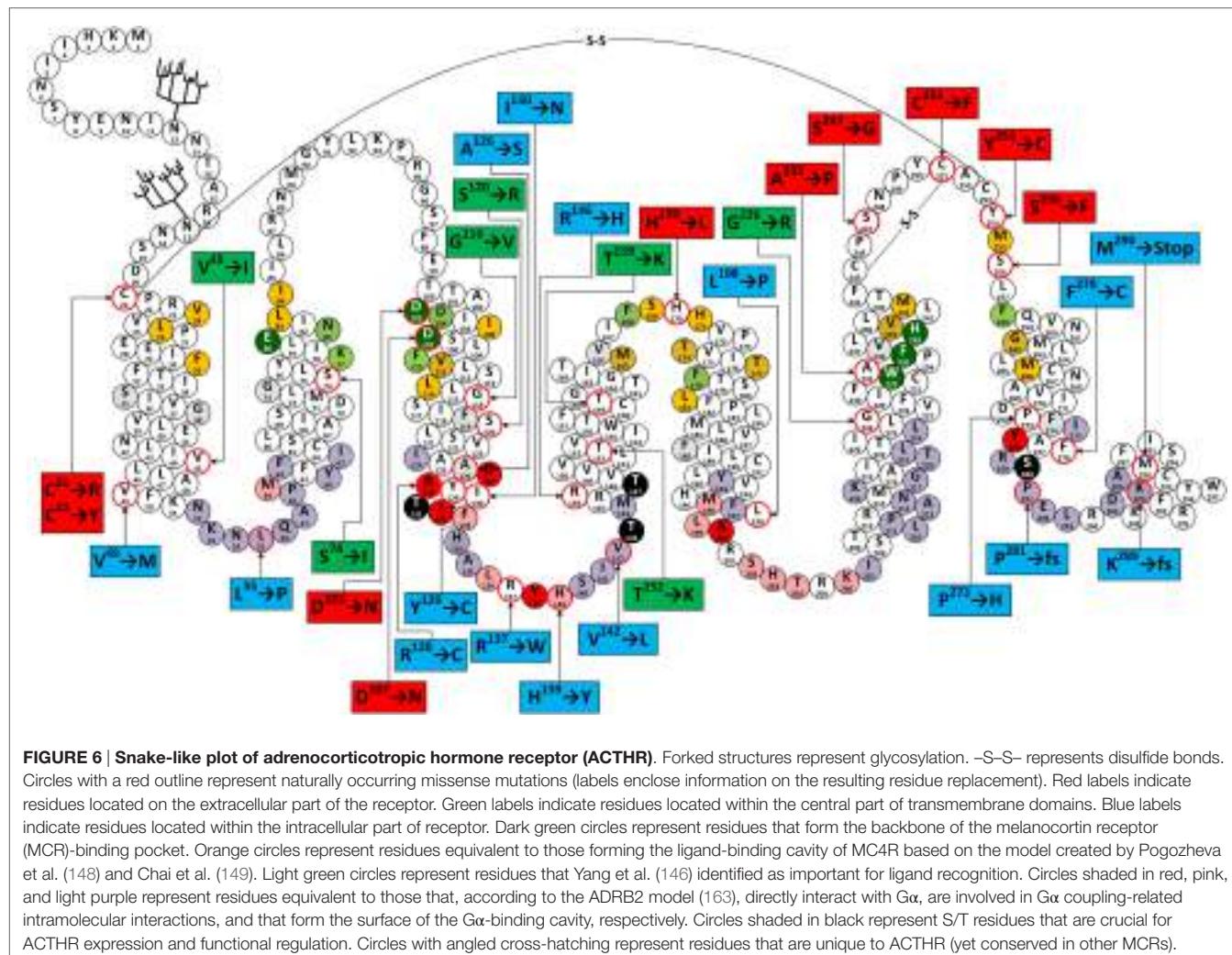
**FIGURE 4 | Amino acid sequence alignment of human melanocortin receptors (MCRs).** +--- and vertical dashes mark transmembrane domain boundaries, acquired from GPCRdb (<http://gpcrdb.org/>). \*Indicates residues that form the “backbone” of the MCR-binding pocket. ^Indicates residues that are unique to adrenocorticotrophic hormone receptor (ACTHR). Red boxes indicate amino acid residues that, according to the model created by Pogozheva et al. (148) and Chai et al. (149), form the ligand-binding cavity of MC4R.



**FIGURE 5 | Cartoon representation of the backbone of the MC4R model created by Pogozheva et al. (148) and Chai et al. (149).** (A) View from the side and top on the surface of the ligand-binding cavity that contains several acidic (red) and multiple aromatic residues (blue) located in groups at opposite sides of the cavity and forming acidic- and aromatic-binding pockets, respectively. (B) Close-up view of the binding cavity without the surface layer.

MRAP1) (156) was severely reduced in comparison to wild-type (WT) ACTHR. As expected, the discovery of this mutation was soon followed by others, and at present 35 different coding

sequence alleles have been associated with adrenocortical disorders (detailed information on these mutations is summarized in Table 2; Figure 6).



**FIGURE 6 | Snake-like plot of adrenocorticotrophic hormone receptor (ACTHR).** Forked structures represent glycosylation. –S–S– represents disulfide bonds. Circles with a red outline represent naturally occurring missense mutations (labels enclose information on the resulting residue replacement). Red labels indicate residues located on the extracellular part of the receptor. Green labels indicate residues located within the central part of transmembrane domains. Blue labels indicate residues located within the intracellular part of receptor. Dark green circles represent residues that form the backbone of the melanocortin receptor (MCR)-binding pocket. Orange circles represent residues equivalent to those forming the ligand-binding cavity of MC4R based on the model created by Pogozheva et al. (148) and Chai et al. (149). Light green circles represent residues that Yang et al. (146) identified as important for ligand recognition. Circles shaded in red, pink, and light purple represent residues equivalent to those that, according to the ADRB2 model (163), directly interact with G $\alpha$ , are involved in G $\alpha$  coupling-related intramolecular interactions, and that form the surface of the G $\alpha$ -binding cavity, respectively. Circles shaded in black represent S/T residues that are crucial for ACTHR expression and functional regulation. Circles with angled cross-hatching represent residues that are unique to ACTHR (yet conserved in other MCRs).

From a functional point of view, these mutations can be divided into three groups: (1) those located within the intracellular part of the receptor (**Table 2**; blue labels in **Figure 6**) that are most likely to affect G-protein interactions; (2) those located on the extracellular part of the receptor (**Table 2**; red labels in **Figure 6**) that are most likely to affect receptor–ligand interactions; (3) those located within the central regions of TM domains (**Table 2**; green labels in **Figure 6**) that are most likely to affect the overall conformation of the receptor. Despite the fact that this division is quite general, it is in good agreement with data from modeling of the MCR-binding pocket (148) and also the crystal structure of the  $\beta_2$  adrenergic receptor–G $_s$  protein complex (163).

The majority of extracellular mutations altered either the previously mentioned “backbone” MC-binding residues (dark green in **Figure 6**), as is the case for D<sup>103</sup> and D<sup>107</sup>, or residues that are adjacent to other MC ligand cavity-forming residues (orange in **Figure 6**). According to the model, MC4R-S<sup>259</sup> (ACTHR-A<sup>233</sup>) and the conserved MC4R-P<sup>260</sup> (ACTHR-P<sup>234</sup>) are located between aromatic-binding pocket-forming residues MC4R-W<sup>258</sup> (ACTHR-W<sup>232</sup>) and MC4R-F<sup>261</sup> (ACTHR-F<sup>235</sup>) (**Figures 4** and **6**). The presence of conserved P within the TM domain is

rather unusual, since due to its chemical structure it is unable to participate in the formation of an  $\alpha$ -helix, and when present it introduces a “kink”-like structure that is often employed for the proper positioning of surrounding reactive aa residues. Indeed, this appears to be exactly the case for MC4R-P<sup>260</sup> (ACTHR-P<sup>234</sup>), and it is logical to presume that additional proline located adjacent to this conserved one, as is the case of ACTHR-A<sup>233</sup> → P, would not only disrupt the binding pocket but also alter the overall conformation of the receptor to the extent that it is recognized as misfolded and retained within intracellular compartments and ultimately degraded.

According to both the model and other experimental data, there are two structure-stabilizing disulfide bonds within the extracellular region of MCRs (148, 183, 184). It is presumed that in the case of ACTHR these are formed between C<sup>245</sup> and C<sup>251</sup>, and between C<sup>21</sup> and C<sup>253</sup> (**Figure 6**). Both the MC4R model and predictions using the NetTurnP 1.0 server (185) indicate that the bond between this first pair of residues “locks” the conserved –P–Q–N–P–Y– motif of extracellular loop (EL) 3 into a  $\beta$ -turn-like structure, thus exposing its N and Y residues to the entrance of the binding pocket, and this also limits the physical distance

**TABLE 2 | Amino acid residue replacements of naturally occurring *ACTHR* gene missense mutations and their effects on receptor function.**

Mutation	Binding	Cyclic adenosine 3',5'-monophosphate (cAMP)	Basal cAMP activity	Membrane trafficking	Reference
EC C <sup>21</sup> → R	↓		↔		(164, 165)
EC C <sup>21</sup> → Y					(166)
TM V <sup>45</sup> → I	↔	↔			(159)
IC V <sup>49</sup> → M					(153)
IC L <sup>55</sup> → P				↓	(156)
TM S <sup>74</sup> → I	↓	↓		↓	(97, 107, 108, 156–158, 161, 162)
EC D <sup>103</sup> → N	↓	↓	↑	↔	(107, 156, 167–170)
EC D <sup>107</sup> → N	↓	↓		↔	(156, 159, 171–173)
TM G <sup>116</sup> → V		↓		↓	(156, 174)
TM S <sup>120</sup> → R				↓	(151, 156)
IC A <sup>126</sup> → S		↓			(160)
IC R <sup>128</sup> → C	↓	↓		↔	(107, 156, 158)
IC Y <sup>129</sup> → C		↓		↓	(156, 175)
IC I <sup>130</sup> → N				↓	(156, 176)
IC R <sup>137</sup> → W		↓		↓	(108, 156)
IC H <sup>139</sup> → Y				↓	(156, 176)
IC V <sup>142</sup> → L		↓			(167–169)
IC R <sup>146</sup> → H	↓	↓	↑	↓	(107, 156–158, 166, 177)
TM T <sup>152</sup> → K				↓	(156, 178)
TM T <sup>159</sup> → K	↓	↓	↑	↓	(107, 156, 179)
EC H <sup>170</sup> → L		↓		↔	(156)
IC L <sup>198</sup> → P				↓	(156, 176)
TM G <sup>226</sup> → R				↓	(156)
EC A <sup>233</sup> → P		↓		↓	(156, 168, 169)
EC S <sup>247</sup> → G	↓		↔		(164, 165)
EC C <sup>251</sup> → F		↓		↓	(156, 159, 171, 172)
EC Y <sup>254</sup> → C		↓		↓	(108, 156, 180, 181)
EC S <sup>256</sup> → F				↓	(156)
IC P <sup>273</sup> → H		↓		↓	(156, 162)
IC F <sup>278</sup> → C	↔	↔	↑	↔	(109, 175)
IC P <sup>281</sup> → fs					(173)
IC K <sup>289</sup> → fs				↓	(182)
IC M <sup>290</sup> → Stop				↓	(182)
EC C <sup>21</sup> → R + EC S <sup>247</sup> → G			↑		(164)
IC Y <sup>129</sup> → C + IC F <sup>278</sup> → C		↓		↓	(175)

EC, located within the extracellular part; IC, located within the intracellular part; TM, located within the central part of the TM domain; fs, frameshift; ↔, functional properties were unaffected; ↑, functional properties were improved; ↓, functional properties were hampered; empty field, no data available.

between TM6 and TM7. Although data on the role of “locked” aa residues in MCR ligand recognition is ambiguous, the disruption of this disulfide bond ( $C^{251} \rightarrow F$ ) apparently prevents proper receptor folding and results in intracellular retention. From this perspective, it is hard to explain the negative effect of the  $S^{247} \rightarrow G$  mutation, because according to model, the side chain of the residue homologous to ACTHR-S<sup>247</sup> in MC4R is located on the exterior surface of the receptor and thus cannot be involved in ligand recognition. Perhaps the function of this  $\beta$ -turn-like structure is to serve as a physical barrier that restricts the movement of the ligand while it occupies the cavity, or as a physical support that keeps the cavity entrance open. Thus, replacement of  $S^{274}$  with most other residues would not have any effect, but replacement with the highly flexible G allows it to adopt an alternative conformation that does not provide the necessary physical support. The disulfide bond between  $C^{21}$  and  $C^{253}$  connects TM1 and TM7 domains and locks the TM domain bundle in a “closed” conformation, which reduces flexibility and the number of available conformations. This effect appears to be crucial for formation of a functionally active receptor, as the disruption of this bond by  $C^{21} \rightarrow R$  or  $C^{21} \rightarrow Y$  mutations resulted in receptors incapable of binding ACTH. One possible explanation is probably connected to fact that the surface of the MCR ligand-binding cavity is predominantly formed from the TM domains. Another mutation that falls within the disulfide bond category is  $Y^{254} \rightarrow C$ , since it introduces an additional C residue within the EL3 region that could interfere with the formation of one or other of the previously mentioned disulfide bonds. Therefore, unsurprisingly, the  $Y^{254} \rightarrow C$  mutant was intracellularly retained, similar to the  $C^{251} \rightarrow F$  mutant.

The effects of the last two extracellular mutations ( $S^{256} \rightarrow F$  and  $H^{170} \rightarrow L$ ) are harder to explain. Alignment of MCR coding sequences reveals that residue  $S^{256}$  is somewhat conserved (present in three out of five human MCRs), while the model suggests that the side chain of MC4R-S<sup>282</sup> (ACTHR-S<sup>256</sup>) is oriented toward the external surface of the receptor and thus cannot directly interact with the ligand. However, it is located adjacent to MC4R-M<sup>281</sup> (ACTHR-M<sup>255</sup>) that lines the binding cavity, and in close proximity to the disulfide bond forming MC4R-C<sup>279</sup> (ACTHR-C<sup>253</sup>). Thus, replacement of the small, polar, and hydrophilic S with a bulky, non-polar, and hydrophobic F could affect these functionally important residues by misfolding the extracellular region, resulting in the retention of the receptor in the interior of the cell. The mechanisms of this effect may vary; for example, to minimize contact with water molecules, the side chain of F could reorient from the exterior surface to the internal cavity of the receptor, or as predicted by TMpred software (186), increase the length of TM7 by introducing an additional turn so that it is embedded within the lipid bilayer of the membrane.

Although difficult to explain, the  $H^{170} \rightarrow L$  variant is perhaps the most interesting mutation of on the extracellular side of ACTHR because it is located within EL2, which is the shortest of the three loops. In most MCRs, this region is well conserved and formed from one acidic residue (E or D) and a number of hydrophilic and/or polar residues (Figure 4), but within ACTHR there are two basic H and one hydrophilic S residue. The conservation of acidic residues and the participation of both flanking

residues in the formation of the binding cavity of MC4R are indicative of a functional purpose. Perhaps it works as “bait” for basic residues on the MC pharmacophore to recruit/direct ligand to the cavity entrance. Alternatively, it could interact with the basic -K-K-R-R-P- motif of ACTH, thus modulating its receptor activation ability. Determination of the true purpose clearly requires additional research. Nevertheless, such speculation is at least partially untrue in the case of ACTHR because the basic  $H^{170}$  is in the exact position occupied by an acidic residue in other MCRs, yet the loss of functional activity observed by Clark’s group (156) shows that it is still important. Peculiarly, the Clark group also observed that  $H^{170} \rightarrow L$ ,  $D^{103} \rightarrow N$ ,  $D^{107} \rightarrow N$ , and  $R^{128} \rightarrow C$  were the only 4 out of 22 functionally hampered receptors that were effectively transported to the cell membrane. Unifying the other three mutations was their location and functional purpose, since all are located deep within the cavities of ACTHR, and all participate in ligand–receptor or receptor–G-protein interactions. Thus, it can be presumed that these substitutions altered only the internal surface and not the overall structure of ACTHR. In light of this knowledge, it is tempting to speculate that  $H^{170} \rightarrow L$  could have similar effects. However, being located on the edge of a binding cavity, this residue cannot participate in interactions with the MC consensus pharmacophore and must instead interact with either the second ACTH pharmacophore (-K-K-R-R-P-) or with MRAP1.

According to the MC4R model, mutations in the central part of the TM domains are located either on the external surface of ACTHR and thus in contact with the lipid bilayer ( $S^{74} \rightarrow I$ ,  $T^{152} \rightarrow K$ ,  $T^{159} \rightarrow K$ , and  $G^{226} \rightarrow R$ ) or buried within the tightly packed core of the TM bundle ( $S^{120} \rightarrow R$  and  $G^{116} \rightarrow V$ ). Therefore, it was not surprising that all native residues were small and/or hydrophobic, and mutated residues were hydrophilic ( $S^{120} \rightarrow R$ ,  $T^{152} \rightarrow K$ ,  $T^{159} \rightarrow K$ , and  $G^{226} \rightarrow R$ ) or significantly larger than native residues ( $S^{74} \rightarrow I$  and  $G^{116} \rightarrow V$ ). The mechanisms underlying the changes in receptor activity for all these substitutions thus appear to be very simple. In the case of hydrophobic → hydrophilic alterations, the introduced residue would likely induce a rotational shift of the TM domain to minimize exposure to the hydrophobic environment, while for small → large alterations, the surrounding structure would have to shift to accommodate the larger residue. Either type of rearrangement would likely result in misfolding and/or altered functional cavities.

Analogous to mutations located on the extracellular side of receptor, those on the intracellular side likely affect receptor–G-protein interactions. The crystal structure of the  $\beta_2$  adrenergic receptor–Gs protein complex identified a number of receptor residues that are involved in interactions between both partners (163). Although the  $\beta_2$  adrenergic receptor (ADRB2) and ACTHR are relatively distantly related (~26% sequence identity), they both couple with the  $G\alpha_s$  subunit, thus the ACTHR surface that interacts with  $G\alpha$  should be similarly folded to that of ADRB2 (Figure 6). For this very reason, we based our subsequent analysis described in the next paragraphs on this receptor–ligand complex crystal structure (PDB ID: 3SN6).

Given that there are a significantly larger number of GPCR subtypes than G-protein subtypes, the correct folding of the intracellular parts of receptors is presumably rigorously monitored

and misfolded molecules intracellularly retained and degraded. Indeed, this was observed by the Clark group (156) who characterized a number of intracellular mutations located within ACTHR regions that either directly interact with G $\alpha_s$  (R<sup>128</sup> → C) or are homologous to residues that form the surface of ADRB2 G $\alpha_s$  coupling cavity (L<sup>55</sup> → P, H<sup>139</sup> → Y, V<sup>142</sup> → L, P<sup>281</sup> → fs, and K<sup>289</sup> → fs). The effect of R<sup>128</sup> → C can be easily explained because it is located within the highly conserved rhodopsin GPCR family -D-R-Y- motif, which is involved in signal transduction. The retention of L<sup>55</sup> → P, H<sup>139</sup> → Y, and V<sup>142</sup> → L is most probably caused by relevant changes in the properties of the residues. Mutation to P often restricts the backbone of the polypeptide chain and prevents the adoption of its native conformation, while the replacement of more hydrophilic H and V to more hydrophobic Y and L generally causes local structural rearrangements because the introduced residues tend to get buried deeper within the protein or lipid bilayer to minimize contact with water molecules. In the case of V<sup>142</sup>, these local rearrangements and the increased size of the hydrophobic side chain could possibly affect phosphorylation of the adjacent T<sup>143</sup> because the homologous position (T<sup>157</sup>) within MC1R is critical for receptor functional activity (86, 187, 188). Peculiar, however, were the P<sup>281</sup> and K<sup>289</sup> frameshifts and the M<sup>290</sup> → Stop mutation affecting located within the small C-terminal intracellular  $\alpha$ -helix (often referred as H8). Since neither structural data nor experimental results have illuminated the role of this region in signal transduction via G-protein binding, the retention caused by its loss seems interesting. Several research groups have undertaken efforts to uncover the purpose of this structural element, but results are ambiguous (189–191). Its primary role could be to properly orient the GPCR within the plasma membrane, so that it can be effectively accessed by both ligand and G-protein.

Results from mutations located outside the binding cavity are harder to interpret, yet some appear relatively straightforward. Substitution of Y<sup>129</sup> → C, located within the previously mentioned -D-R-Y- motif, can be interpreted based on the ADRB2 structure, which suggests this particular residue is oriented toward the exterior of the molecule, and a role in signal transduction has been demonstrated in other MCRs (192). The A<sup>126</sup> → S and I<sup>130</sup> → N replacements involve changes from hydrophobic to hydrophilic residues. In both cases, the side chains of native residues are predicted to be located on the surface of the receptor and oriented toward the lipid bilayer; therefore, these replacements most probably induce some structural rearrangements so that the mutated residues have limited interaction with the hydrophobic environment of the membrane. The opposite is likely true of R<sup>137</sup> → W, in which the hydrophilic R is replaced with the hydrophobic W. R<sup>137</sup> (ADRB2-K<sup>140</sup>) is located between Y<sup>138</sup> (ADRB2-Y<sup>141</sup>), and L<sup>136</sup> (ADRB2-F<sup>139</sup>), and the first of these two residues interacts with D in the -D-R-Y- motif that orients the second residue toward the hydrophobic pocket of the G-protein. It appears the purpose of the basic R<sup>137</sup> is to serve as a kind of a “lever” that keeps this otherwise hydrophobic region at the surface of the cell membrane. Its replacement would likely cause this region to adopt a different conformation and possibly “sink” deeper in to the membrane. As already mentioned, replacements

involving P are often associated with large changes in the protein backbone, and this appears to be the case for P<sup>273</sup> → H and L<sup>198</sup> → P. P<sup>273</sup> (ADRB2-P<sup>323</sup>) is one of the most highly conserved residues within rhodopsin GPCRs, where it introduces a kink in TM7 that allows the highly conserved Y<sup>276</sup> (ADRB2-Y<sup>326</sup>) to interact with G $\alpha_s$ . Introduction of H at this position would likely disrupt the native conformation. The opposite is true of L<sup>198</sup> which is located at the base of TM5 and followed by IL3, which plays a crucial role in receptor-G-protein interactions. Introduction of P and an associated  $\alpha$ -helical kink would alter the orientation of the entire loop, resulting in a misfolded receptor with hampered signal transduction ability.

Of all the intracellular mutations, the effects of V<sup>49</sup> → M and R<sup>146</sup> → H are hardest to interpret, because in both cases the native residue is replaced with a residue that has similar properties. Nevertheless, closer examination involving analysis of the ADRB2 structure can shed light on the underlying mechanisms. Replacement of V<sup>49</sup> → M involves only a change in size and the introduction of a sulfur atom. V<sup>49</sup> (ADRB2-I<sup>58</sup>) is located on the surface at the base of TM1 and being hydrophobic, it does not protrude outwards but is instead oriented along the surface of the ACTHR molecule toward the TM2 domain and the receptor interior. V is a small residue compared with M, therefore the size difference could account for the observed effects. However, in ADRB2, the same position is occupied by I, which is also larger than V, yet this receptor has high activity, indicating that the introduction of a sulfur atom cannot be excluded. Interestingly, both sulfur-containing residues (C and M) and H are able to bind metal ions, and if several similar residues are present in a given location, they may simultaneously bind single metal ion to form a bridge-like structure. According to the crystal structure of ADRB2, the base of both TM1 and TM2 is located in close proximity, and there is an additional M<sup>59</sup> at the base of the TM2 domain in ACTHR that may be sufficiently close to form a metal-binding site with the mutated M<sup>49</sup> (V<sup>49</sup> → M). Based on the structural similarity with ADRB2, this second M<sup>59</sup> (ADRB2-T<sup>68</sup>) is likely located within the functionally important region that interacts with the previously mentioned Y<sup>138</sup> (ADRB2-Y<sup>141</sup>) and D<sup>127</sup> (ADRB2-D<sup>130</sup>) from the -D-R-Y- motif, hence the formation of this putative metal bridge-like structure could alter the ability of the receptor to activate the G-protein. Although this theory seems highly speculative, in our previous studies, we successfully introduced similar albeit artificial metal-binding sites into the structure of MC4R (147).

The R<sup>146</sup> → H replacement involves residues that both are basic, suggesting the observed decrease in receptor’s ability to bind ligand, induce functional response and reach cellular surface is due to size differences, aromatic interactions, or the ability to bind metal ions rather than the loss of a reactive group. R<sup>146</sup> (ADRB2-K<sup>149</sup>) is located on the surface of the receptor at the base of the TM4 domain. Being hydrophilic, this residue is oriented toward the surface of the molecule, thus structural alterations due to size differences are unlikely to affect receptor function. Coincidentally, as with the TM1 domain, the base of this domain is also located in close proximity to the TM2 domain. Therefore, the formation of a M<sup>59</sup>-H<sup>146</sup> metal-binding pocket

that alters the conformation of the intracellular region is also plausible. Additionally, within the TM2 domain, approximately one  $\alpha$ -helical turn above M<sup>59</sup> is located F<sup>62</sup> (ADRB2-F<sup>71</sup>), which is conserved in rhodopsin GPCRs. The side chain of this residue is oriented toward the TM4 domain, suggesting that abnormal aromatic interactions could prohibit ACTHR from adopting its native conformation in a similar way to that proposed for the putative metal-binding pockets described above.

In addition to mutations that hamper receptor activity, the intracellular part of ACTHR includes two constitutive activity mutations F<sup>278</sup> → C and the double mutation C<sup>21</sup> → R + S<sup>247</sup> → G. The first of these mutations was discovered in a patient with ACTH-independent Cushing's syndrome, a disease typically caused by excess ACTH. Functional analysis revealed that this mutated receptor was effectively transported to the cell surface and displayed functional activity parameters that were comparable with WT ACTHR. The only difference was the elevated basal activity. Further research revealed that this effect was due to deficient receptor desensitization, and site-directed mutation of the phosphorylation site in the S<sup>280</sup> → A variant resulted in a receptor with similar properties (109). The authors of this article concluded that the F<sup>278</sup> → C mutation somehow masks the presence of the phosphorylation site. However, the mechanism of this masking is still unknown and hard to interpret. Some years later, a patient was identified that in addition to F<sup>278</sup> → C also carried an inactivating Y<sup>129</sup> → C mutation (as described in previous paragraphs). As a result, this double mutant receptor was intracellularly retained.

Although considered to be rare, this C<sup>21</sup> → R + S<sup>247</sup> → G double mutant was identified in another ACTH hypersensitivity patient (164). However, unlike in the previous patient, this mutant displayed only basal activity, and cAMP accumulation was not observed upon stimulation with ACTH. Expressed separately, both mutations generated functionally inactive receptors (as described in previous paragraphs), thus the authors speculated that within the double mutant these residues are located in close proximity and are required to maintain the receptor in an activated conformation.

Taken together, the knowledge gained from naturally occurring mutations is in good agreement with the findings of Pogozheva et al. (148), the MC4R-binding pocket model, and the crystal structure of the  $\beta_2$  adrenergic receptor–Gs protein complex of Rasmussen et al. (163). Thus, both models appear to be useful for evaluation of the effects caused by single residue mutations.

## SITE-DIRECTED MUTAGENESIS

Although analysis of naturally occurring mutations has provided us with a significant amount of functional data, the nature of this type of research can be somewhat random and unfocused. Thus, in order to pursue a broader knowledge on ACTHR specificity and the underlying molecular mechanisms, several research groups employed site-directed mutagenesis and subsequent functional characterization of mutated ACTHRs. Due to the difficulties to express functionally active ACTHR (193), majority of such studies were performed only after the

characterization of Y6 and OS3 adrenal cell lines (106) and/or discovery of MRAP1 (39).

One of the first to perform systematic mutagenesis of ACTHR was Yang and colleagues who had already performed similar analyses on other MCRs (138, 139, 142). They introduced 16 point mutations within various ACTHR regions to assess their functional role in ligand binding and receptor activation. The repertoire of mutations covered both residues that were known to alter the activity of MC1-3R, and residues that, according to sequence alignment, were unique to ACTHR. The results of these analyses are summarized in Table 3 (146). In Section “Discussion,” the authors concluded that the general organization of the ACTHR ligand-binding cavity is similar to that of other MCRs, as it also consists of acidic- and aromatic-binding pockets formed by the same “backbone” residues (E<sup>80</sup>, D<sup>103</sup>, D<sup>107</sup>, F<sup>235</sup>, and H<sup>238</sup>). However, there are also significant differences. Compared with other MCRs, the role of D<sup>103</sup> in ligand binding and receptor activation is significantly diminished, as its alteration caused only a 2.8-fold decrease in ACTH (1–24) affinity and a 7.3-fold decrease in potency. The role of this residue appears to be compensated by the adjacent D<sup>104</sup> that is unique to ACTHR, since replacement of this residue had a greater effect (10.2-fold and 18.3-fold decrease, respectively). A number of aromatic residues unique to ACTHR were also tested, and their replacement with alanine had a significant (F<sup>168</sup>) or even tremendous (F<sup>110</sup> and F<sup>178</sup>) effect on ligand binding and receptor activation. This led to speculation that the binding cavity of ACTHR could be “broader” than that of other MCRs and could accommodate additional ligand residues that would interact with some of the identified functionally relevant unique residues (146). However, work based on the MC4R-binding pocket model (148) partially counters this speculation, because homologous positions of these high effect aromatic residues (F<sup>110</sup> and F<sup>178</sup>) were located in close proximity to aromatic residues of the MC consensus pharmacophore (F<sup>235</sup> and W<sup>232</sup>, respectively), thus indicating their role in ligand binding and receptor activation. Nevertheless, this “broader” binding cavity remained possible because homologous position of the basic ACTHR-K<sup>77</sup> within MC4R model resides within the densely packed and hydrophobic TM domain bundle region adjacent to the acidic D<sup>103</sup>. Being charged and larger than N (in MC1, MC3, and MC4R) or S (in MC5R), this residue could conceivably thread through this region toward the surface of the binding cavity and interact with D<sup>103</sup> to alter (broaden) the cavity. In light of the previously discussed naturally occurring H<sup>170</sup> → L mutation it was particularly interesting to test F<sup>168</sup> which, like H<sup>170</sup>, is located within EL2 at the entrance to the binding cavity, and, like H<sup>170</sup>, it affects ligand binding, although apparently to a lesser extent. This provided further evidence of the role of this region in receptor functional activity.

Although very informative, this work by Yang and colleagues was not their first involving ACTHR. In their preceding study (published only a few months earlier), they used chimeric MC4/ACTHR receptors to probe TM domain residues of MC4R that are responsible for the high affinity for the synthetic ligand NDP-MSH (195). Due to the high ligand recognition specificity, they

**TABLE 3 | Site-directed mutants of adrenocorticotrophic hormone receptor (ACTHR) and their effects on receptor function.**

Mutation	Binding ( $\times$ wtACTHR)	Cyclic adenosine 3',5'-monophosphate ( $\times$ wtACTHR)	Membrane trafficking ( $\times$ wtACTHR)	Rate of internalization (% of initial rate)	Reference
D <sup>70</sup> → A	X	X	X		(146)
*K <sup>77</sup> → A	$K_i = 4.5 \times \uparrow$	$EC_{50} = 7.8 \times \uparrow$	$1.4 \times \downarrow$		
E <sup>80</sup> → A	$K_i = 11.6 \times \uparrow$	$EC_{50} = 9.8 \times \uparrow$	$1.3 \times \downarrow$		
*N <sup>81</sup> → A	$K_i = 1.8 \times \uparrow$	$EC_{50} = 2.4 \times \uparrow$	$1.1 \times \downarrow$		
D <sup>103</sup> → N	$K_i = 2.8 \times \uparrow$	$EC_{50} = 7.3 \times \uparrow$	$1.5 \times \downarrow$		
*D <sup>104</sup> → N	$K_i = 10.2 \times \uparrow$	$EC_{50} = 18.3 \times \uparrow$	$1.3 \times \downarrow$		
D <sup>107</sup> → N	$K_i > 178.6 \times \uparrow$	$EC_{50} > 1250.0 \times \uparrow$	$1.5 \times \downarrow$		
*F <sup>110</sup> → A	$K_i > 116.3 \times \uparrow$	$EC_{50} > 208.3 \times \uparrow$	$1.5 \times \downarrow$		
*T <sup>164</sup> → A	$K_i = 1.2 \times \downarrow$	$EC_{50} = 1.3 \times \downarrow$	$1.1 \times \downarrow$		
*F <sup>168</sup> → A	$K_i = 25.3 \times \uparrow$	$EC_{50} = 68.3 \times \uparrow$	$1.8 \times \downarrow$		
*F <sup>178</sup> → A	$K_i > 178.6 \times \uparrow$	$EC_{50} > 1250.0 \times \uparrow$	$1.5 \times \downarrow$		
F <sup>235</sup> → A	$K_i = 17.5 \times \uparrow$	$EC_{50} = 270.0 \times \uparrow$	$1.2 \times \downarrow$		
H <sup>238</sup> → A	$K_i > 178.6 \times \uparrow$	$EC_{50} > 1250.0 \times \uparrow$	$1.4 \times \downarrow$		
*F <sup>244</sup> → A	$K_i = 1 \times \leftrightarrow$	$EC_{50} = 1.1 \times \uparrow$	$1.1 \times \downarrow$		
F <sup>258</sup> → A	$K_i = 13.9 \times \uparrow$	$EC_{50} = 233.8 \times \uparrow$	$1.3 \times \downarrow$		
D <sup>272</sup> → A	X	X	X		
N <sup>12</sup> N <sup>13</sup> → QQ		$EC_{50} = \sim 1.4 \times \uparrow$	$\leftrightarrow$		(194)
N <sup>17</sup> N <sup>18</sup> → QQ		$EC_{50} = \sim 2.1 \times \uparrow$	$\leftrightarrow$		
N <sup>12</sup> N <sup>13</sup> N <sup>17</sup> N <sup>18</sup> → QQQQ		$EC_{50} = \sim 4.5 \times \uparrow$	$\sim 3.2 \times \downarrow$		
wtACTHR	$R_{max} = 1 \times \leftrightarrow$		$1 \times \leftrightarrow$	33.1	(86)
T <sup>131</sup> → A	$R_{max} = 1.1 \times \downarrow$		$1.27 \times \uparrow$	<0.1	
T <sup>131</sup> → D	$R_{max} = 4.5 \times \downarrow$		$1.3 \times \uparrow$	<0.1	
S <sup>140</sup> → A	$R_{max} = 1.1 \times \downarrow$		$1.1 \times \downarrow$	25.5	
S <sup>140</sup> → D	$R_{max} = 1.2 \times \downarrow$		$1.25 \times \uparrow$	43.2	
T <sup>143</sup> → A	$R_{max} = 33.3 \times \downarrow$		$100 \times \downarrow$	22.2	
T <sup>143</sup> → D	$R_{max} = 1 \times \leftrightarrow$		$3.1 \times \downarrow$	16.3	
T <sup>143</sup> → S	$R_{max} = 1.1 \times \uparrow$		$2.3 \times \downarrow$	27.7	
T <sup>143</sup> → G	X		$11.1 \times \downarrow$	X	
T <sup>143</sup> → K	$R_{max} = 2.9 \times \downarrow$		$12.5 \times \downarrow$	X	
T <sup>147</sup> → A	$R_{max} = 1.2 \times \uparrow$		$1.2 \times \uparrow$	23.6	
T <sup>147</sup> → D	$R_{max} = > 100 \times \downarrow$		$> 100 \times \downarrow$	<0.1	
S <sup>202</sup> → A	$R_{max} = 1 \times \leftrightarrow$		$1.5 \times \uparrow$	40.6	
S <sup>202</sup> → D	$R_{max} = 1.1 \times \uparrow$		$1.1 \times \downarrow$	60.7	
S <sup>204</sup> → A	$R_{max} = 1.2 \times \uparrow$		$1 \times \leftrightarrow$	18.8	
S <sup>204</sup> → D	$R_{max} = 1 \times \leftrightarrow$		$1.5 \times \downarrow$	39.4	
S <sup>208</sup> → A	$R_{max} = 1.1 \times \uparrow$		$1.1 \times \downarrow$	22.6	
S <sup>208</sup> → D	$R_{max} = 1.1 \times \downarrow$		$1.1 \times \downarrow$	56.7	
T <sup>209</sup> → A	$R_{max} = 1.1 \times \uparrow$		$1 \times \leftrightarrow$	32.4	
T <sup>209</sup> → D	$R_{max} = 1.1 \times \downarrow$		$1.2 \times \downarrow$	20.1	
S <sup>280</sup> → A	$R_{max} = 1 \times \leftrightarrow$		$1.4 \times \downarrow$	50.8	
S <sup>280</sup> → D	$R_{max} = 1.5 \times \downarrow$		$1.1 \times \downarrow$	<0.1	
S <sup>294</sup> → A	$R_{max} = 1 \times \leftrightarrow$		$1.3 \times \downarrow$	36.4	
S <sup>294</sup> → D	$R_{max} = 1.4 \times \downarrow$		$1.3 \times \downarrow$	49.0	
N <sup>12</sup> N <sup>13</sup> N <sup>17</sup> N <sup>18</sup> → QQQQ		$R_{max} = 1.2 \times \downarrow$	$3.0 \times \downarrow$	80.5	

X, fold difference compared with wtACTHR;  $\leftrightarrow$ , value unaffected;  $\uparrow$ , value increased;  $\downarrow$ , value decreased; X, not determined; empty field, no data available.

intended to use the TM domains of ACTHR for loss of function studies, and in order to localize the alterations and simplify data interpretation, they replaced only one TM domain (TM2, TM3, TM4, TM5, or TM6) at a time without disruption of adjacent intracellular or ELs. The results revealed that in majority of cases these replacements had little or no effect on receptor surface expression, NDP-MSH recognition or receptor activation efficiency. The exception was the replacement of TM3 (hMC4R/TM3 hMC2R); although this receptor was transported to the cell surface, it displayed significantly reduced ligand affinity and potency (a 12.7-fold and 40-fold decrease, respectively; Table S1 in Supplementary Material). Therefore, the authors concluded that non-conserved residues in this region determine the ability of MC4R to effectively bind NDP-MSH (195). However, based on the above knowledge, we believe that these observations are more likely due to peculiarities of ligand recognition within ACTHR rather than MC4R, in particular binding pocket disturbance caused by the presence of an additional acidic (D<sup>104</sup>) residue, and also possibly the aromatic F<sup>110</sup> residue (195).

The successful application of a chimeric receptor strategy for various MCRs has been reported on multiple occasions (195–198). Since this initial work number of groups including our own group has used this platform for functional research on ACTHR (199–202). However, since the results of these studies are to a degree speculative, and because different analytical methods were employed, the findings are quite difficult to summarize and so are reviewed in chronological order.

Although many groups were working in parallel, we were the first group to publish in this area. During the initial stages of our research (199), we were more interested in exploring the structural elements that determine the selectivity of ACTHR membrane expression rather than its ligand recognition selectivity. Therefore, since it was already known that posttranslational modification of the N-terminus affected the membrane transport of cell-surface glycoproteins, in our first set of chimeric receptors, we substituted this region in both ACTHR and MC4R. The results partially confirmed our hypothesis; the membrane expression of MC4R with the ACTHR N-terminus (Ch1 in Table S1 in Supplementary Material) was significantly reduced compared with wt MC4R. The results also demonstrated that the N-terminus is not the only region of ACTHR that facilitates its intracellular retention, because ACTHR with the N-terminus of MC4R (Ch2 in Table S1 in Supplementary Material) was not trafficked to the cell membrane. Thus, to explore further, we created additional sets of chimeric receptors by replacing ACTHR TM domains with corresponding regions of MC4R in various combinations (Ch3–Ch15 in Table S1 in Supplementary Material). Subsequent evaluation of the membrane transportation efficiency revealed that retention occurred only when both TM3 and TM4 from ACTHR were present simultaneously, suggesting this region, or elements of this region, is responsible for intracellular retention. In addition to membrane transportation, we also carried out binding and cAMP response analyses. Although, due to their sensitivity, the initial purpose of these assays was to enable the detection of low-level membrane trafficking, the results also identified regions that determine ACTHR

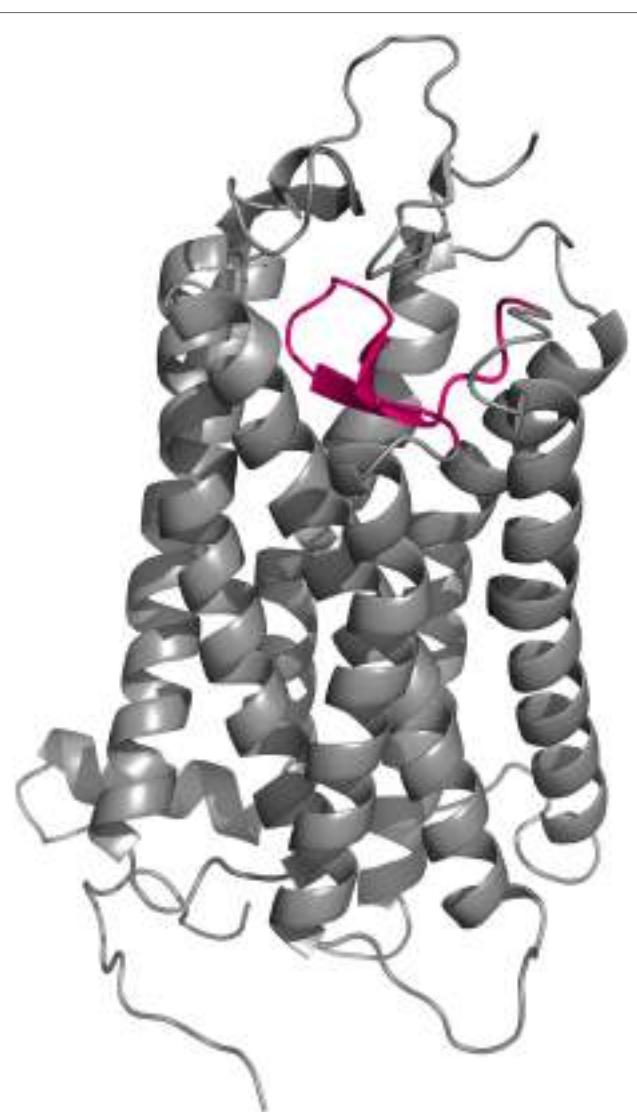
ligand-binding specificity. MC4R-based chimeric receptors were able to bind ligands when TM4–5 and TM6–7 were replaced with the corresponding parts of ACTHR, but binding was not observed when both regions were replaced simultaneously. Thus, we speculated that there must be some kind of interaction between these two elements, which disrupts the conformation required for formation of the MC consensus pharmacophore (M-X-H-F-R-W) binding pocket (199).

A year after publication of our report, Hinkle and colleagues reported on employment of remarkably similar strategy (201). Indeed, several of the chimeric receptors were identical in spite of being independently created. However, they employed distinct methods for characterization of the chimeric receptor properties (i.e., measurement of surface transport and activation efficiency), and they also evaluated the effects of co-expression with MRAP1. As in our study, the chimeric receptors were generated in sets. The first set included ACTHR-based chimeric receptors with a variety of TM domains replaced to corresponding regions from MC4R (2C1–2C6 in Table S1 in Supplementary Material), while the second set mirrored these so that the same TM domains within MC4R were replaced with the corresponding regions from ACTHR (4C1–4C6 in Table S1 in Supplementary Material). Functional analysis confirmed that co-expression with MRAP1 promoted the cell-surface trafficking of ACTHR-based chimeric receptors, but this was decreased with MC4R-based receptors. However, the ACTHR-based receptor with only the N-terminus, TM1 domain, and IL1 replaced to the corresponding regions of MC4R (2C1 in Table S1 in Supplementary Material) was an exception, since it was located on the cell surface even in the absence of MRAP1, but despite its successful transportation it was functionally inactive. To investigate this further, Hinkle and colleagues created an additional set of chimeric receptors (C2C1a, C2C1b, and C2C1c in Table S1 in Supplementary Material), and subsequent evaluation of their properties narrowed the region responsible for intracellular retention to TM1 alone, because the corresponding receptor variant (C2C1b in Table S1 in Supplementary Material) was both effectively transported to the cell plasma membrane, and functionally active in the presence of MRAP1. Functional analysis of receptors from the main set also revealed another surprising discovery; when co-expressed with MRAP1, ACTHR-based chimeric receptor with TM2, EL1, and TM3 replaced to the corresponding regions of MC4R (2C2 in Table S1 in Supplementary Material) was able to induce intracellular response upon stimulation with both NDP-MSH and ACTH and also displayed substantial constitutive activity. Since both of these traits are known to be characteristic of MC4R, the authors attempted to pinpoint the responsible region by undertaking the creation of another set of C2C2 derivative chimeric receptors (C2C2a, C2C2b, C2C2c, and C2C2d in Table S1 in Supplementary Material), but functional data showed that none shared the properties of the parental chimeric or wt receptors. Based on this and other studies, Hinkle and colleagues proposed that a more likely explanation for these observations was the general misfolding of ACTHR that, without the assistance of MRAP1, is unable to pass the rigorous quality control system in the ER and is subsequently degraded. Regarding our article, the

authors simply remarked that although they did not study exactly the same chimeras, but according to their data introduction of the TM2-TM3 or TM4-TM5 regions of ACTHR receptor had little effect on MC4R surface expression (201).

Based on confidence in our own results and given that the action of ACTHR remained largely unexplained, we decided to further investigate the matter. In our following study (200), we generated additional sets of ACTHR-based chimerical receptors. Given our previously proposed hypothesis that the N-terminus and TM3-TM5 region of ACTHR was important for receptor transportation and ligand recognition specificity, we replaced various elements of these regions with the corresponding elements of MC4R (Ch16–Ch22 in Table S1 in Supplementary Material). Similarly to the Hinkle group, we tested all receptors for their ability to reach the cell plasma membrane and induce intracellular cAMP responses upon stimulation with ACTH and  $\alpha$ -MSH during both standalone expression and co-expression with MRAP. The results were somewhat unexpected because they indicated that the integrity of the extracellular part of receptor and not the separate TM domains was of paramount importance for the formation of ACTHR-specific and MRAP1-dependent arrest signal. Although initially these observations seemed to conflict with the conclusions of our former publication, in-depth reanalysis of data from both studies revealed a good agreement. In most cases, when the N-terminus, EL1 and EL2 of ACTHR were present the chimera was retained, but when two of the three elements were present, transport of the chimera to the membrane was hampered (Table S1 in Supplementary Material). Intrigued by this finding, we decided to verify and investigate it in more detail by making smaller (two to five aa) replacements within the TM3-TM5 region of Ch2, a chimera from our previous study, which is essentially ACTHR with the N-terminus of MC4R (Table S1 in Supplementary Material). While performing the same analyses as carried out on previous sets of receptors, we observed that in most cases these replacements increased the membrane export efficiency during standalone expression, but replacement of residues located within the central part of TM3 and the intracellular part of TM4 had no effect (Ch24, Ch27, and Ch28 in Figure S1 in Supplementary Material). Co-expression with MRAP1 revealed a whole spectrum of effects, including receptors that were unaffected by MRAP1 and still retained (Ch28 in Figure S1 in Supplementary Material), receptors unaffected by MRAP1 and still effectively transported to the membrane (Ch26, Ch32, and Ch35 in Figure S1 in Supplementary Material), receptors with improved transportation efficiency comparable with ACTHR (Ch24, Ch27, Ch30, Ch31, and Ch34 in Figure S1 in Supplementary Material), and receptors with decreased transportation efficiency comparable to MC4R (Ch23, Ch25, Ch29, and Ch33 in Figure S1 in Supplementary Material). As observed with large-scale replacements, these results were unexpected because we believed we finally understood ACTHR specificity. Nevertheless, like Hinkle and colleagues, we came to the conclusion that the overall structure of ACTHR must be somewhat attuned for misfolding as changes within various regions could avert this effect. Thus, we speculated that similarly to bovine rhodopsin, the extracellular part of ACTHR might form

a lid-like structure over the ligand-binding pocket (Figure 7), but rather than being permanent like in rhodopsin, it must be able to undergo MRAP1- and -K-K-R-R-P- pharmacophore-induced conformational changes that result in opening of the -M-X-H-F-R-W- binding cavity. In addition, this lid-like structure could also serve as an arrest signal, since being located on the surface of the cell membrane, it is readily accessible to components of the ER quality control system. However, it also appeared that the correct formation of this structure requires a precise alignment of the TM domains, as even the slightest changes were able to disrupt its formation. Although this study, like the previous one, mainly focused on identification of mechanisms underlying the ACTHR membrane transportation



**FIGURE 7 | Cartoon representation of the crystal structure of bovine rhodopsin (150).** EL2 (magenta) forms a lid-like structure over the ligand-binding pocket that prevents any external molecular interference with the covalently bound ligand.

specificity, we were also able to gain some insight regarding structures that determine its ligand recognition specificity. Since all chimeric receptors created during this study were ACTHR based, it was not surprising that the majority of them were able to induce cAMP responses only when co-expressed with MRAP1 and stimulated with ACTH; therefore, the most interesting in this respect were the functionally inactive Ch24 and Ch31 (Figure S1 in Supplementary Material). The functional inactivity of Ch24 was rather easy to explain, since all affected residues were located in close proximity to residues known to form the -M-X-H-F-R-W- acidic-binding pocket. However, residues replaced within Ch31 were far more interesting because they are located within EL2 that forms the edge of the binding cavity, as described above regarding the naturally occurring H<sup>170</sup> → L mutation. Since none of the adjacent alterations displayed a similar effect, and because selected residues were substituted with corresponding ones from MC4R, we concluded that this region must be either involved in the formation of the -K-K-R-R- binding pocket or functional interaction between ACTHR and MRAP1 that mediates receptor activation. In an attempt to explain this observation, we also compared the properties of the original and substituted residues, which showed that H<sup>170</sup> and H<sup>171</sup> (substituted with D and S, respectively) were the most plausible candidates for residues with a functional role because their replacement had the most significant effect on properties of this region, and due to its potential positive charge, propensity for hydrogen-bonding, and aromatic nature, histidine is often a functionally important residue (200).

The latest in a series of publications on chimeric ACTH/MC4 receptors was published by Yang and colleagues (202). This study assessed the binding affinity and potency of the synthetic ligand [D-Phe<sup>7</sup>]ACTH(1–24) on chimera receptors that were very similar to those described in their previous report in 2007 (195) (Table S1 in Supplementary Material). Their main conclusion was also very similar to their former conclusion that TM3 of ACTHR is critical for ligand selectivity and potency. Therefore, due to their lack of novelty, these results are not reviewed in detail, although there is one aspect that must be mentioned. In this article, the authors also described the creation of five ACTHR-based receptors with single TM domain substitutions to corresponding regions of MC4R (TM2, TM3, TM4, TM5, or TM6; Table S1 in Supplementary Material). In the experimental procedures section, the authors indicated that expression of chimeric receptors was performed in both OS3 and HEK cell lines, the former of which is known to support functional expression of ACTHR. However, during the experiments, they observed that all ACTHR-based chimeric receptors were transported to the cell surface at very low levels, and they therefore failed to assess their ligand-binding affinity and potency (202). Although co-expression with MRAP1 in HEK293 or CHO cell lines and the OS3 cell line may not be directly comparable, in our view, these results are somewhat contrary to the observation made by us and the Hinkle group that most ACTHR-based chimeras co-expressed of with MRAP1 were able to reach the cell membrane and many were functionally active.

Interpreting the results of studies using ACTH/MC4 chimera receptors can be difficult and even controversial. We believe this

is due to using a small-scale approach such as few chimeric receptors with single domain replacements to investigate large-scale problems such as the molecular structure and intermolecular interactions of ACTHR (i.e., overinterpretation of results acquired using the reductionist approach). This could be compared with observing the construction of a building through small holes in the fence that surrounds it. Since each group generates their own hypotheses and is only able to generate and test a limited number of mutant receptors, they see only a small part of the “bigger picture” and thus were viewing the problems from a different perspective. Nevertheless, in spite of these differences, we have attempted to summarize the disparate results in Table S1 in Supplementary Material, and, to our surprise, data obtained by different groups are generally in good agreement. Of course, some seemingly identical receptors displayed very different properties in the hands of different research groups, and differences in the expression systems may account for the apparent discrepancies (for example, Ch5 or Ch6 in our study and 2C6 or 4C4 in the Hinkle group). However, the overall general agreement allowed us to draw some summative conclusions. Firstly, it appears there is no single domain that is responsible for ACTHR intracellular retention, but rather the general structure of ACTHR may be somewhat misfolded and coupling with MRAP1 is required to either correct or mask this. Secondly, the results indicated that the role of the extracellular and possibly even the intracellular parts of ACTHR had been underestimated, because in multiple cases the substitution of even a single loop or terminus was crucial on determining receptor localization and the ability to induce an intracellular response.

While ourselves and others were working on large-scale replacements and searching for domains and regions that determine the membrane transportation and ligand recognition specificity of ACTHR, a group led by Gallo-Payet was taking a more focused approach on ACTHR glycosylation (194) and phosphorylation (86) and their effects on receptor functionality. These simple, elegant studies are reviewed below, again in chronological order.

It has been known for quite some time that glycosylation of cell-surface proteins is often required for their successful transport to the plasma membrane (203, 204). In the case of GPCRs, however, the effects of glycosylation are wider and range from being important for correct receptor folding and maturation, to having no apparent function (205–208). Prediction of potential glycosylation sites within ACTHR using such online tools as NetNGlyc 1.0 Server<sup>1</sup> or GlycoEP<sup>2</sup> identifies two glycosylation sites within the N-terminus of the receptor: N<sup>12</sup>N<sup>13</sup>T<sup>14</sup> and N<sup>17</sup>N<sup>18</sup>S<sup>19</sup> (Figure 6). Since this aspect of ACTHR functionality had not been previously researched in detail, Roy et al. (194) employed site-directed mutagenesis to eliminate these sites and performed functional expression and characterization of the resultant mutated ACTHRs within the HEK293/FRT cell line to evaluate the role of glycosylation. Upon first reading, the results of this study seemed rather confusing, as the authors observed that during standalone expression abolishment of one

<sup>1</sup><http://www.cbs.dtu.dk/services/NetNGlyc/>.

<sup>2</sup><http://www.imtech.res.in/raghava/glycoep/>.

glycosylation site had a moderate effect on the surface transportation of ACTHR, while abolishment of both significantly reduced it. In addition, co-expression with MRAP1 ( $\alpha$ ,  $\beta$ , or C-terminally truncated) resulted in the effective rescue of surface expression for mutants with only one valid glycosylation site, and moderate rescue for mutants with no glycosylation sites (Table 3). The confusing part, of course, was the presence of ACTHR at the cell membrane in the absence MRAP. However, this was later explained by the discovery that this cell line actually expresses low levels of MRAP2 that promotes ACTHR membrane transportation. Although at the first glance the selection of this expression system might seem unfortunate, it actually allowed the researchers to observe the effects of glycosylation in detail because, due to overexpression and MRAP1 rescue effects, the employment of any other MRAP2-less cell line most probably would not have allowed them to distinguish between unhampered and moderately hampered transportation efficiency. The causal reason for the endogenous expression of this accessory protein could be related to the source of the particular human embryonic kidney cell line. Based on MRAP2 expression data available at “The Human Protein Atlas”<sup>3</sup> (209, 210), adult kidney tissue expresses this protein at low levels. Although the same database suggests the parent (HEK293) cell line does not express this protein, it is plausible that this cell line is prone to triggering expression during later passages, hence its use in ACTHR surface expression studies should be carefully considered. In addition to cell-surface transportation measurements, the authors also performed receptor activation experiments, and all mutated receptors were functionally active, but there was a tendency for EC<sub>50</sub> values to increase with the number of mutated glycosylation sites, thus it was concluded that N-glycosylation of ACTHR is not critical but does have a slight influence on receptor activity (Table 3) (194).

Another type of posttranslational modification that often plays a major role in protein functionality is phosphorylation. In the case of GPCRs, it is usually associated with receptor desensitization and internalization, and it is performed by a group of serine/threonine protein kinases known as G-protein-coupled receptor kinases (211–215). Since this aspect of ACTHR action was not previously researched in detail, Roy and coworkers (86) introduced point mutations that altered all S and T residues located within the intracellular part of the receptor to the non-phosphorylatable residue A, or to the negatively charged D that can mimic phosphorylation. Additionally, T<sup>143</sup> was also mutated to S, G, and K because, as already mentioned above, the homologous position (T<sup>157</sup>) within MC1R is critical for receptor export to the plasma membrane and thus functional activity (86, 187, 188). Detailed activation, surface expression, and receptor internalization analyses both standalone and during co-expression with MRAP1 $\beta$  in the HEK293/FRT cell line generated a large number of results, the most relevant of which (in the context of this review) are presented in Table 3. The authors initially evaluated the internalization of WT ACTHR

and established that ligand binding is a necessary prerequisite for ACTHR internalization. Additionally, MRAP1 was internalized along with ACTHR, further confirming that a functional receptor is formed following the formation of ACTHR and MRAP1 complex. They also revealed that stimulation with low concentrations of ACTH (<0.3 nM) did not induce any apparent internalization, and ~28% of internalized receptor molecules were recycled back to the cell surface. Having established a baseline and confirmed the necessity of further investigations on the role of intracellular S/T residues, Roy et al. (86) carried out similar analyses on all mutated receptors, and the results showed that T<sup>143</sup> → A and T<sup>147</sup> → D replacements disrupted transportation to the cell surface and abolished the ability to respond to stimulation with ACTH, while the internalization of receptors carrying mutations T<sup>131</sup> → A, T<sup>131</sup> → D, and S<sup>280</sup> → D was essentially undetectable. Thus, it was concluded that these four residues (T<sup>131</sup>, T<sup>143</sup>, T<sup>147</sup>, and S<sup>280</sup>) are crucial for ACTHR expression and functional regulation (86).

## CONCLUSION

Data presented in this review are rather voluminous and reflect the heterogeneity of available information and applied research methodology. However, despite the difficulties, we hope that condensing this large body of work has proved useful and shall provide some novel insight. At the beginning of this article, we implied that functional ACTHR was discovered only in 2005 by Metherell et al. (39), while 1992 marks the discovery of the main subunit (38). This statement is clearly at odds with the currently used nomenclature, and the name ACTHR is even more at odds (the HGNC accepted name is MC2R). But, through this review, we have come to the conclusion that using term “receptor” is inappropriate because without MRAP1 ACTHR/MC2R does not bind to its ligand. Thus, while in this article we have ignored this, we propose that the name ACTHR should not be abandoned entirely but rather used to describe the molecular complex that specifically binds only ACTH (ACTHR-2 × MRAP1).

## AUTHOR CONTRIBUTIONS

Preparation of this article was a joint effort of all authors. DF was coordinating the process of manuscript preparation.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at <http://journal.frontiersin.org/article/10.3389/fendo.2017.00013/full#supplementary-material>.

<sup>3</sup><http://www.proteinatlas.org/>.

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# Dual Topology of the Melanocortin-2 Receptor Accessory Protein Is Stable

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Melanocortin 2 receptor accessory protein (MRAP) facilitates trafficking of melanocortin 2 (MC2) receptors and is essential for ACTH binding and signaling. MRAP is a single transmembrane domain protein that forms antiparallel homodimers. These studies ask when MRAP first acquires this dual topology, whether MRAP architecture is static or stable, and whether the accessory protein undergoes rapid turnover. To answer these questions, we developed an approach that capitalizes on the specificity of bacterial biotin ligase, which adds biotin to lysine in a short acceptor peptide sequence; the distinct mobility of MRAP protomers of opposite orientations based on their N-linked glycosylation; and the ease of identifying biotin-labeled proteins. We inserted biotin ligase acceptor peptides at the N- or C-terminal ends of MRAP and expressed the modified proteins in mammalian cells together with either cytoplasmic or endoplasmic reticulum-targeted biotin ligase. MRAP assumed dual topology early in biosynthesis in both CHO and OS3 adrenal cells. Once established, MRAP orientation was stable. Despite its conformational stability, MRAP displayed a half-life of under 2 h in CHO cells. The amount of MRAP was increased by the proteasome inhibitor MG132 and MRAP underwent ubiquitylation on lysine and other amino acids. Nonetheless, when protein synthesis was blocked with cycloheximide, MRAP was rapidly degraded even when MG132 was included and all lysines were replaced by arginines, implicating non-proteasomal degradation pathways. The results show that although MRAP does not change orientations during trafficking, its synthesis and degradation are dynamically regulated.

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## INTRODUCTION

The hypothalamic/pituitary/adrenal axis comprises a classical endocrine loop in which hypothalamic corticotropin-releasing hormone stimulates synthesis and release of ACTH from corticotropes of the anterior pituitary gland, ACTH stimulates glucocorticoid production in the adrenal gland, and glucocorticoids exert feedback control centrally. ACTH stimulates multiple steps in the glucocorticoid biosynthetic pathway, acting via a receptor coupled to the heterotrimeric Gs protein to activate adenylyl cyclase and elevate intracellular cAMP. ACTH also exerts receptor-dependent but cAMP-independent effects (1).

**Abbreviations:** AP, acceptor peptide; BirAcyt, cytoplasmic biotin ligase; BirAER, ER-targeted biotin ligase-KDEL; Ccyt, cytoplasmic carboxyterminus; Cexo, extracellular carboxyterminus; CRE, cAMP response element; ER, endoplasmic reticulum; MC2R, melanocortin-2 receptor; MRAP, MC2 receptor accessory protein; Ncyt, intracellular aminoterminus; Nexo, extracellular aminoterminus.

The receptor for ACTH was identified almost 25 years ago by Mountjoy et al. (2). The melanocortin 2 (MC2) (ACTH) receptor is one of five structurally related G protein-coupled melanocortin receptors, MC1 through MC5, all of which are activated by peptides derived from pro-opiomelanocortin, or POMC. The MC2 receptor was found to differ from the other four melanocortin receptors in two important ways. First, it could not be activated by  $\alpha$ -,  $\beta$ -, or  $\gamma$ -MSH but only by the longer ACTH peptide. Second, it was not functional unless it was expressed in adrenal or melanoma cells. This led to speculation that the MC2 receptor required an additional protein present in a limited number of cell types.

The nature of the hypothetical accessory protein remained a mystery for more than a decade, when Metherell et al. (3) identified the gene encoding MC2 receptor accessory protein (MRAP) from individuals with familial glucocorticoid deficiency type 2, which is characterized by resistance to ACTH despite normal MC2 receptor genes. MRAP encodes a small protein with a single predicted transmembrane domain. Two splice variants of human MRAP ( $\alpha$  and  $\beta$ ) are identical in the aminoterminal and transmembrane regions. When MRAP was co-expressed with MC2 receptor, the receptor was able to traffic to the plasma membrane and respond to ACTH with an increase in cAMP in cells that were otherwise non-responsive. MRAP and MC2 receptors have been shown to interact closely by a variety of approaches including co-precipitation (4, 5), fluorescence microscopy (6, 7), bimolecular fluorescence complementation (8), and co-internalization in response to ACTH (9). Recent data establish that two MRAP dimers can interact with one MC2 receptor and that two MC2 receptors can interact with one MRAP, but it is unclear whether this happens normally (10). MRAP2, an MRAP paralog, co-precipitates with the MC2 receptor and all other members of the melanocortin receptor family (5) but does not support signaling by MC2 receptors (11).

Using an alanine-scanning mutagenesis approach and focusing on the highly conserved aminoterminal of MRAP, we discovered that MRAP lacking a critical tyrosine-rich region was able to promote MC2 receptor trafficking but not signaling (11). This established that MRAP has two distinct functions, one to enable MC2 receptor trafficking to the cell surface, perhaps because it assists protein folding, and a second to enable signal transduction. Importantly, when MC2 receptors were expressed with a mutated MRAP, receptors were localized on the plasma membrane but completely unable to bind ACTH. As a result, ACTH cannot be expected to exert any effect – cAMP-dependent or otherwise – in cells lacking MRAP.

It is well established that mRNA encoding MC2 receptor undergoes feed-forward regulation, increasing in response to stress or ACTH (12). MRAP mRNA levels also increase, and do so within minutes, in response to either an imposed stress or ACTH (13, 14). MC2 receptor and MRAP mRNAs rise dramatically when cultured adrenal cells are incubated with ACTH, establishing a direct action at the adrenal gland (15). It is not known whether MC2 receptor and MRAP protein levels also change on a rapid time scale. One of the goals of the experiments described here was to characterize MRAP turnover in an isolated cell system.

The architecture of MRAP is extremely unusual. MRAP appears to form antiparallel homodimers. This conclusion is based on the following: (1) immunological findings showing dual topology, where antibodies identify both the amino and carboxyterminal ends of MRAP facing outwards on the cell surface (4); (2) quantitative co-precipitation of differentially tagged MRAPs, pointing to a multimeric structure (4); (3) biochemical experiments showing that approximately half of MRAP undergoes glycosylation at the single predicted site for N-linked glycosylation, a process that takes place in the interior of the endoplasmic reticulum (ER) and Golgi apparatus (4); (4) bimolecular fluorescence complementation (8) and bioluminescence resonance energy transfer (16) in configurations consistent with an antiparallel dimeric structure; and (5) evidence that a concatenated protein made by fusing two MRAPs to the extracellular aminoterminal of the MC2 receptor is ACTH-responsive (10). The latter studies established that the tyrosine-rich domain of the MRAP aminoterminal is necessary on the outer surface of the cell, likely allowing ACTH to bind.

Taken together, these data provide strong evidence that MRAP forms antiparallel homodimers. MRAP and MRAP2 are the only single transmembrane domain proteins thought to exist in this configuration. It is not yet known when MRAP conformation is established, whether MRAP orientation changes during synthesis and trafficking, whether dual topology predominates in adrenal cells, and whether MRAP is stable. We designed the experiments reported here to address these unresolved issues and describe several approaches to probe the topology of the MRAP protein at different times after biosynthesis and estimate its half-life.

## MATERIALS AND METHODS

### Reagents

Melanocortin 2 receptor accessory protein plasmids that have not been described previously (4, 8, 11) were constructed using QuikChange from Stratagene with standard molecular biological techniques and verified by sequencing. MRAP-V5-3xFlag contained mMRAP followed by GKPIP<sup>N</sup>PLLGLDSTGRDY KDHDGDYKDHDIDYKDDDDK at the C-terminus. AP-MRAP contained the minimal AP sequence GLNDIFEAQKIEWHE immediately after the initiating Met followed by mMRAP or mMRAP-V5-3xFlag. MRAP-AP contained the same AP sequence at the C-terminus of mMRAP or mMRAP-V5-3xFlag followed by a stop codon. MRAPs containing the AP sequence but no epitope tags were constructed similarly. On figures, these constructs are shown as AP-MRAP or MRAP-AP and epitope tags are described in the legends. V5-MART1-AP and AP-MART1-V5 were constructed by adding V5 epitope (GKPIP<sup>N</sup>PLLGLDST) and AP sequence (GLNDIFEAQKIEWHE) to opposite ends of mMART1. (4K to R)MRAP refers to V5-mMRAP-3xFlag with the four Lys in native mMRAP mutated to Arg. 3xHA-hMC2 receptor, referred to as MC2 receptor, was from Missouri cDNA Resource Center and plasmid encoding eGFP from Clontech. BirA in pcDNA3 and BirA<sub>ER</sub> in pDisplay vectors were generously donated by Dr. Alice Ting (Massachusetts Institute of Technology) and CRE-luciferase by Dr. George Holz (SUNY Upstate Medical University). Plasmid encoding HA-ubiquitin (HA-Ub) has been described (17). Monoclonal antibodies were

from the following: Sigma (M2 anti-Flag and immobilized M2 anti-Flag), Covance (HA-11 anti-HA), and AbDSerotec (anti-V5). Streptavidin–HRP and streptavidin–agarose were from Thermo Scientific, immobilized Protein A/G beads from Santa Cruz Biotechnology, and HRP-labeled antibody against mouse heavy and light chains from BioRad.

## Cell Growth and Transfection

CHO cells were from American Type Culture Collection and OS3 adrenal cells (18) from Dr. Bernard Schimmer (University of Toronto). CHO and OS3 cells were maintained in DMEM/F12 medium containing 5% fetal bovine serum at 37°C in 5% CO<sub>2</sub>-95% air and passaged with trypsin. Transfection was performed using FugeneHD or Lipofectamine 3000 according to manufacturers' instructions. Stable cell lines were generated by transfecting with plasmids encoding an MRAP, BirA, or BirA<sub>ER</sub> and selecting with 1 mg/ml G418. Pooled cells were used for experiments. Biotin-labeling experiments were performed by adding 2.5 μM biotin to complete medium at the time of transfection unless otherwise noted.

## cAMP Responses

In brief, cells were grown in white 96-well plates and transfected with 40–50 ng total DNA/well, usually with equal parts of MC2 receptor, MRAP construct and CRE-luciferase, a reporter containing multiple cAMP response elements from the rat insulin promoter (19). After 24 h cells were challenged for 4–5 h with hACTH(1–24) or 20 μM forskolin in DMEM with 0.1% BSA. Medium was replaced with Firefly Reagent from Nanolight and luminescence read in a BioTek platereader. Unless noted, results are expressed as percent of the forskolin response measured in the same experiment.

## Immunoprecipitation, SDS-PAGE, and Immunoblotting

Cells were washed with PBS and lysed on ice in Tris/Mg/EGTA buffer (150 mM NaCl, 50 mM Tris-Cl, 1 mM EDTA, and 1% Triton X-100, pH 8.0) containing protease inhibitors. Iodoacetamide (10 mM) was included in lysis buffers when ubiquitin labeling was being assessed. Samples were centrifuged at 10,000 × g for 20 min to remove nuclei and supernatants used for analysis. In some cases, HA- or Flag-labeled proteins were immunoprecipitated by incubating overnight at 4°C with 1:1000 dilutions of antibody and collected on Protein A/G beads. Biotin-labeled proteins were pelleted following overnight incubation with streptavidin–agarose beads. Samples were taken up in NuPage LDS sample buffer with a final concentration of 50 mM dithiothreitol. For analysis by SDS-PAGE samples were run on Lonza PagR or BioRad TGX gels. Proteins were transferred to nitrocellulose, blocked in TBST (Tris-buffered saline with 0.05% Tween 20) with 5% non-fat dry milk, and incubated overnight at 4°C in either monoclonal anti-V5, anti-HA, or anti-Flag antibody at 1:5000 in TBST/milk. After washing, antibody blots were incubated in 1:5000 HRP-anti-mouse heavy and light chain, washed and visualized using Western Lightning chemiluminescent reagent. To visualize biotin-labeled proteins, blots were incubated in 1:10,000 HRP-streptavidin in TBST with 0.5–1% BSA, because

milk contains biotin, washed and developed. In some cases, blots were subsequently incubated in 0.1% sodium azide to inactivate HRP and blotted with antibody as described above.

## Cell Surface ELISA

Expression of proteins on the plasma membrane was quantified as described previously (4, 11). Cells grown in 12- or 24-well plates were fixed with 2 or 3% paraformaldehyde for 10–20 min at room temperature, washed with PBS, and incubated for ~1 h with 1:5000 monoclonal anti-HA, anti-V5, or anti-Flag antibodies in PBS containing 5% non-fat dry milk. Cells were then washed extensively and incubated for ~1 h with 1:5000 HRP-labeled anti-mouse IgG, and washed prior to the addition of tetramethylbenzidine substrate (Sigma) and measurement of absorbance at 450 nm.

## Fluorescence Microscopy

For microscopy, CHO cells stably expressing BirA<sub>cyt</sub> or BirA<sub>ER</sub> were grown on glass coverslips and transfected with AP-tagged MRAP constructs. After overnight incubation, live cells were incubated with anti-Flag antibody (1:250 in serum-containing medium) for 30 min at 37°C, washed, and then incubated with 1:500 Alexa488-labeled anti-mouse M2 and 1:500 Alexa555-labeled streptavidin and incubated for 30 min at room temperature. Cells were washed, fixed in paraformaldehyde, washed again and mounted in Prolong Gold (InVitrogen), and viewed in a Nikon epifluorescence microscope with a 40× oil objective. Images showing Flag and streptavidin were obtained using 500 and 2500 ms exposures, respectively. All Flag and all streptavidin micrographs were processed identically.

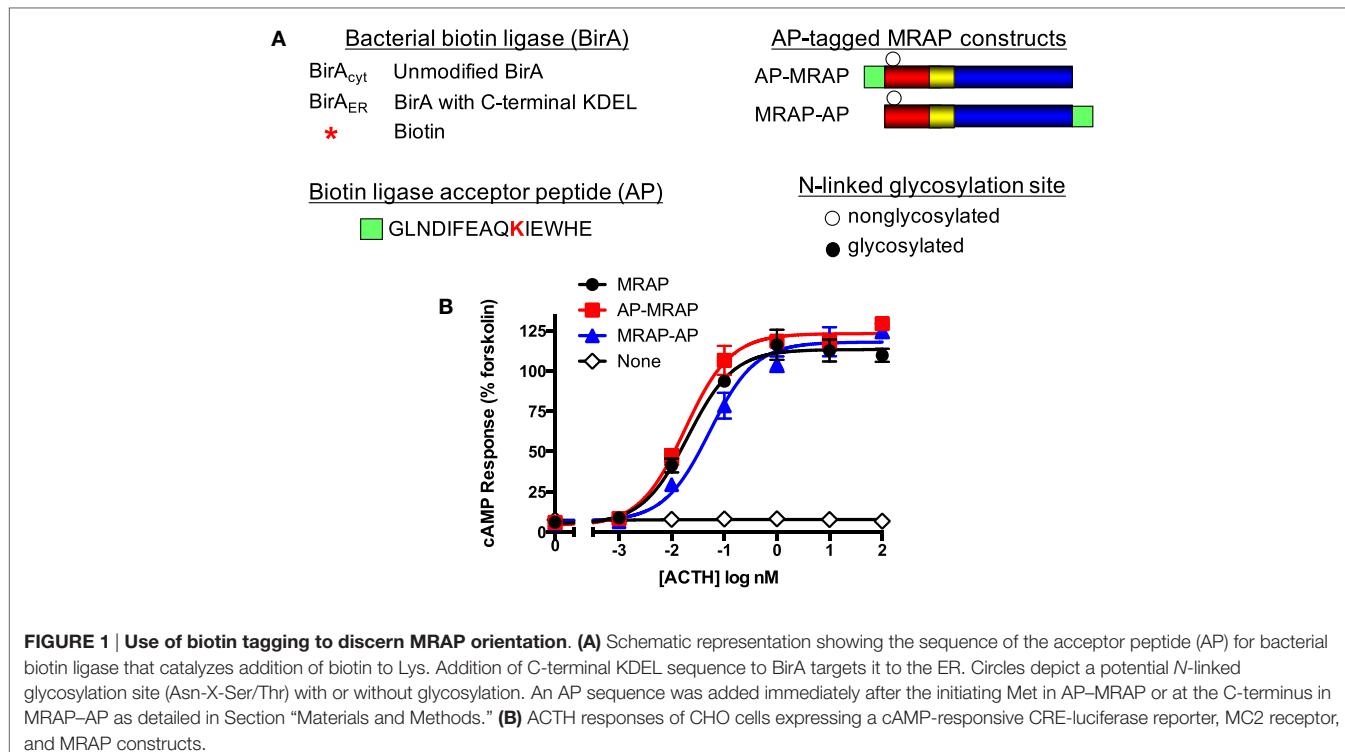
## Miscellaneous

To deglycosylate proteins, cell lysates were denatured and incubated with PNGaseF from New England Biolabs following manufacturer's instructions; control lysates were incubated identically in reactions lacking enzyme. All experiments were repeated a minimum of two times. Error bars indicate mean and range or SE of duplicate or triplicate determinations in representative experiments. Significance of differences between two values was determined by Student's *t*-test.

## RESULTS

### When Is MRAP Topology Established?

The strategy used to determine whether MRAP is first synthesized in dual orientations is outlined schematically in **Figure 1A**. Biotin labeling of a protein modified to contain a decarboxylase biotin acceptor domain was used previously to characterize membrane insertion of bacterial lactose permease (20). The biotin-labeling approach described here uses a small acceptor sequence and exogenous BirAs to provide a new means to analyze MRAP architecture. The bacterial enzyme biotin ligase (BirA) attaches a biotin residue to the single Lys residue in a specific acceptor peptide sequence (AP). When expressed in mammalian cells, bacterial biotin ligase is localized in the cytoplasm; addition of a carboxyterminal KDEL sequence targets the enzyme to the ER (21, 22). Because there are relatively low concentrations of



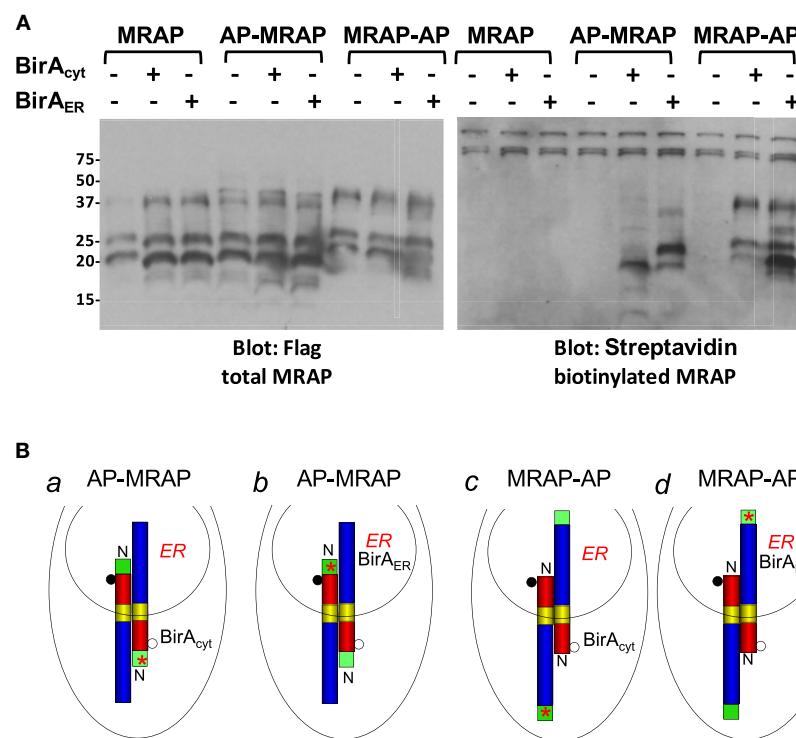
**FIGURE 1 | Use of biotin tagging to discern MRAP orientation.** **(A)** Schematic representation showing the sequence of the acceptor peptide (AP) for bacterial biotin ligase that catalyzes addition of biotin to Lys. Addition of C-terminal KDEL sequence to BirA targets it to the ER. Circles depict a potential N-linked glycosylation site (Asn-X-Ser/Thr) with or without glycosylation. An AP sequence was added immediately after the initiating Met in AP-MRAP or at the C-terminus in MRAP-AP as detailed in Section “Materials and Methods.” **(B)** ACTH responses of CHO cells expressing a cAMP-responsive CRE-luciferase reporter, MC2 receptor, and MRAP constructs.

biotin in standard serum-supplemented tissue culture media, it is straightforward to initiate biotin labeling of a protein tagged with an AP sequence by adding exogenous biotin. Mouse MRAP contains a single N-linked glycosylation site at Asn3 of the aminoterminal and N-glycosylation occurs exclusively in the inside of the ER and Golgi apparatus, which is topologically equivalent to the exoplasmic face of the plasma membrane. Glycosylated MRAP, which migrates more slowly than non-glycosylated MRAP on SDS-PAGE, must, therefore, be in the N<sub>exo</sub>/C<sub>cyt</sub> orientation unless it has changed orientations during trafficking. We engineered MRAPs with an AP sequence at either the aminoterminal or carboxyterminal end of the protein; each of these MRAP constructs also contained a Flag epitope. As shown in **Figure 1B**, control MRAP, AP-MRAP (N-terminal AP sequence), and MRAP-AP (C-terminal AP sequence) all supported comparable ACTH-stimulated increases in a cAMP reporter.

MRAP, AP-MRAP, and MRAP-AP were expressed in CHO cells stably expressing either cytoplasmic BirA or ER-targeted BirA<sub>ER</sub>. The upper left panel of **Figure 2** shows a Flag immunoblot identifying total MRAP. In every case, monomeric glycosylated and non-glycosylated MRAP species ran as a doublet at ~20 and 25 kDa. Enzymatic degradation with the glycosidase PNGaseF confirmed that the two major bands represented MRAP with and without N-linked glycosylation (4) and **Figure 11** below. Higher MW bands are likely MRAP multimers and their mobility shifts following PNGaseF treatment (not shown). The upper right panel of **Figure 2A** shows the same blot probed with streptavidin to detect only biotin-labeled proteins. Despite having identical molecular weights, AP-MRAP and MRAP-AP consistently displayed different mobilities on SDS-PAGE, as do AP-MART1

and MART1-AP (**Figure 3** below). It is possible that the proteins undergo different post-translational modifications but the behavior of membrane proteins on SDS-PAGE is not always predictable.

Endogenous biotinylated carboxylases are visible in all lanes at high molecular weight (75 kDa and above) in the blot on the upper right. These metabolically labeled proteins do not depend on transfected BirA. The three control lanes shown on the left of each blot in **Figure 2** demonstrate that MRAP lacking the biotin acceptor peptide was expressed well but not labeled with biotin. Cytoplasmic BirA catalyzed the addition of biotin to the faster-migrating, non-glycosylated form of AP-MRAP (situation *a* in **Figure 2B**), whereas ER-targeted BirA<sub>ER</sub> added biotin to the higher molecular weight glycosylated form of AP-MRAP (situation *b*). The reverse was found when cytoplasmic BirA and ER-targeted BirA<sub>ER</sub> were expressed with MRAP-AP. In this case, BirA<sub>cyt</sub> preferentially labeled glycosylated MRAP-AP and BirA<sub>ER</sub> preferentially labeled non-glycosylated MRAP-AP (situations *c* and *d*, respectively). These results support the conclusion that the great majority of MRAP molecules achieve an antiparallel orientation when first synthesized. The finding of some biotin-labeled but non-glycosylated AP-MRAP with BirA<sub>ER</sub> could result from (1) biotin labeling of AP-MRAP that is incompletely glycosylated, (2) incomplete import of overexpressed BirA<sub>ER</sub> into the ER leaving some active enzyme in the cytoplasm, or (3) a change in orientation of a small portion of MRAP after synthesis. The finding of a biotin-labeled and glycosylated MRAP-AP band with BirA<sub>ER</sub> is consistent with incomplete BirA<sub>ER</sub> import. After overnight incubation in biotin-supplemented media, essentially all AP-tagged MRAP could be collected on streptavidin-agarose beads, implying nearly quantitative biotinylation.



**FIGURE 2 | Biotin labeling confirms dual topology of MRAP.** **(A)** CHO cells stably expressing BirA<sub>cyt</sub> or BirA<sub>ER</sub> were transfected with MRAP, AP–MRAP, or MRAP–AP; these plasmids also contained a Flag epitope. Following detergent solubilization, proteins were resolved on SDS-PAGE and probed with monoclonal anti-Flag antibody and HRP-anti-mouse IgG or HRP-streptavidin. **(B)** Expected localization of BirA and predicted orientation of newly synthesized MRAPs. Filled and open circles depict glycosylated and non-glycosylated MRAP and red stars represent biotin.

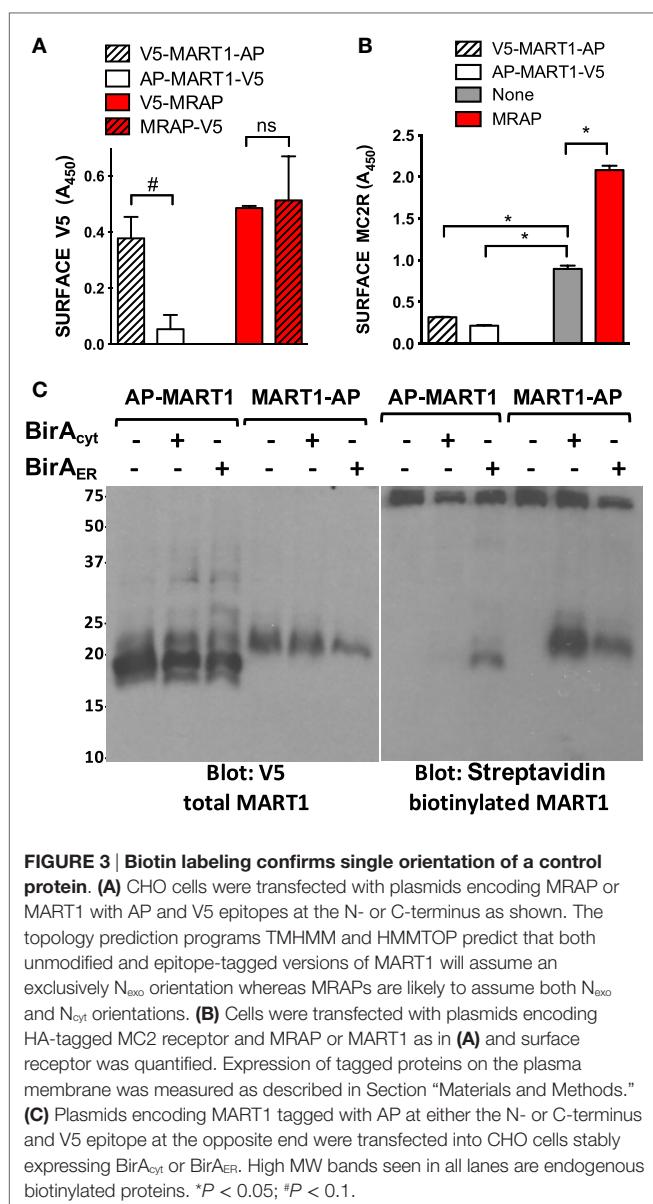
As an additional control for this approach, we studied MART1 (also called Melan-A), a melanosome protein with a single transmembrane domain, strongly predicted N<sub>exo</sub>/C<sub>cyt</sub> orientation, and size close to that of mouse MRAP (18 vs. 14 kDa without epitopes or carbohydrate). We engineered plasmids encoding MART1 with AP tags at one end or the other and V5 tags at the opposite ends. We used a previously described fixed cell ELISA protocol (4) in which antibodies were added to intact cells to detect epitopes localized on the outside of the plasma membrane (**Figure 3A**). MART1 was found in the predicted N<sub>exo</sub>/C<sub>cyt</sub> orientation, whereas MRAPs with the AP sequence at either end were found in dual N<sub>exo</sub>/C<sub>cyt</sub> and N<sub>cyt</sub>/C<sub>exo</sub> orientations. MRAP but not MART1 increased surface expression of co-expressed MC2 receptor (**Figure 3B**). MART1 was then tested in an experiment identical to that shown in **Figure 2** for MRAP. AP–MART1 was expressed strongly but labeled only by ER-localized BirA<sub>ER</sub> and labeling was rather weak (**Figure 3C**). By contrast, MART1–AP was expressed weakly but labeled strongly by cytoplasmic BirA and slightly by BirA<sub>ER</sub>. Labeling by BirA<sub>ER</sub> probably occurred because of incomplete import of BirA<sub>ER</sub> into the ER, which left some biotin ligase in the cytoplasm.

## Does MRAP Change Orientation after Biosynthesis?

To isolate MRAP that had trafficked to the plasma membrane, we localized cell surface biotin-labeled MRAP by fluorescence

microscopy. Cells stably expressing cytosolic or ER-targeted BirA were transiently transfected to express either AP–MRAP–Flag or MRAP–Flag–AP. After incubation with biotin, live cells were stained with monoclonal anti-Flag antibody or fluorescent streptavidin. Because MRAP forms an antiparallel structure, both AP–MRAP–Flag and MRAP–Flag–AP were detectable on the cell surface with anti-Flag antibody, as expected (**Figure 4A**). On the other hand, if AP–MRAP–Flag and MRAP–Flag–AP did not change orientations following biosynthesis, only ER-localized BirA<sub>ER</sub> would be able to label acceptor peptide in the ER lumen and, after trafficking, the extracellular side of the plasma membrane (**Figure 4B**). This result was obtained, with cell surface MRAP clearly labeled in all configurations but cell surface biotin-labeled MRAP visible only in cells expressing BirA<sub>ER</sub>. The results of this experiment are not confounded by incomplete import of BirA<sub>ER</sub> into the ER.

A kinetic approach was also undertaken to determine whether MRAP changes orientation after biosynthesis (**Figure 5**). To avoid the ambiguity present in experiments using BirA<sub>ER</sub>, cells were transfected with cytosolic BirA and either AP–MRAP or MRAP–AP and incubated overnight in the absence of added biotin. During this period MRAP synthesis took place with very little biotin labeling (zero time point). Cells were then treated with added biotin for times up to 24 h. Immunoblotting for total AP–MRAP and MRAP–AP with anti-Flag antibodies is shown in the left panels. As seen previously (**Figure 2A**), cytosolic



**FIGURE 3 |** Biotin labeling confirms single orientation of a control protein. **(A)** CHO cells were transfected with plasmids encoding MRAP or MART1 with AP and V5 epitopes at the N- or C-terminus as shown. The topology prediction programs TMHMM and HMMTOP predict that both unmodified and epitope-tagged versions of MART1 will assume an exclusively N<sub>exo</sub> orientation whereas MRAPs are likely to assume both N<sub>exo</sub> and N<sub>cyt</sub> orientations. **(B)** Cells were transfected with plasmids encoding HA-tagged MC2 receptor and MRAP or MART1 as in **(A)** and surface receptor was quantified. Expression of tagged proteins on the plasma membrane was measured as described in Section “Materials and Methods.” **(C)** Plasmids encoding MART1 tagged with AP at either the N- or C-terminus and V5 epitope at the opposite end were transfected into CHO cells stably expressing BirA<sub>cyt</sub> or BirA<sub>ER</sub>. High MW bands seen in all lanes are endogenous biotinylated proteins. \*P < 0.05; #P < 0.1.

BirA labeled the faster-moving non-glycosylated AP-MRAP band. Increased biotin labeling could be detected as early as 6 min after biotin supplementation and labeling continued over time; after 24 h, there was only a trace amount of a higher molecular weight biotin-labeled AP-MRAP (Figure 5A). This result does not support the idea that MRAP flips orientations after synthesis. In the case of MRAP-AP, a minor fraction of non-glycosylated biotin-labeled MRAP was visible but the proportion did not increase with time, indicating that MRAP does not gradually flip but rather that a small amount of N<sub>exo</sub>/C<sub>cyt</sub> MRAP does not become fully glycosylated (Figure 5B).

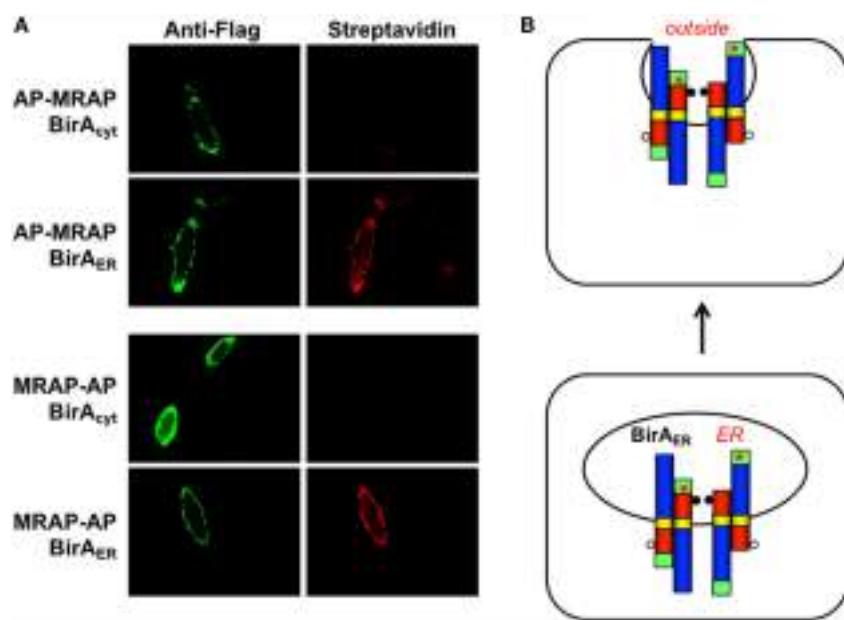
To characterize MRAP topology in its natural setting, we took advantage of the OS3 line of mouse adrenal cells that does not express MC2 receptors. ACTH responses were measured in parallel in OS3 and CHO cells transfected with the cAMP reporter CRE-luciferase with or without MC2 receptor and

MRAP (Figures 6A,B). ACTH stimulated a 26-fold increase in the cAMP response in CHO cells expressing MRAP and MC2 receptor. Mock-transfected OS3 cells did not respond to ACTH, consistent with the reported absence of MC2 receptors (18). When the adrenal cells were transfected with MRAP and MC2 receptor, ACTH produced a striking 273-fold increase in cAMP reporter activity. OS3 cells must express MRAP, because MC2 receptors are functional when transfected alone. Interestingly, responses to forskolin were also exceptionally strong in OS3 cells. Forskolin acts directly on adenylyl cyclase and amplifies both basal and Gs-stimulated enzyme activity. Together, these findings suggest that adrenal OS3 cells have unusually efficient signal transduction at the G protein/adenylyl cyclase level.

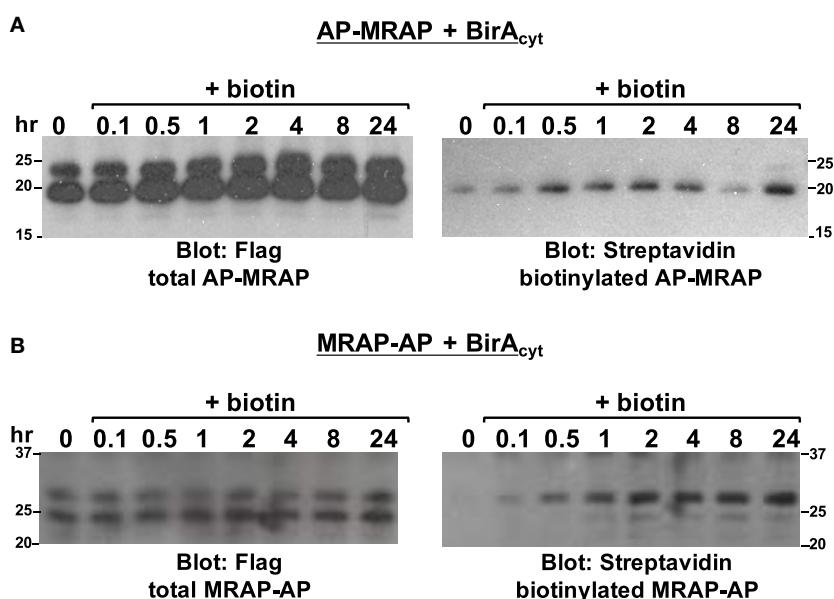
We also explored MRAP orientation and the effects of MC2 receptor and ACTH (Figures 6C,D). OS3 cells and parallel cultures of CHO cells were transfected with BirA and AP-tagged MRAPs with or without MC2 receptors and incubated overnight with or without 10 nM ACTH. For reasons that are not clear, expression of AP-MRAP was higher when MC2 receptor was co-expressed and that of MRAP-AP was lower. ACTH had little effect. Biotin labeled the non-glycosylated AP-MRAP band, as predicted if topology does not change. In OS3 cells, there was a non-specific biotin-labeled protein at ~37 kDa that did not correspond to any MRAP band. MRAP-AP was weakly expressed in these cells and biotin-labeled species were partially obscured by the non-specific band.

## Is the MRAP Protein Stable?

Having established that MRAP topology is stable, we examined the overall stability of the accessory protein. The stability of MRAP was estimated in CHO cells stably expressing Flag-tagged MRAP together with GFP or MC2 receptor. Cultures were treated for 4 h with the protein synthesis inhibitor cycloheximide (CHX), the proteasome inhibitor MG132 or both (Figure 7A). MRAP was quite labile, with little protein remaining 4 h after protein synthesis blockade. Surprisingly, MG132 by itself caused an enormous increase in the concentration of MRAP. On the other hand, MG132 did little to protect MRAP from degradation when CHX was present. A similar pattern was seen with the MC2 receptor, although the rate of degradation was slower (half-life between 3 and more than 8 h in different experiments) and effect of MG132 alone was much less marked. Mature MC2 receptor, which contains multiple sites for N-linked glycosylation (23, 24), ran as a broad band at high molecular weight, whereas immature core glycosylated and non-glycosylated receptor ran in more distinct bands close to the predicted molecular weight of 3xHA-hMC2 receptor, 37.3 kDa. Figure 7B shows a similar experiment demonstrating the rapidity of CHX and MG132 effects. The half-life of MRAP was estimated in similar experiments in which CHO cells were incubated with CHX from 0 to 5 h and MRAP levels were quantified densitometrically in immunoblots assuming first order kinetics. In most experiments, glycosylated MRAP was lost less rapidly than the non-glycosylated form, but this was not entirely consistent. The half-life of total MRAP averaged  $1.7 \pm 0.5$  h ( $n = 7$ ). The half-life of MRAP was short when it was transiently expressed with or without MC2 receptors in CHO cells but longer in adrenal OS3 cells, which grow less



**FIGURE 4 | Only ER-targeted BirA labels MRAP on the outer surface of the plasma membrane.** (A) CHO cells stably expressing BirA<sub>cyt</sub> or BirA<sub>ER</sub> were grown on coverslips and transiently transfected with plasmids encoding AP–MRAP or MRAP–AP, each containing a Flag epitope in the C-terminal domain. Live cells were incubated with monoclonal anti-Flag epitope followed by Alexa488-labeled anti-mouse IgG (green) and Alexa555-labeled streptavidin (red), washed and imaged. Barely visible untransfected cells not expressing MRAPs demonstrate low background staining. (B) Schematic representation of N<sub>exo</sub>/C<sub>cyt</sub> and N<sub>cyt</sub>/C<sub>exo</sub> MRAPs before and after trafficking to the plasma membrane assuming that orientation does not change.

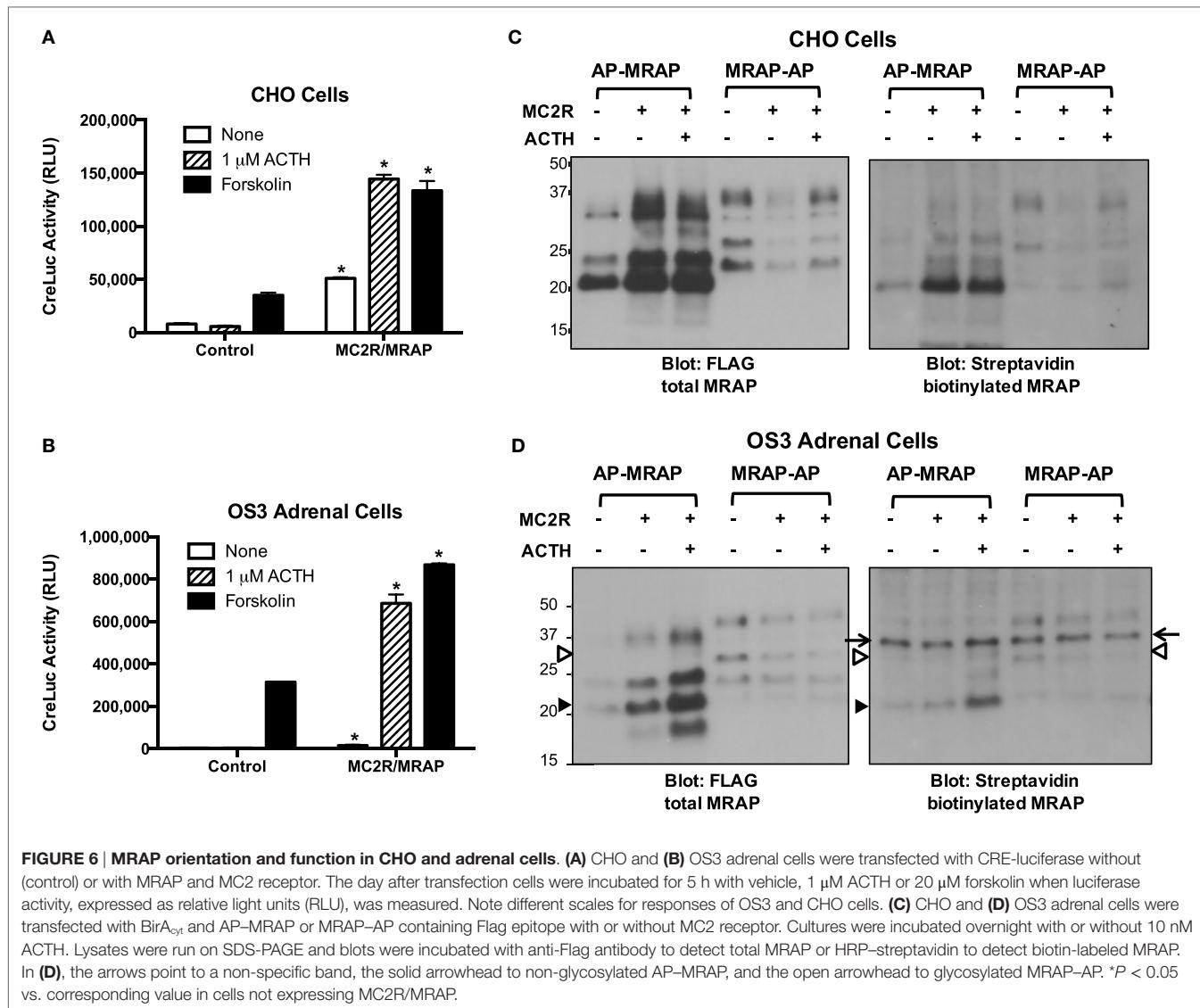


**FIGURE 5 | Kinetics of biotin labeling.** CHO cells were transfected with BirA<sub>cyt</sub> and either (A) AP–MRAP or (B) MRAP–AP, both containing Flag epitope. After overnight incubation in medium without added biotin, 2.5  $\mu$ M biotin was added for the times shown. MRAPs were immunoprecipitated with anti-Flag antibody, resolved on SDS-PAGE and blotted with either anti-Flag antibody, to identify total MRAP, or HRP–streptavidin, to identify biotin-labeled protein.

rapidly than CHO cells (Figure 7C). To study turnover of MRAP on the plasma membrane, CHO cells were incubated for 1 h with protein synthesis or proteasome inhibitors and then either lysed or incubated with antibody to label cell surface MRAP selectively

(Figure 7D). The effects of CHX and MG132 were not as extreme but clearly present when plasma membrane MRAP was isolated.

The use of a global protein synthesis inhibitor to study degradation of a particular protein is complicated by potential

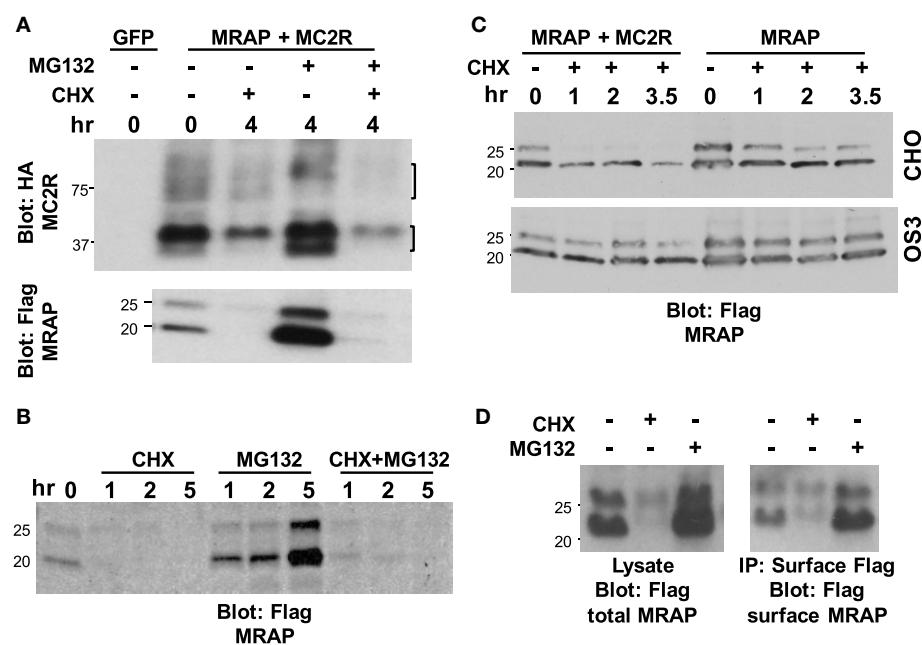


**FIGURE 6 | MRAP orientation and function in CHO and adrenal cells. (A)** CHO and **(B)** OS3 adrenal cells were transfected with CRE-luciferase without (control) or with MRAP and MC2 receptor. The day after transfection cells were incubated for 5 h with vehicle, 1 μM ACTH or 20 μM forskolin when luciferase activity, expressed as relative light units (RLU), was measured. Note different scales for responses of OS3 and CHO cells. **(C)** CHO and **(D)** OS3 adrenal cells were transfected with BirA<sub>cyt</sub> and AP-MRAP or MRAP-AP containing Flag epitope with or without MC2 receptor. Cultures were incubated overnight with or without 10 nM ACTH. Lysates were run on SDS-PAGE and blots were incubated with anti-Flag antibody to detect total MRAP or HRP-streptavidin to detect biotin-labeled MRAP. In **(D)**, the arrows point to a non-specific band, the solid arrowhead to non-glycosylated AP-MRAP, and the open arrowhead to glycosylated MRAP-AP. \*P < 0.05 vs. corresponding value in cells not expressing MC2R/MRAP.

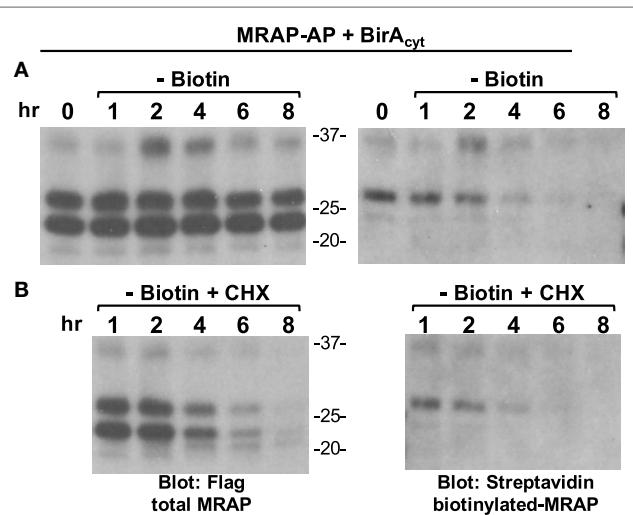
effects on proteins other than the one under study. To avoid this problem, we studied cells stably expressing cytoplasmic BirA and then measured the amount of biotinylated-MRAP-AP at intervals after the removal of extracellular biotin with or without addition of CHX (Figure 8). Since biotin cannot be removed from a protein until it has been thoroughly degraded, this approach is conceptually equivalent to a radiolabeling pulse-chase experiment. For a low abundance protein, the method has the advantage of labeling only the protein of interest that can be concentrated on streptavidin beads prior to analysis. A limitation is the delay caused by the time required for washout of intracellular biotin. Following biotin withdrawal, total MRAP pools were quite stable over 20 h while normal synthesis and degradation were ongoing. The half-life of biotin-labeled MRAP was only 2.8 h, however, indicating that MRAP has a short half-life. In the presence of CHX, the corresponding values were 2.8 h for the total MRAP pool and 2.2 h for biotin-labeled MRAP. Because the half-lives measured by metabolic labeling and protein synthesis inhibition

were strikingly similar, rapid turnover of MRAP was not an indirect effect of CHX.

In light of the effect of MG132, we investigated the possibility that MRAP itself undergoes ubiquitin-mediated degradation. In the canonical pathway ubiquitin is added to Lys residues and mouse MRAP has only four lysines, all located on the N-terminal side of the transmembrane helix in the juxtamembrane region. To test the importance of Lys to overall MRAP stability, we mutated these four residues to Arg (4K to R), retaining positive charge but removing lysine ε-amino groups that can be ubiquitylated. Arg-substituted MRAP was tagged with a V5 epitope on the N-terminus and a Flag epitope on the C-terminus, enabling analysis of its topology. Although (4K to R)MRAP was not expressed as highly as wild-type MRAP, it still assumed dual topology with both V5 and Flag epitopes on the plasma membrane (Figure 9A). Despite a lower level of MC2 receptor on the plasma membrane, cells expressing (4K to R)MRAP responded to ACTH with a cAMP increase equivalent to that supported by wild-type MRAP



**FIGURE 7 | Stability of MRAP and MC2 receptor.** **(A)** CHO cells stably expressing Flag-tagged MRAP were transfected with either GFP or HA-tagged MC2 receptor and then incubated for 4 h with no drug, 10 µg/ml cycloheximide (CHX), 50 µM MG132, or both. Cell lysates were run on SDS-PAGE and blotted for either (*upper*) MC2 receptor or (*lower*) MRAP. Brackets on the right show glycosylated MC2 receptor and core and non-glycosylated receptor. **(B,D)** CHO cells stably expressing Flag-tagged MRAP were incubated with 10 µg/ml cycloheximide (CHX), 50 µM MG132, or both for **(B)** times shown or **(D)** 1 h. In **(B)**, cells were lysed and immunoprecipitation with anti-Flag antibody performed whereas in **(D)** cells were incubated for 15 min with 1:1000 anti-Flag antibody and washed extensively before lysis and collection of Flag-labeled proteins on Protein A/G beads to isolate cell surface MRAP. **(C)** CHO and OS3 adrenal cells were transiently transfected with MRAP or MC2 receptor and MRAP, then incubated with 100 µg/ml cycloheximide for the times shown, lysed and blotted for MRAP.

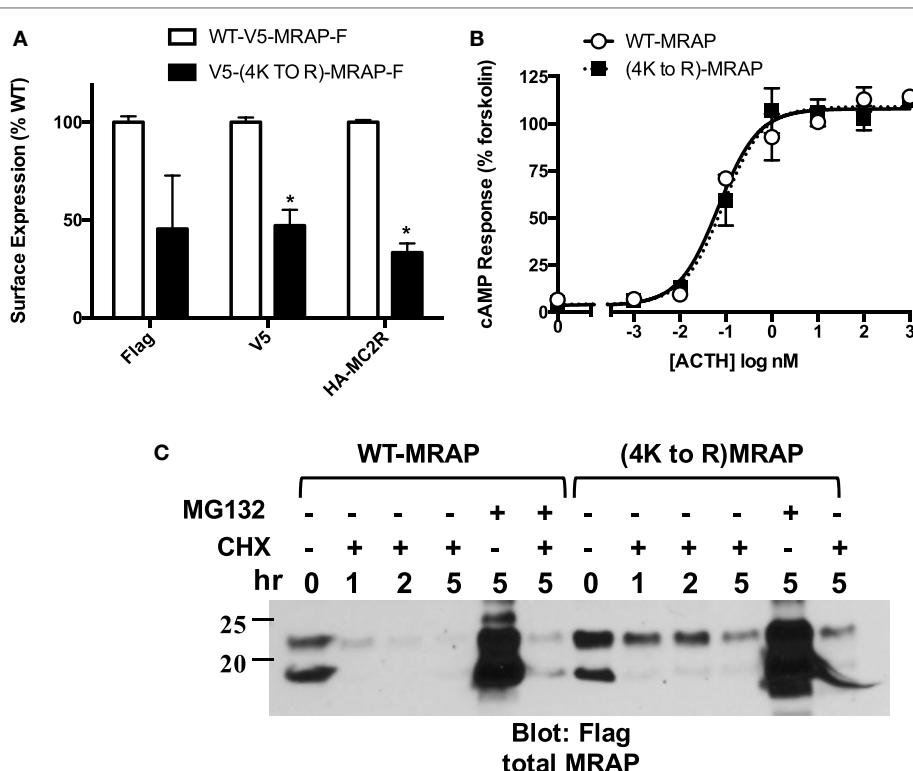


**FIGURE 8 | Half-life of biotinylated MRAP is short with or without cycloheximide.** **(A,B)** CHO cells stably expressing BirA<sub>cyt</sub> were transfected with MRAP-AP containing a Flag sequence, and incubated overnight in medium supplemented with biotin. Cells were then washed twice and incubated for 1–8 h in medium lacking biotin, **(A)** without or **(B)** with 10 µg/ml cycloheximide (CHX), when lysates were prepared and proteins resolved on SDS-PAGE and blots probed for either Flag epitope or biotin. The glycosylated form of MRAP-AP is labeled by cytoplasmic BirA.

(Figure 9B). MG132 by itself caused an enormous increase in (4K to R)MRAP concentration just as it did with wild-type. Replacing all of the MRAP Lys residues with Arg delayed MRAP degradation by at most threefold (Figure 9C).

We tested whether ubiquitin is directly added to MRAP lysines by co-expressing HA-ubiquitin with either wild-type or (4K to R)MRAP in CHO cells and incubating for 3 h with MG132 to allow accumulation of any ubiquitylated species. Co-immunoprecipitation and immunoblotting revealed that Flag-tagged MRAP undergoes ubiquitin modification (Figure 10A). Multiple ubiquitylated MRAP bands were observed and only one of them was lost in the Arg-substituted mutant, suggesting that ubiquitin is added to Lys and also to one of the amino acids less frequently ubiquitylated: the aminoterminal amino group, the hydroxyl group of Ser or Thr, or the sulphydryl group of Cys (25). On average, ubiquitylated MRAP species totaled ~5% of total MRAP after incubation for 3 h with a proteasome inhibitor.

To rule out the possibility that MRAP ubiquitylation was occurring on one of the Lys residues in the Flag sequence, we tested AP-MRAP and AP-(4K to R)MRAP that contained the biotin acceptor peptide sequence but no epitope tag in cells expressing cytoplasmic BirA (Figure 10B). Once labeled with biotin, the AP tag has no lysine amino group available for ubiquitin addition. Once again, multiple ubiquitylated MRAP bands were observed and only one was lost in the Arg-substituted



**FIGURE 9 | Importance of MRAP Lys residues for function, orientation, and stability.** CHO cells were transfected with no accessory protein, wild-type MRAP or (4K to R)MRAP with the four native Lys residues mutated to Arg. **(A)** Surface expression of the N-terminal V5 and C-terminal Flag epitopes of MRAP and (4K to R)MRAP, and surface expression of co-transfected HA-tagged MC2 receptor. Results are normalized to values with wild-type MRAP. **(B)** cAMP responses to ACTH in cells co-transfected with MC2 receptor and CRE-luciferase. **(C)** Cells expressing MRAP or (4K to R)MRAP were incubated for the times shown with 10 µg/ml cycloheximide (CHX), 50 µM MG132 or both. Lysates were run on gels and blotted for Flag epitope. \*P < 0.05 vs. corresponding value in cells expressing wild-type MRAP.

mutant, suggesting that ubiquitin was added to MRAP at an atypical site.

The glycosylation status of various MRAP bands was analyzed in lysates of cells expressing HA-Ub and MRAP before and after enzymatic deglycosylation with PNGaseF. None of the ubiquitylated MRAP bands was lost following PNGaseF treatment, whereas the slower-migrating MRAP bands collapsed to the mobility of the faster-migrating non-glycosylated species (Figure 11).

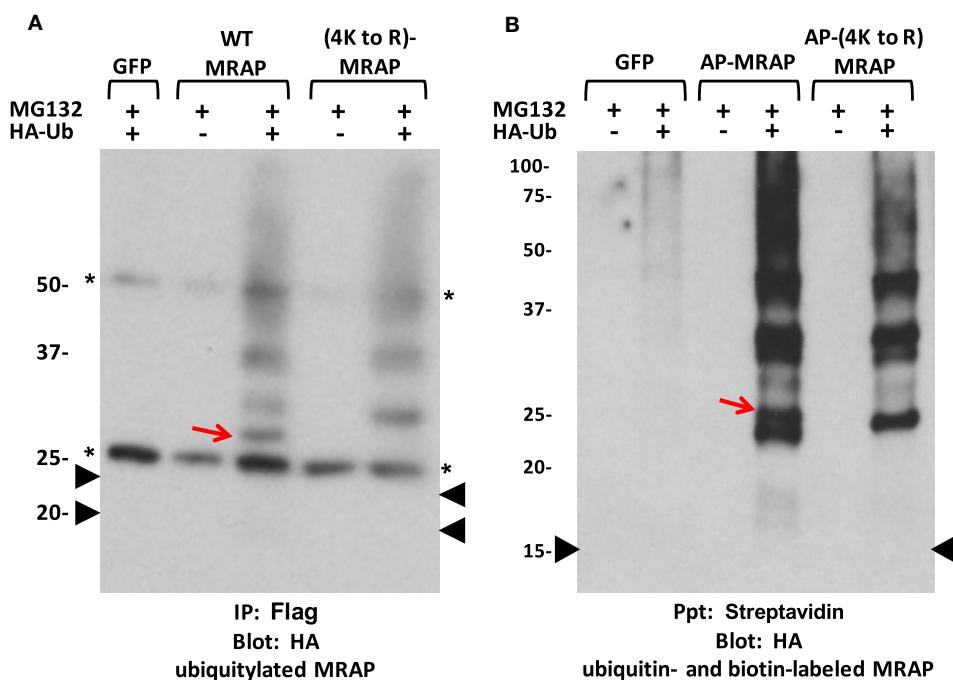
## DISCUSSION

The unusual antiparallel dimer structure of MRAP been described only for MRAP and its paralog MRAP2. These two proteins are evolutionarily ancient (26–28) and have retained features that result in dual topology, including highly conserved aminoterminal and transmembrane domains and basic residues in the juxtamembrane region (29). The experiments described here do not explain what advantages are conferred by the conserved MRAP structure but they do establish that MRAP topology is stable from the time of MRAP biosynthesis to the time of MRAP degradation.

It has been particularly difficult to study MRAP topology because each side of the protein faces each direction in the antiparallel homodimer. Many of the methods used to assign

membrane orientation require the addition of bulky fluorescent proteins or enzymes that are themselves larger than MRAP. Biotin labeling avoids this issue but has other drawbacks. Analysis of biotin-labeling patterns depended on the ability to distinguish between  $N_{\text{exo}}$  and  $N_{\text{cyt}}$  MRAPs based on glycosylation. Protein glycosylation never reaches 100%, and in our experience the extent of MRAP glycosylation varies with MRAP species, epitope tags, and expression levels. In addition, some BirA activity appeared to remain in the cytoplasm when ER-targeted BirA was overexpressed. Finally, backgrounds on HRP-streptavidin blots tended to be high when low abundance proteins were being detected. Despite these limitations, some situations were unambiguous. Once it has left the ER and Golgi apparatus, MRAP cannot undergo further glycosylation. Once it has been biotinylated by cytoplasmic BirA, MRAP cannot be orientated with biotin facing the outside of the cell unless it flips. The important point is that changes in the orientation of MRAP were not observed in any of configurations examined.

When protein synthesis was blocked, MRAP protein levels declined with a half-life that varied in different experiments but averaged under 2 h. Use of biotin labeling to study MRAP stability was again instructive, confirming that rapid MRAP turnover was not an artifact of CHX treatment. Although it is not known how



**FIGURE 10 | MRAP is ubiquitylated.** **(A)** CHO cells were transfected with plasmids encoding GFP, Flag-tagged MRAP or (4K to R)MRAP, with or without HA-labeled ubiquitin (HA-Ub). After overnight incubation, cells were treated for 3 h with 50  $\mu$ M MG132. MRAP proteins were immunoprecipitated with anti-Flag antibody and resolved on SDS-PAGE; blots were probed for HA. Asterisks mark IgG bands and black arrowheads show the position of the two MRAP bands from a parallel gel blotted for Flag. **(B)** CHO cells stably expressing cytosolic BirA were transfected with plasmids encoding GFP or AP-MRAP or AP-(4K to R)MRAP, both lacking any epitope tag, with or without HA-ubiquitin (HA-Ub). After overnight incubation in biotin-supplemented media, 50  $\mu$ M MG132 was added for 3 h. Biotin-labeled proteins were collected on streptavidin beads and resolved on gels; blots were probed for HA. The arrowhead shows the position of biotin-labeled non-glycosylated AP-MRAP found on a parallel gel probed with streptavidin. The red arrows point to bands present in WT but not Lys-substituted MRAP. AP-MRAPS without epitope tags run at lower MWs than their longer tagged counterparts.

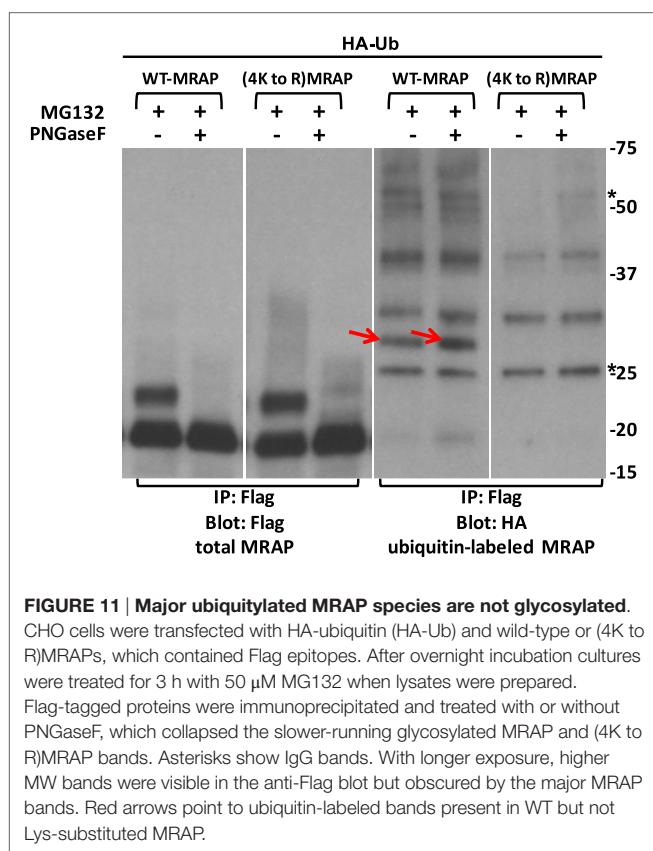
quickly intracellular biotin declines when biotin is removed from the medium, this must happen quickly because biotin-labeled MRAP disappeared at roughly the same rate as total MRAP during protein synthesis blockade. A biotin-labeling approach has the potential to measure the half-life of any protein that can be modified by the addition of a biotin ligase acceptor peptide. It avoids the cost and risk of radioisotopes, is applicable for proteins with amino acid compositions that render them unsuitable for  $^{35}$ S-Met/Cys labeling, and can be used for proteins expressed at low abundance because of the ease of enriching on streptavidin beads.

Despite the presence of an array of chaperones that assist with protein folding in the ER, it is believed that a sizable fraction of newly synthesized protein fails to fold or assemble correctly. The accumulation of misfolded proteins in the ER can have disastrous outcomes, preventing synthesis of other membrane or secretory proteins. Cells contain elaborate and essential systems to remove terminally misfolded proteins from the ER, a process termed ER-associated degradation or ERAD (30, 31). ERAD involves recognition of misfolded protein, retrotranslocation from the ER, polyubiquitylation, and proteasomal degradation. Proteins are deglycosylated prior to degradation.

The involvement of proteasomal pathways in MRAP degradation was investigated here. One puzzling finding was that MG132

caused a very striking increase in the amount of MRAP when protein synthesis was ongoing but provided almost no stabilization when protein synthesis was blocked. Assuming that MG132 effectively inhibited proteasome activity, the results indicate that an alternative pathway for MRAP degradation exists and is responsible for the majority of MRAP degradation. The ability of MG132 to increase MRAP may be explained if the proteasome inhibitor increases the efficiency of MRAP biosynthesis, perhaps by inducing molecular chaperones (32).

Although the results with MG132 suggest that proteasomal degradation is not the major pathway for MRAP turnover, ubiquitylated MRAP was detected following a 3-h incubation with MG132. One ubiquitylated MRAP band had a molecular weight 8–10 kDa higher than the comparable non-glycosylated MRAP band and was lost in Arg-substituted MRAP, indicating that it represents MRAP with a single ubiquitin added to a lysine. At least two heavier ubiquitylated MRAP bands were present in MRAP lacking any lysines and must, therefore, represent ubiquitylation at some other amino acid but based on their migration on gels, these did not form a classical ubiquitin ladder. Cooray et al. demonstrated that MGRN1, an E3 ubiquitin ligase implicated in MC1 receptor function, is present in adrenal fasciculata and capable of adding ubiquitin to the MC2 receptor. They did not detect ubiquitylation of human MRAP in HEK293 cells in the absence of



**FIGURE 11 | Major ubiquitylated MRAP species are not glycosylated.**

CHO cells were transfected with HA-ubiquitin (HA-Ub) and wild-type or (4K to R)MRAPs, which contained Flag epitopes. After overnight incubation cultures were treated for 3 h with 50  $\mu$ M MG132 when lysates were prepared. Flag-tagged proteins were immunoprecipitated and treated with or without PNGaseF, which collapsed the slower-running glycosylated MRAP and (4K to R)MRAP bands. Asterisks show IgG bands. With longer exposure, higher MW bands were visible in the anti-Flag blot but obscured by the major MRAP bands. Red arrows point to ubiquitin-labeled bands present in WT but not Lys-substituted MRAP.

a proteasome inhibitor (24). Taken together, published work and the experiments described here indicate that polyubiquitylation and proteasomal degradation do not account for most MRAP degradation.

The stability of MRAP may be of interest in tissues outside of the adrenal gland. MC2R mRNA is highly expressed in adrenal and testicular tissue, whereas MRAP $\alpha$  mRNA has a somewhat broader distribution with high levels in the adrenal gland and testes but substantial amounts in fat and brain, among other tissues (3, 7). To date, there is no information about the relative

expression of the protein products of these genes. hMRAP $\alpha$  coprecipitates with all five melanocortin receptors (5) and has modest effects on signaling by MC1 and MC3–5 receptors (7), making it plausible that MRAP has a role outside of the adrenal cortex. Patients who have glucocorticoid deficiency due to mutations in MC2 receptors and those who have mutations in MRAP display rather similar phenotypes (33), but comparison of MC2 receptor and MRAP knockout mice may provide insight into non-adrenal actions of MRAP in the future. A paralog of MRAP termed MRAP2 is widely distributed and influences signaling by MC4 receptors (34, 35) and at least one receptor outside the melanocortin receptor family (36) to regulate energy metabolism.

Not only is it uncertain how many G protein-coupled receptors interact with MRAPs, it is also uncertain whether MRAP actions are limited to receptors. The work detailed above supports previous findings of an antiparallel homodimer structure for MRAP and indicates that partners in the MRAP dimer remain in a fixed orientation during trafficking and expression on the plasma membrane. The finding that MRAP turns over rapidly in CHO cells needs to be interpreted cautiously because of the heterologous cell system, protein overexpression, and likely imbalance of accessory protein and receptor. An important question that remains to be addressed is whether MC2 receptor and MRAP protein levels change on a rapid time scale *in vivo*. Fortunately, ever more powerful mass spectrometry techniques are being developed and information about the stability membrane signaling proteins in a native environment should be forthcoming in the near future.

## AUTHOR CONTRIBUTIONS

ZM, LJ, SM, and PH designed and conducted experiments, and PH wrote the manuscript.

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# Hypothesis and Theory: Revisiting Views on the Co-evolution of the Melanocortin Receptors and the Accessory Proteins, MRAP1 and MRAP2

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The evolution of the melanocortin receptors (MCRs) is closely associated with the evolution of the melanocortin-2 receptor accessory proteins (MRAPs). Recent annotation of the elephant shark genome project revealed the sequence of a putative MRAP1 ortholog. The presence of this sequence in the genome of a cartilaginous fish raises the possibility that the *mrap1* and *mrap2* genes in the genomes of gnathostome vertebrates were the result of the chordate 2R genome duplication event. The presence of a putative MRAP1 ortholog in a cartilaginous fish genome is perplexing. Recent studies on melanocortin-2 receptor (MC2R) in the genomes of the elephant shark and the Japanese stingray indicate that these MC2R orthologs can be functionally expressed in CHO cells without co-expression of an exogenous *mrap1* cDNA. The novel ligand selectivity of these cartilaginous fish MC2R orthologs is discussed. Finally, the origin of the *mc2r* and *mc5r* genes is reevaluated. The distinctive primary sequence conservation of MC2R and MC5R is discussed in light of the physiological roles of these two MCR paralogs.

**Keywords:** melanocortin receptors, MRAP1, MRAP2, MC2R, MC5R, evolution

## INTRODUCTION

In many respects, the features of the melanocortin receptor (MCR) gene family (i.e., *mc1r*, *mc2r*, *mc3r*, *mc4r*, *mc5r*) are rather straightforward. These G Protein-coupled receptors are only found in chordates (1), and the proliferation of paralogous genes in this family has been influenced by the two genome duplication events that occurred during the early evolution of the chordates (2–4). In addition, these receptors appear to be predominately coupled to a cAMP/PKA pathway at their respective target cells (5). Finally, all of the MCRs are activated by one or more of the melanocortin-related peptides (i.e., ACTH, α-MSH, β-MSH, γ-MSH, or δ-MSH), which are derived from the precursor protein, POMC in gnathostomes (6), and the precursors POM or POC in lampreys (7).

There are also features of this gene family that are somewhat unique. For example, some of the MCRs interact with the accessory proteins melanocortin-2 receptor accessory protein (MRAP)1 and MRAP2 (8, 9), and these interactions can affect receptor trafficking and activation. In addition, for teleosts and tetrapods, the MC2R paralog has exclusive ligand selectivity for ACTH as compared to the more permissive ligand selectivity of the other MCR paralogs for ACTH and the MSH-sized ligands (10). Finally, while it is assumed that two genome duplications should yield four paralogous genes,

there are five paralogous genes present in this family. Hence, the origin of the fifth gene and the physiological significance of the fifth gene are another issue that will be revisited.

## PHYLOGENY AND PROPOSED EVOLUTION OF THE MRAPs

Following the initial cloning of the five MCRs, pharmacology studies for each receptor were done in heterologous non-adrenal cortex-derived mammalian cell lines with one exception – MC2R (6). Mountjoy et al. (11) found that in order to examine the ligand selectivity of human MC2R, the receptor cDNA needed to be expressed in Cloudman S91 melanoma cells; a cell line that endogenously expresses the *Mclr* gene. Subsequent studies would show that mammalian MC2R orthologs could be functionally expressed in cell lines derived from adrenal cortex cells, but not in non-adrenal mammalian cell lines (12–15). These observations contributed to the discovery of the accessory protein, MRAP (16).

Melanocortin-2 receptor accessory protein is a single chain polypeptide with one membrane-spanning domain. This transmembrane (TM) protein forms a homodimer at the endoplasmic reticulum in which the two monomers are oriented in an anti-parallel manner [reverse topology; for reviews, see Ref. (8, 9)]. In the human genome, there are two paralogous *MRAP* genes, *MRAP* or *MRAP1* (16), and *MRAP2* (17). For this discussion, “*mrap*” will be used to refer to the ancestral accessory protein gene, and *mrap1* and *mrap2* will be used to designate the two paralogous members of the gene family. As a reference for the discussion that will follow, **Table 1** summarizes the observations from Chan et al. (17) with respect to the effects of human MRAP1 $\alpha$  and human MRAP2 on the activation and trafficking of the five human MCRs.

The salient features of the MRAPs are illustrated by mouse MRAP1 and MRAP2 (**Figure 1**). For MRAP1, the LKANKH motif is required for reverse topology (18), and the corresponding reverse topology motif in mouse MRAP2 is LKAHKY, [(8); **Figure 1**]. Reverse topology motifs are also apparent in the chicken and zebrafish MRAP1 and MRAP2 orthologs (**Figure 1**).

The TM domain of mouse Mrap1 is required for the trafficking of MC2R to the plasma membrane (18), and the corresponding sequence in mouse Mrap2 (**Figure 1**) has 43% amino acid sequence identity with the TM domain of mouse Mrap1. Among the MRAP1 orthologs presented in **Figure 1**, the amino acid sequence identity between mouse Mrap1 and chicken MRAP1, and mouse Mrap1 and zebrafish MRAP1 is 74 and 48%, respectively. This level of sequence identity is apparently adequate since both chicken MC2R (19) and zebrafish MC2R (20) can be activated when co-expressed in heterologous mammalian cells with their respective MRAP1 ortholog. It is interesting that the amino acid sequence identity for the TM region of the MRAP2 orthologs presented in **Figure 1** is 74%. Since MRAP2 is expressed in brain and adrenal cortex cells, there appears to be selection pressure to maintain the TM sequences of MRAP2 orthologs. The physiological roles of the MRAP2 orthologs will be discussed later in this section.

Given the preceding comments on primary sequence similarity, the most striking difference between mouse Mrap1 and Mrap2 is the activation motif present in Mrap1 that is conspicuously absent in Mrap2 (**Figure 1**). As a result, although MC2R will move to the plasma membrane in the presence of MRAP2, activation of the receptor following an ACTH-binding event is barely detectable at concentrations of ACTH of  $10^{-8}$ M or less (8, 9, 21). Conversely, in the presence of MRAP1, the activation of MC2R is robust following an ACTH-binding event [(16); **Table 1**]. It would appear then, that when a mammalian MC2R ortholog is expressed alone, the receptor mis-folds, and is non-functional (22). When co-expressed with MRAP1, MC2R assumes an active conformation, and the MC2R/MRAP1 complex can be activated by ACTH. Interaction with an MRAP1 paralog to achieve functional expression is a strict requirement for teleost and tetrapod MC2R orthologs (20, 23).

Sebag and Hinkle (18) found that if the activation motif (LDYI) in the N-terminal domain of mouse Mrap1 (**Figure 1**) was replaced with alanine residues, the activation of MC2R was blocked. Furthermore, a single alanine substitution of the Y residue in the mouse Mrap1 LDYI motif resulted in a 50% drop in activation. Note that similar activation motifs are present in the N-terminal domains of the chicken and zebrafish MRAP1

**TABLE 1 | Summary of the interactions between human melanocortin receptors and human MRAP1 $\alpha$  and human MRAP2.**

		MRAP1 $\alpha$	MRAP2	
	Trafficking	Activation	Trafficking	Activation
MC1R	Not required	Not required	Not required	Lowers
MC2R	Facilitates	Required	Facilitates	Required
MC3R	Not required	Lowers	Not required	Lowers
MC4R	Restricts	Lowers	Restricts	Lowers
MC5R	Restricts	Lowers	Restricts	Lowers

Chan et al. (17) expressed individual human melanocortin receptors in CHO cells either in the presence or absence of either human MRAP1 $\alpha$  or human MRAP2, and measured either trafficking to the plasma membrane or activation with human ACTH (1–39) (MC2R; single dose  $10^{-8}$ M) or NDP-MSH (MC1R, MC3R, MC4R, MC5R; single dose  $10^{-9}$ M). For the trafficking experiments, “not required” indicates that co-expression with an MRAP had no negative or positive effect on trafficking to the plasma membrane relative to CHO cells transfected with only the melanocortin receptor. “Facilitates” indicates that the receptor did not translocate to the plasma membrane in the absence of the MRAP. “Restricts” indicates that there was a decline in trafficking to the plasma membrane when the receptor was co-expressed with an MRAP. For activation experiments, “not required” indicates that co-expression with an MRAP had no negative or positive effect on activation relative to CHO cells transfected with only the melanocortin receptor. “Requires” indicates that the receptor could not be activated when expressed alone in CHO cells. “Lowers” indicates that there was a statistically significant drop in activation when the receptor was co-expressed with an MRAP.

**MRAP1**

[-----N-Terminal-----] [-----TM-----]  
 es M-ADVEAFTNSSEDILRLNHNSNEYRFYEY-EYE--V  
 zf M-----KNSS-----EYVWGYEYYYDYIDP  
 c M-----ANRT-----NSSEYFWSYEYYWDYIDPIP  
 m M-----ANGT-----DASVPLTSYEYYLDYIDLIPV  
 Activation      Reverse      Trafficking  
 Motif      Topology Motif      Motif

**MRAP2**

m M-EMSAQRLLASNRTSPQSPSNSDYTWEYEY---EIGPVSFEGLKAHNSIVIGFWVGLAVFVIFMFFVLT  
 c M---SALRLISNRTSQQALSNSDYTWEYEY---EYGPVSFEGLKAHKYSIVIGFWVGLAVFVIFMFFVLT  
 zf MPRMSENNPVVNKTTHPGFNNDYTWGYEY---DYGPVSFEGLKAHRYSIVIGFWVGLAVFVIFMFFVLT  
 es M---SENNPVVNKTTHPGFNNDYTWGYEY---DYGPVSFEGLKAHRYSIVIGFWVGLVVFVIFMFFVLT  
 lp      LNGSERASARTPGSNRSKEYYWDFEY---DYEPISFEGLRAHRYSIVIGFWVGIASFVLFMFFILVM

**MRAP1**

[-----C-Terminal-----]  
 es RPQSLLDNTILLPGGVNP  
 zf ISHSGQLPRGPRVKSGLPIMKGYASSQ  
 c MSRSGSNPVKQVVVRNRVEESSNSEQPHGDNLSSPFDPVAPGTPSCLFDHSGIHGSI  
 m MSWSGPSQMRHSPQPQPICSWTHSFNLPLCLRRASLQTTEEPGRAGTDQWLTTQQSPS

**MRAP2**

m LTKTGAPHQDNAESSERRFRMNSFVSDFGKPLESDKVFSRQNEESRSLFHCYINEVEH.....  
 c LTKTGAPHQENTESSEKRFRMNSFVADFGRPLESERVFSRQIAEESRSLFHCINEVEH.....  
 zf LTKTGAPHPEAAEPYEKRMRLTSCADGLGRQRETDGRTGLSRPLLEESRSLSLFHCYINEE.....  
 es LTKTGAPHQENVDLPAKQHRMNGFSVGYPMLQKPDCAFVHRVSEESRSLSLFHCYVNEGH.....  
 lp LVRSGV

**FIGURE 1 | Amino acid sequence alignment of MRAP1 and MRAP2 paralogs.** The amino acid sequences of mouse (m) MRAP1 (NP\_084120.1), zebrafish (z) MRAP1 (XP001342923.2), chicken (c) MRAP1 (XR\_001470382) elephant shark (es) MRAP1 (XM\_007903550.1), mouse (m) MRAP2 (XP\_006511239.1), chicken (c) MRAP2 (XP\_015140201), zebrafish (zf) MRAP2a (XP\_001342923.4), elephant shark (es) MRAP2 (XP\_007906624.1), and lamprey (lp) MRAP2 (FAA00710.1) were aligned to the sequences of mouse MRAP1 and mouse MRAP2, respectively. Note that only a partial sequence for lamprey (*Petromyzon marinus*) MRAP2 has been reported (1). In addition, only the partial C-terminal sequences for the MRAP2 orthologs are presented. Predicted N-linked glycosylation sites are underlined. Note that there are two potential N-linked glycosylation sites in the putative elephant shark MRAP1 amino acid sequence. The amino acids in the activation motif for mouse MRAP1 are highlighted in red. Conserved amino acid positions in the proposed activation motifs of the chicken, zebrafish, elephant shark MRAP1 orthologs are also highlighted in red. The amino acids in the reverse topology motif of mouse MRAP1 were highlighted in green. Conserved amino acid positions in the reverse topology motif of chicken, zebrafish, elephant shark, mouse, and lamprey MRAP1 and MRAP2 sequences, respectively, were also highlighted in green. Finally, the amino acids in the transmembrane domain of mouse MRAP1 are highlighted in blue, and the conserved amino acid positions in the chicken, zebrafish, elephant shark, mouse and lamprey MRAP1 and MRAP2 sequences, respectively, were also highlighted in blue.

orthologs (Figure 1). In addition, a recent study indicated that alanine substitution of the DY residues in the YDYV motif of zebrafish MRAP1 blocks activation of MC2R (24).

While it appears that the direct interaction of mammalian MRAP2 orthologs with mammalian MC2R orthologs might be pharmacological rather physiological [Table 1; (8, 9, 21)], there are cells such as mouse adipocytes (25) and embryonic mouse adrenal cortex cells (26) that co-express Mrap2 as well as Mc2r and Mc5r. Sebag and Hinkle (27) found that when human MC5R

and mouse Mrap2 were co-expressed in CHO cells, the trafficking of human MC5R to the plasma membrane decreased. The implication of these experiments was that by decreasing the number of MC5 receptors on the plasma membrane, Mrap2 would make the target cells more selective for stimulation by ACTH, rather than  $\alpha$ -MSH. Since, MC2R and MC5R are co-expressed in chicken adrenal cortex cells (28), frog interrenal tissue (23), and rainbow trout interrenal tissue (29), an interaction between MC5R and MRAP2 could have physiological implications for

non-mammalian vertebrates as well. Hence, an evaluation of the pharmacological interactions of MC5R and MRAP2 orthologs with respect to trafficking to the plasma membrane and ligand selectivity in these species is warranted.

When considering a physiological role for MRAP2, a promising area of study has been the interaction between MRAP2 and MC4R in the modulation of feeding behavior by neurons in the hypothalamus (30). In both zebrafish (31) and mice (32), endogenous MRAP2 orthologs appear to play roles in the ligand sensitivity of the MC4 receptor. For example, Asai et al. (32) observed that when mouse Mc4r and Mrap2 are co-expressed in HEK-293 cells, Mc4R has a higher sensitivity for  $\alpha$ -MSH; an outcome that would decrease feeding activity *in vivo*. Conversely, in mice in which the *Mrap2* gene was selectively deleted from neurons in the hypothalamus, the result was an obese phenotype. While the implications of these results are intriguing from a biomedical perspective, it appears that for mammals there may be species specific difference in the regulation of MC4R. For example, Kay et al. (33) observed that co-expression of human MC4R and human MRAP2 in HEK-293 cells had no effect on ligand sensitivity. However, a shift in ligand sensitivity was observed when human MC4R was co-expressed with human MRAP $\alpha$ . Clearly, studies are needed on other tetrapods (i.e., amphibians, reptiles, and birds) are needed to determine the role that MRAP2 may play in modulating the ligand selectivity of MC4R in the regulation of feeding behavior in these organisms.

A more complex mechanism for the role of MRAP2 in regulating feeding behavior has been observed for the zebrafish (31, 34). As a result of a teleost-specific genome duplication [3R event; (35)], two paralogs of the *mrap2* gene (*mrap2a* and *mrap2b*) are present in the zebrafish genome (20). Furthermore, the expression of these paralogs appears to be developmentally regulated. Sebag et al. (31) report that during the larval stage of development, MRAP2a lowers the ligand sensitivity of zebrafish MC4R (as measured by Vmax), and as a result the animal eats more; an outcome that would favor growth. During larval development, the expression levels of the zebrafish *mrap2b* gene are low. Conversely in the adult stage, zebrafish *mrapb* gene expression is elevated, and zebrafish *mrapa* gene expression declines. Sebag et al. (31) also report that co-expression of zebrafish MC2R and zebrafish MRAP2 in HEK-293 cells increases the sensitivity of zebrafish MC4R for  $\alpha$ -MSH. These results are interpreted as giving the adult zebrafish fine control over food consumption; a trait that would be considered adaptive (31). Hence, it would appear that zebrafish MRAPb is functioning in a manner analogous to mouse Mrap2 (32). However, Aguijero et al. (34) observed that co-expression of zebrafish MC4R and zebrafish MRAP2a in HEK-293 cells resulted in a higher sensitivity of the zebrafish MC4R for ACTH as compared to  $\alpha$ -MSH. The latter study proposes that ACTH may be playing a role in the control of feeding behavior, and that role can be influenced by the expression levels of zebrafish MRAP2a. In the later study, the *in vitro* effect of zebrafish MRAP2b on zebrafish MC4R ligand selectivity was not apparent. Finally, zebrafish MRAP2a had no negative or positive effect on the trafficking of zebrafish MC4R to the plasma membrane (31, 34); whereas zebrafish MRAP2b appeared to increase the surface expression of zebrafish MC4R (31). Hence,

there appears to be species-specific differences in way the MC4R orthologs respond to interaction with MRAP2 (See Table 1). That said, at the molecular level it is not clear which domain(s) of MRAP2 (MRAP2a or MRAP2b) are making contact with MC4R to alter ligand selectivity. The possibility of an “activation motif” in MRAP2 orthologs, analogous to the activation motif in MRAP1 orthologs, has not been investigated.

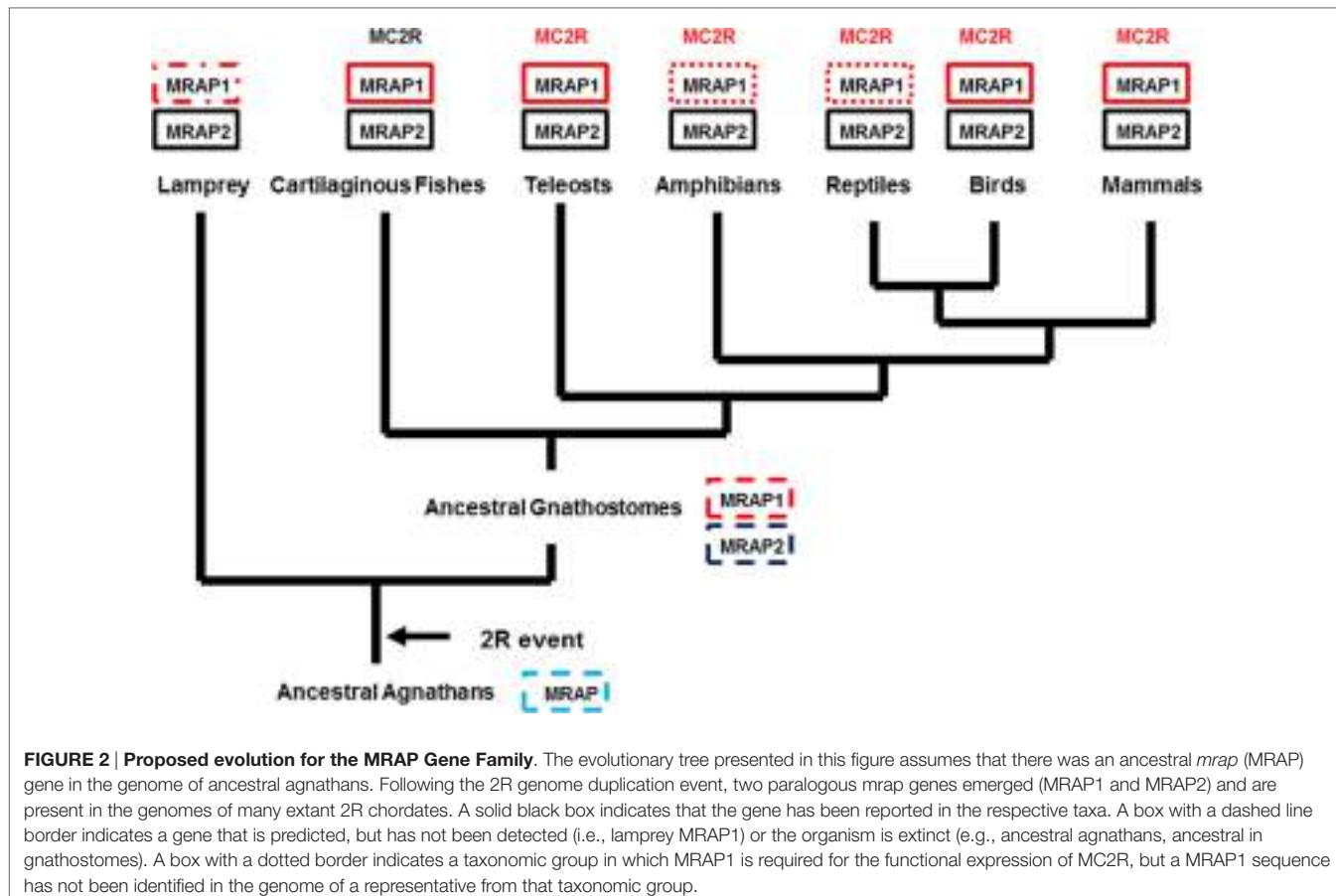
In terms of the evolution of the MRAP gene family, an earlier review concluded that MRAP2 was the ancestral “MRAP” (1). This conclusion was based on the apparent absence of MRAP1 orthologs in the genomes of a cartilaginous fish (*Callorhinchus milii*, the elephant shark) and the lamprey (*Petromyzon marinus*), and the presence of MRAP2 orthologs in both these species. In this scenario, the duplication of the ancestral *mrap* gene may have occurred in the bony fishes following the divergence of the ancestral cartilaginous fishes and the ancestral bony fishes (36) over 420 million years ago. However, recent annotation of the elephant shark genome project<sup>1</sup> revealed a cDNA (accession number: XM\_007903550.1) that Blast analysis<sup>2</sup> has identified as an MRAP1 ortholog. The deduced amino acid sequence of the putative elephant shark MRAP1 ortholog is presented in Figure 1. The putative elephant shark MRAP1 has a reverse topology motif (LQVNKY), and the TM region has 39% amino acid sequence identity with the mouse MRAP1 TM region. The C-terminal domain of the putative elephant shark MRAP1 is very short relative to the other MRAP1 orthologs. However, Sebag and Hinkle (18) have shown that the C-terminal of mouse MRAP1 is not required for either trafficking or activation of mammalian MC2R orthologs. The N-terminal domain of the putative elephant shark MRAP1 is nearly 43% longer than the other MRAP1 orthologs in Figure 1. By inserting gaps, it was possible to align these sequences and identify a putative activation motif (EYE) in the putative elephant shark MRAP1. The presence of the Y residue in this domain is particularly interesting, given the importance of this residue for mammalian and teleost MRAP1 orthologs (18, 24).

From a phylogenetic/evolutionary perspective, the detection of the putative cartilaginous fish MRAP1 ortholog fills a gap. The elephant shark is in Subclass Holocephali (Class Chondrichthyes), and it is very probable that *mrap1* orthologs are present in the genomes of members of Subclass Elasmobranchii (i.e., sharks and rays). Hence, *mrap1* and *mrap2* paralogs may have been present in the genome of the ancestral gnathostomes (Figure 2). Given these assumptions, the evolution of the *mrap* gene family may have involved the following scenario. In the ancestral agnathan vertebrates that underwent the 2R genome duplication event, the ancestral *mrap* gene would have been duplicated to yield the *mrap1* and *mrap2* genes, and these paralogous genes presumably would have been distributed on separate chromosomes. Currently, *mrap1* and *mrap2* genes have been found on separate chromosomes in the various gnathostome genome databases where chromosomes maps are available.<sup>3</sup> Among extant 2R

<sup>1</sup><http://esharkgenome.imcb.a-star.edu.sg>

<sup>2</sup><https://blast.ncbi.nlm.nih.gov/Blast.cgi>

<sup>3</sup><http://ensemble.org>



**FIGURE 2 | Proposed evolution for the MRAP Gene Family.** The evolutionary tree presented in this figure assumes that there was an ancestral *mrap* (MRAP) gene in the genome of ancestral agnathans. Following the 2R genome duplication event, two paralogous *mrap* genes emerged (MRAP1 and MRAP2) and are present in the genomes of many extant 2R chordates. A solid black box indicates that the gene has been reported in the respective taxa. A box with a dashed line border indicates a gene that is predicted, but has not been detected (i.e., lamprey MRAP1) or the organism is extinct (e.g., ancestral agnathans, ancestral in gnathostomes). A box with a dotted border indicates a taxonomic group in which MRAP1 is required for the functional expression of MC2R, but a MRAP1 sequence has not been identified in the genome of a representative from that taxonomic group.

vertebrates (Figure 2), an *mrap1* ortholog has not been detected in the current version of the lamprey genome project<sup>4</sup> (Figure 2). Whether the absence of this ortholog represents the incomplete state of the lamprey genome project, or a secondary loss of the ortholog cannot be determined at this time. In addition, *mrap1* orthologs have not been detected in the genomes of either the frog, *Xenopus tropicalis* or the reptile, *Anolis carolinensis*. However, the MC2R orthologs for both species require co-expression with a tetrapod MRAP1 ortholog for functional expression in CHO cells (21, 37). It would appear that either the *X. tropicalis* and *A. carolinensis* genome projects are not complete, or some other accessory protein is utilized in these species.

From a pharmacological perspective, the presence of the putative elephant shark MRAP1 ortholog is perplexing. An earlier study had shown that the elephant shark MC2R ortholog could be functionally expressed in CHO cells in the absence of co-transfection of an exogenous *mrap1* cDNA (38). Elephant shark MC2R could be stimulated in a dose-dependent manner by either human ACTH (1–24) or by dogfish (*Squalus acanthias*) ACTH (1–25). More recently, a MC2R cDNA cloned from the genome of the stingray, *Dasyatis akajei*, was also functionally expressed in CHO cells in the absence of co-transfection of an exogenous *mrap1* cDNA (39). The stingray MC2R ortholog also

could be stimulated by stingray ACTH (1–24) and stingray Des-Acetyl- $\alpha$ -MSH. Hence, there are several issues with respect to the putative elephant shark MRAP1 that need to be resolved. It will be important to determine whether the elephant shark *mrap1* mRNA is expressed in the same cells as elephant shark *mcr* mRNAs. In addition, pharmacological studies are needed to determine whether co-expression of cartilaginous fish MCR orthologs with the putative elephant shark MRAP1 ortholog have any effect on either trafficking of the MCR orthologs to the plasma membrane or sensitivity to melanocortin ligands.

## LIGAND SELECTIVITY OF MC2R ORTHOLOGS

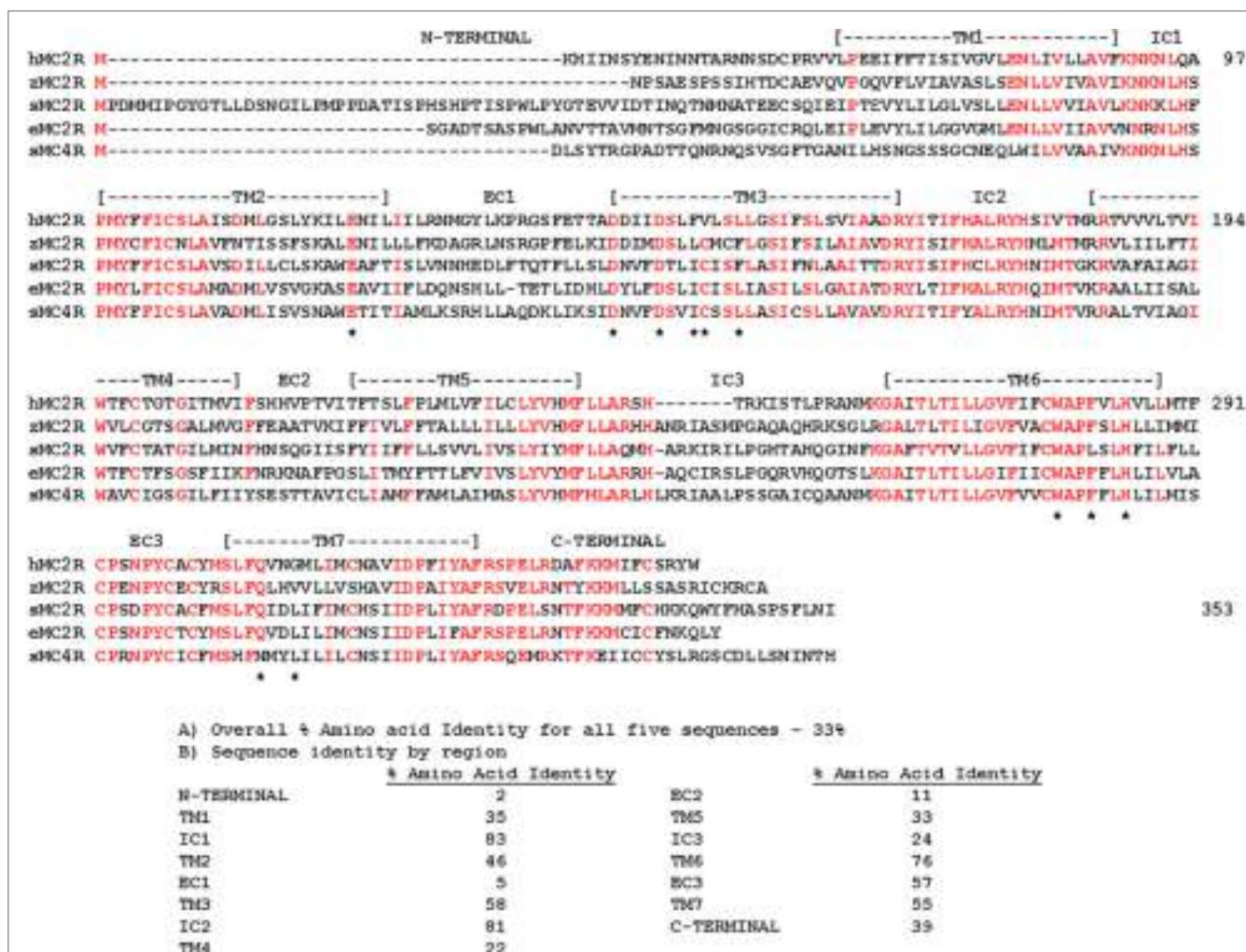
Several studies have shown that the MC2R orthologs of teleosts and tetrapods (Figure 2) require co-expression with a corresponding MRAP1 ortholog. Perhaps as a result of this interaction, and the intrinsic tertiary features of these MC2R orthologs, all of these receptors can only be activated by ACTH, and not by any MSH-sized ligand (19–21, 37, 40). Nearly 40 years ago, analog studies on mammalian ACTH sequences revealed the dual importance of the H<sup>6</sup>F<sup>7</sup>R<sup>8</sup>W<sup>9</sup> motif and the K<sup>15</sup>K<sup>16</sup>R<sup>17</sup>R<sup>18</sup> (tetrabasic) motif in ACTH for the activation of the “ACTH” (MC2R) receptor on mammalian adrenal cortex cells (41). These same features are required for the activation of MC2R on the interrenal and

<sup>4</sup>[http://www.ensembl.org/Petromyzon\\_marinus/Info/Index](http://www.ensembl.org/Petromyzon_marinus/Info/Index)

adrenal cortex cells of non-mammalian tetrapods and teleosts as well (42). Although, teleost and tetrapod  $\alpha$ -MSH sequences have the H<sup>6</sup>F<sup>7</sup>R<sup>8</sup>W<sup>9</sup> motif, these ligands lack the tetrabasic motif, and as a result are incapable of activating either teleost or tetrapod MC2R orthologs. By contrast, the teleost and tetrapod MC1R, MC3R, MC4R, and MC5R paralogs can be activated by either ACTH or the MSH-sized polypeptides derived from POMC with varying potencies (10, 42, 43). It would appear that teleost and tetrapod MCR paralogs all have an HFRW-binding site, and MC2R orthologs have an addition R/KKRR-binding site. These generalizations apply for the ligand selectivity properties of the MCRs of cartilaginous fishes (class Chondrichthyes) with one notable exception.

Studies on the ligand selectivity of dogfish, *Squalus acanthias* (order Squaliformes, subclass Elasmobranchii), MC3R, MC4R, and MC5R paralogs (44–46), and the MC1R, MC3R, MC4R, and MC5R paralogs of the stingray, *D. akajei* [order Rajiformes,

subclass Elasmobranchii; (39)] found that these MCR paralogs could be activated by either ACTH or MSH-sized ligands in a manner analogous to the corresponding MCR paralogs in teleosts or tetrapods. Hence, these paralogs have an HFRW-binding site. However, the MC2R ortholog of the stingray, *D. akajei*, and the MC2R ortholog from the elephant shark, *C. milii* (order Chimaeriformes, subclass Holocephali) could also be activated by either ACTH or MSH-sized ligands (38, 39), and as noted in Phylogeny and Proposed Evolution of the MRAPs, both of these MC2R orthologs could be functionally expressed in CHO cells without co-expression of an exogenous *mrap1* cDNA. The two cartilaginous fish MC2R orthologs, from different subclasses of the cartilaginous fishes, have ligand selectivity properties more similar to MC4R paralogs, and most likely have only a HFRW-binding pocket. This apparent feature for the cartilaginous fishes MC2R orthologs would be quite distinct from teleost and tetrapod MC2R orthologs. Whether the ligand selectivity properties



**FIGURE 3 | Amino Acid Alignment of MC2R Orthologs.** The amino acid sequences of human (h) MC2R (NP\_001278840.1), zebrafish (z) MC2R (XP\_00518229.1) stingray (s) MC2R (LC108747), elephant shark (e) MC2R (FAA704.1), stingray (s) MC4R (LC108749) were aligned, and amino acid positions in which four of the five sequences were identical are marked in red. The position of critical amino acids in the HFRW-binding site of MC4R orthologs (47) are marked with a star. The overall percent primary sequence identify (**A**) and the percent identity within each domain (**B**) are presented.

of the cartilaginous fishes MC2R orthologs are an ancestral trait or a derived trait unique to the cartilaginous fishes is not clear at this time.

In any event, there should be distinct sites within the teleost/tetrapod MC2R orthologs and the cartilaginous fishes MC2R orthologs that can account for the ligand selectivity properties of these receptors. In this regard, a comparison of MC2R orthologs with an MC4R paralog may reveal these potential sites. As shown in **Figure 3**, the human, zebrafish, elephant shark, and stingray MC2R amino acid sequences could be aligned to the stingray MC4R sequence by inserting a minimum of two gaps. The positions of critical residues in TM2, TM3, TM6, and TM7 that correspond to the HFRW-binding site for a MC4R ortholog (47) are marked with a star. For the teleost and tetrapod MC2R orthologs, six of these sites are conserved, and for the cartilaginous fishes MC2R orthologs eight of these positions are conserved. Overall only 33% of the positions in this alignment are identical in at least four of the five sequences, however, there are very clear regions of primary sequence identity, which serve as markers for MCR-related sequences. The highly conserved regions (sequence identify greater than 50%) include: IC1, TM3, IC2, TM6, EC3, and TM7. Moderately conserved regions (sequence identity greater than 35%) include: TM1, TM2, and the C-terminal domain. The highest primary sequence divergence (<15%) was observed for the N-terminal domain, EC1, and EC2.

Previous studies used chimeric proteins of human MC2R and human MC4R to analyze the functions of these regions. For example, Fridmanis et al. (48) observed that replacing the N-terminal domain of human MC4R with the N-terminal domain of human MC2R inhibited trafficking of the chimeric MC4R protein to the

plasma membrane. However, since the N-terminal of stingray MC4R is nearly the same length as the human MC2R domain (**Figure 3**), and the stingray receptor could be functionally expressed in CHO cells (39), length alone may not be a factor in influencing trafficking to the plasma membrane. Hinkle et al. (49) observed that exchanging the TM2/EC1/TM3 region of human MC2R with the corresponding region of human MC4R resulted in a chimeric MC2R protein that could be activated by either ACTH or NDP-MSH. Presumably making a similar chimeric protein for human MC2R, but using the TM2/EC1/TM3 region of either elephant shark or stingray MC2R should yield the same outcome. Finally, Fridmanis et al. (48) observed that substitution of the TM4 and TM5 domains in human MC2R affected ACTH activation, and this region of human MC2R may be the KKRR-binding site. In support of the later conclusion, studies on naturally occurring mutations in the TM4/EC2/TM5 domain of human MC2R, and alanine substitution experiments point to the TM4/EC2/TM5 region as playing an important role in the activation of human MC2R [for review see Ref. (50)]. It would now seem advantageous to extend the chimeric protein paradigm to the cartilaginous fishes MC2R orthologs to determine whether exchanging the TM2/EC1/TM3 and TM4/EC2/TM5 domains of teleost/tetrapod MC2R orthologs with the corresponding domains in the elephant shark MC2R orthologs would make the cartilaginous fish MC2R chimeric proteins exclusively selective for ACTH, and in the converse experiments, would the teleost/tetrapod chimeric MC2R proteins have more permissive ligand selectivity properties. Given Malik et al. (51) observations on the importance of extracellular domains in human MC2R for interaction with mouse MRAP1, co-expression of these MC2R

<b>A</b>	
	N-TERMINAL [-----TM1-----] IC1 [-----TM2-----]
- MC2R H-----AVNETDCKEVKIPNEIFFPAITMVSLENILLLVVAVIKNKQHLHSEMYCFCISLALPNWLSSTVSKAL 93	
- MC5R HNSSDYMLNQGPLATSS SNSQSNPTD LPGKTKPTACEQVNIAATEVPLTLGLIVSILENTLVICAIVNQNLHSEHYFVCSLAVADMLVSVSNRW	
-----] EC1 [-----TM3-----] IC2 [-----TM4-----] EC2	
- MC2R ENTHL-VETDAAGRLD SRQKPETKTD DQVMDTLLCMSPSTS IFSLSAIAVDRYITIFPMAFLRYNNIMTMRVAVI LOSINTPCAOSOVVHIIFFRAATV 186	
- MC5R STIVIYLNNHQQLIMEDNPIRQVDNVFDMSMICISVVSAMCSLLAIAVDRYTFIPEALRYNNIMTVRRAGPIIAGINTPCTCGGIVFIIYSDTT	
-----] TM5 ] IC3 [-----TM6-----] EC3 [---	
- MC2R IMTCPIALPLVSLVLILILYVHIFPQLARHTHWKIASLPG-NRSRQHNNNSGAIITLTILFGVPIICWSPEFFFHLLILMVCPLNPyICBCYRSLPFQV 279	
- MC5R VIICLISMFFGHLVLHQASLYSHMFLARSHVVKRIAAALPGYNINHQRAASHKAIAVFTLTILLGIFIVCWAPPFLHLILMISCPRLNYCVCFCMHSFNM	
-----] TM7 -----] C-TERMINAL	
- MC2R HVILLMCNAVIDPVITAFRSAKLRTTLLRKMPFCGSGTREIPVS IICCDNQSVLSSLSI LPFLMKQDMPT 344	
- MC5R YLILIMCNNSVIDPLIYAFRSQE-----MRKTFKE IICCCYSLRNACGLPSKY	
<b>B</b>	
	N-TERMINAL [-----TM1-----] IC1 [-----TM2-----]
- MC4R RDTTHHHH0LIHNYHHSRNPFSSQAPTVDNDSNEKDSSSOCYEQLLISTEVPLTLQTVSLLLENILVIAAIIKNQNLHSEMYFFCISLAVADMLVSVS 93	
- MC5R RQISS-----DQMLHQGQPLATSS SNSQSNPTD LPGKTKPTACEQVNIAATEVPLTLGLIVSILENTLVICAIVNQNLHSEHYFVCSLAVADMLVSVS	
-----] EC1 [-----TM3-----] IC2 [-----TM4-----] EC2	
- MC4R RAEWETIVNALITSOHLTIQONLIIQDMDNVFDSMICSSLLASICSLLAIAVDRYTFIPEALRYNNIMTMRVAVI ITTIDWAPCTISGILPFIYSE 186	
- MC5R RAEWETIVIYLNNHQQLIMEDNPIRQVDNVFDMSMICISVVSAMCSLLAIAVDRYTFIPEALRYNNIMTVRRAGPIIAGINTPCTCGGIVFIIYSD	
-----] EC2 [-----TM5-----] IC3 [-----TM6-----] EC3	
- MC4R STTIVIICLISMFFGHLVLHQASLYS10MELLARSHVVKRIAAALPGYNINHQRAASHKAIAVFTLTILLGIFIVCWAPPFLHLILMIACPRLNYCVCFCMS 278	
- MC5R TTPVIICLISMFFGHLVLHQASLYS10MELLARSHVVKRIAAALPGYNINHQRAASHKAIAVFTLTILLGIFIVCWAPPFLHLILMISCPRLNYCVCFCMS	
-----] TM7 -----] C-TERMINAL	
- MC4R HFNNMYLILIMCNNSVIDPLIYAFRSQEMLKTFKEIFCWYGLPQPCVCBLPSKY 330	
- MC5R HFNNMYLILIMCNNSVIDPLIYAFRSQEMLKTFKEIICCYSLRNAC--GLPSKY	

**FIGURE 4 | Amino Acid alignment of Gar MC2R, MC4R, and MC5R.** The amino acid sequences of gar MC2R (ENSLCT00000011667), gar MC4R (ENSLCT00000022303), and gar MC5R (ENSLCG00000018340) were aligned and positions that were identical are marked in red. **(A)** alignment of gar MC2R and gar MC5R; **(B)** alignment of gar MC4R and gar MC5R.

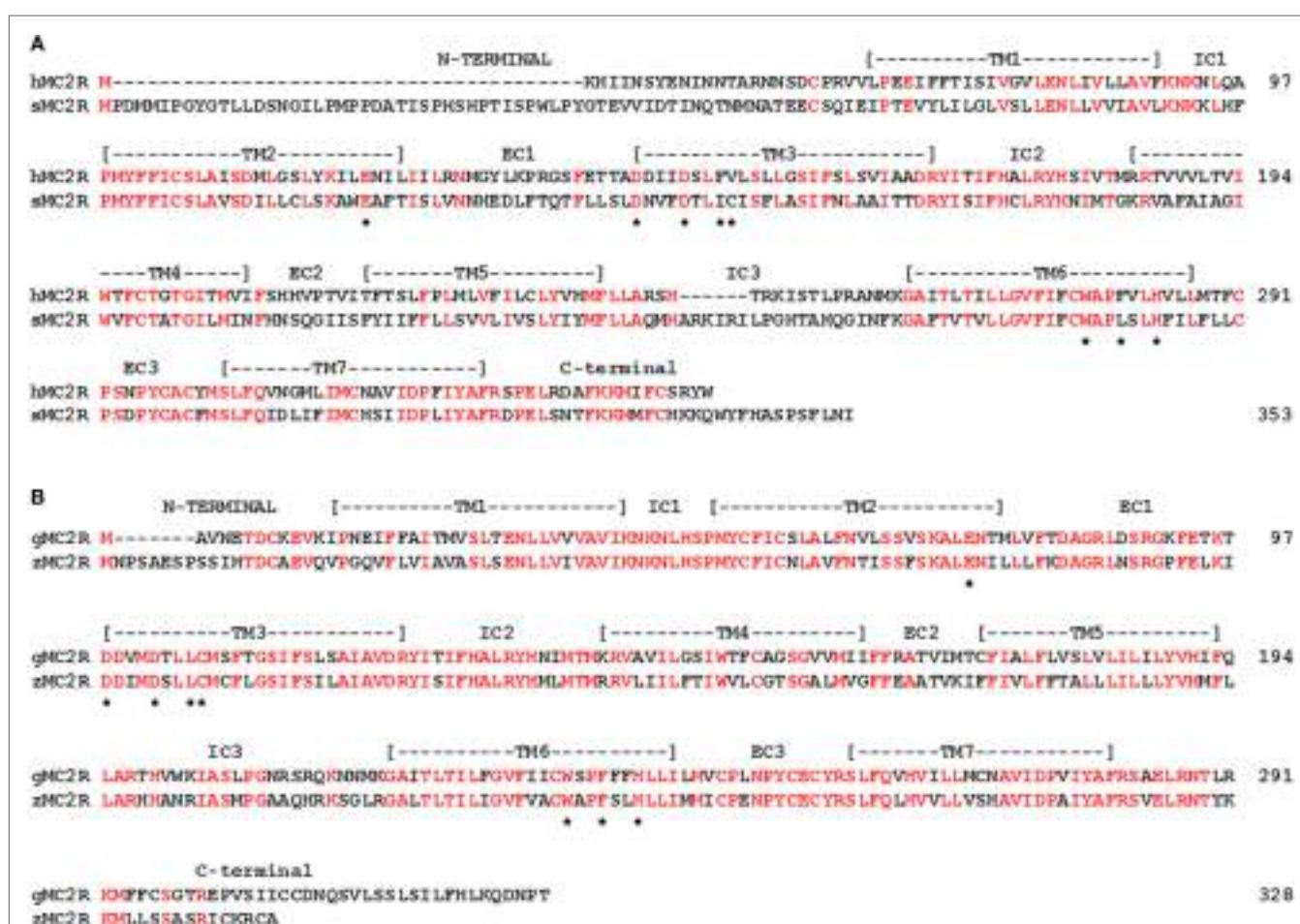
chimeric proteins with a class-specific MRAP1 ortholog may reveal the domain within the MC2R orthologs that makes contact with MRAP1.

## MELANOCORTIN RECEPTOR GENOME AND GENE DUPLICATIONS

The successive genome duplications during the radiation of the chordates theoretically should yield four paralogous genes in the genomes of extant cartilaginous fishes, non-teleost ray finned fishes, and tetrapods. However, in the genomes of the Japanese stingray (39), the spotted gar<sup>5</sup>, or the mouse (6), there are five paralogous MCR genes. The conclusion drawn from these observations is that one of the paralogous *mcr* genes underwent a local gene duplication (52). While there is general agreement that the *mc5r* gene was the result of the local gene duplication, the original *mcr* paralog that was duplicated has not been resolved. The issues

associated with the origin of the *mc5r* gene can be seen in the spotted gar (sg) genome. Chromosome mapping indicates that the *sgmc1r* gene is located on chromosome 21, the *sgmc2r* gene is located on chromosome 11, the *sgmc3r* gene is located on chromosome 18, the *sgmc4r* gene is located on chromosome 9, and the *sgmc5r* gene is also located on chromosome 11. The presence of paralogous genes on different chromosomes is considered an indication of a genome duplication event(s) (4). The presence of two genes on the same chromosome is generally construed as a result of a local gene duplication. In this regard, synteny studies found that the *mc2r* and the *mc5r* genes were on the same chromosome in the genomes of teleost fishes, the chicken (*Gallus gallus*), and several mammals (43, 52, 53). Based on these observations, it seemed reasonable to conclude that the *mc2r* and *mc5r* genes were the result of a local gene duplication (54, 55). In this scenario, the paralogous *mc2r* and *mc5r* genes would accumulate mutations independently, and based on selection pressures diverge in terms of amino acid sequence, and perhaps in terms of function. The divergence in amino acid sequence can be seen from an alignment of gar MC2R and gar MC5R (Figure 4A). The amino acid identity for the two paralogs is 42%. From an evolutionary

<sup>5</sup>[http://www.ensembl.org/Lepisosteus\\_oculatus/Info/Index](http://www.ensembl.org/Lepisosteus_oculatus/Info/Index)



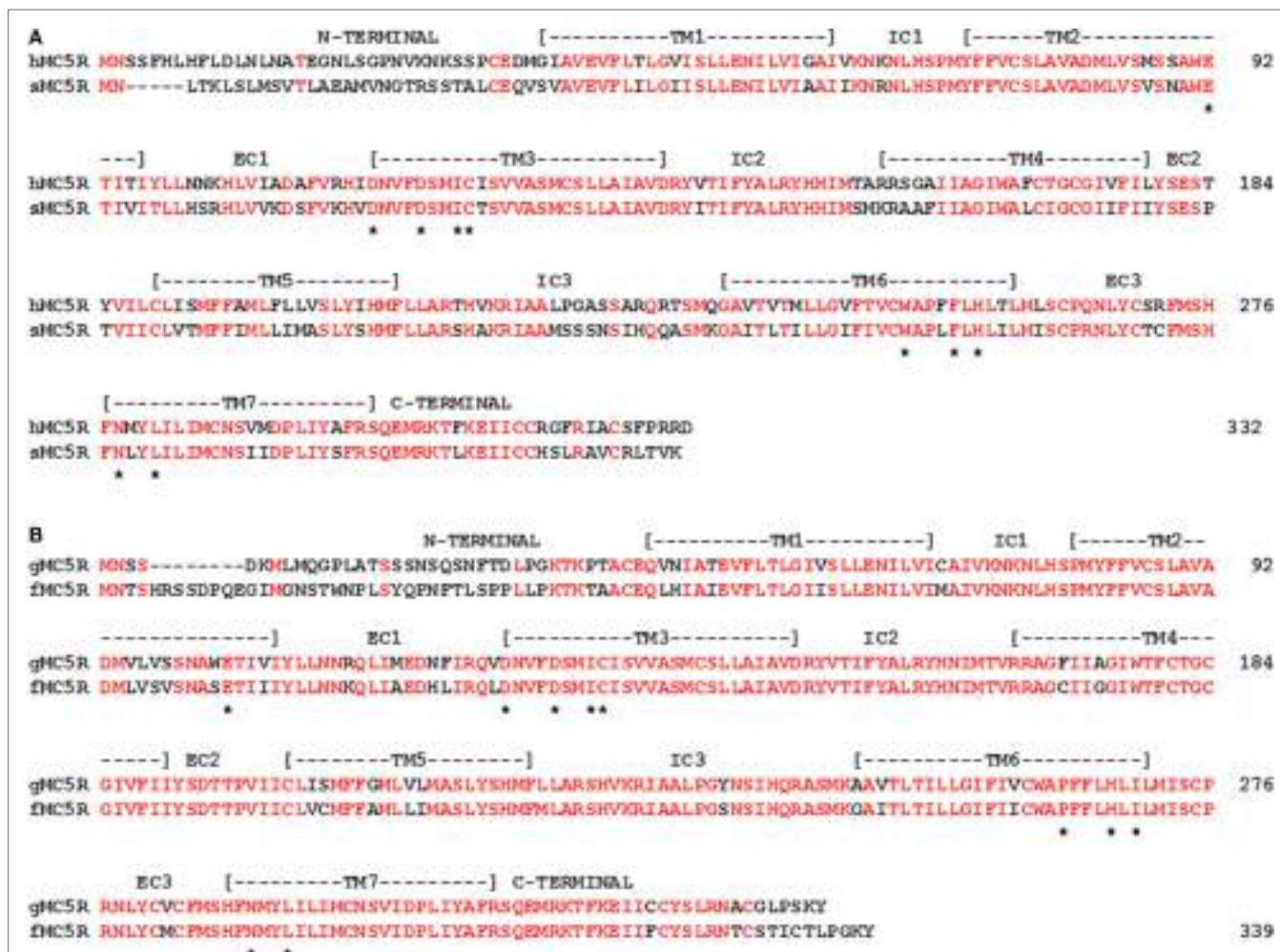
**FIGURE 5 | Amino acid sequence identity of MC2R orthologs. (A)** The amino acid sequences of human (h) MC2R and stingray (s) MC2R were aligned. **(B)** The amino acid sequences of gar (g) MC2R and zebrafish (z) MC2R (XP\_005158229) were aligned. The position of critical amino acids in the proposed HFRW-binding site (47) are marked with a star. Amino acid positions that are identical are marked in red.

perspective, the local gene duplication may have occurred after the 2R genome duplication event in the ancestral gnathostomes prior to the divergence of the ancestral cartilaginous fishes and the ancestral bony fishes.

However, Vastermark and Schioth (1) have pointed out that in phylogenetic analyses, MC5R orthologs form a clade with MC4R orthologs, and do not form a clade with MC2R orthologs. These observations have led to the conclusion that the *mc5r* gene was the result of a duplication of the *mc4r* gene (1). In support of this conclusion, an alignment of gar MC4R and gar MC5R indicates 70% sequence identity (**Figure 4B**). In this scenario, the presence of the *mc2r* and *mc5r* paralogous genes on the same chromosome of extant teleost and tetrapods could have been the result of an exchange of chromosome fragments in the last common ancestor to the ancestral ray-finned fishes (Class Actinopterygii) and the ancestral lobe-finned fishes (Class Sarcopterygii), the lineage which gave rise to the tetrapods, approximately 410 million years ago (56).

While either scenario (i.e., MC2R/MC5R or MC4R/MC5R) can be supported by the current evidence, there are at least two issues that neither scenario adequately addresses. As shown in **Figure 5A**, a comparison of stingray MC2R and human MC2R, vertebrates that last shared a common ancestor over 420 million years ago, the amino acid sequence identity is 37% (positions in red). Given the apparent role of the Hypothalamus/Pituitary/Adrenal (HPA) axis and the Hypothalamus/Pituitary/Interrenal (HPI) axis in maintaining the fitness of vertebrates (57–60), this lack of primary sequence conservation is difficult to comprehend. While divergence is expected, this degree of divergence is difficult to rationalize. It would appear that during the radiation of the gnathostomes, the *mc2r* gene sequence has drifted to the current state, while still maintaining functional capabilities.

For ray-finned fishes, such as the gar or zebrafish (Class Actinopterygii), MC2R primary sequence conservation is higher (55%; **Figure 5B**), and this condition may reflect the close interaction with MRAP1. That interaction most likely



**FIGURE 6 | Amino Acid Sequence Identity of MC5R Orthologs.** **(A)** The amino acid sequences of human (h) MC5R (NP\_005904.1) and stingray (s) MC5R (AY562212) are aligned. **(B)** The amino acid sequences of gar (g) MC5R and *Takifugu rubripes* (f) MC5R (AA06553.1; fugu) were aligned. The position of critical amino acids in the proposed HFRW-binding site (47) is marked with a star. Amino acid positions that are identical are marked in red.

started in the ancestral bony fishes, and while the interaction may not have “rescued” MC2R functionality, the interaction appears to have stabilized the functional capabilities of teleost and tetrapod MC2R orthologs. Tetrapod MC2R orthologs show a similar level of primary sequence conservation. As a result, selection pressures on teleost and tetrapod MC2R orthologs may involve maintaining the close interaction between MRAP1 and MC2R. For the cartilaginous fishes, the MRAP1/MC2R relationship is unclear or may not exist, and the selection pressures to maintain MC2R primary sequence identity does not appear to be as strong. For example, in a recent study, on stingray MCRs (39), *mc2r* and *mc5r* mRNA levels were detected in the interrenal tissue of this species. However, the EC<sub>50</sub> value of the stingray MC5R for ACTH (1–24) was in the 10<sup>-9</sup>M range, whereas the EC<sub>50</sub> value for stingray MC2R was in the 10<sup>-7</sup>M range. In this example, MC5R rather MC2R may be the “ACTH” receptor in the HPI axis of the stingray.

The diminished primary sequence conservation for MC2R orthologs is in sharp contrast to the higher degree of primary sequence conservation for stingray and human MC5R orthologs (55%; **Figure 6A**). In addition, for two very distantly related bony fish MC5R orthologs (gar and fugu) the sequence identity was 73% (**Figure 6B**). These observations beg the question of the functional significance of the stability of MC5R orthologs during the radiation of the gnathostomes. For mammals, MC5R plays a role in exocrine gland secretion (61). However, the role of the MC5R receptor in non-mammalian vertebrates is largely unknown. Perhaps a renewed focus on the distribution of MC5 receptors in various tissues of non-mammalian vertebrates will yield some answers.

## CONCLUSION

While it has been nearly 25 years since the cloning of the first MCRs (6, 11), and nearly 40 years since the structure/function studies on ACTH (41), there are still many aspects of the pharmacology and physiology of the melanocortin peptides and the

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MCRs that have not been resolved. One of the interesting facets of this receptor family is the interaction with the MRAPs (8, 9, 16, 17). The presence of a *mrap1* ortholog in the genome of a cartilaginous fish suggests that the *mrap1* and the *mrap2* paralogous genes were the result of the 2R genome duplication event (2). There is still a considerable amount of work to be done to clarify the physiological roles of the MRAP1 and MRAP2 in non-mammalian vertebrates, and the contact sites between MRAPs and the MCRs that can influence ligand selectivity.

When considering the functional activation of the MCRs, the paralogs MC1R, MC3R, MC4R, and MC5R are activated through an HFRW-binding site on these receptors that appears to be highly conserved. However, the MC2R orthologs of teleosts and tetrapods appear to utilize an additional binding site for the R/KKRR motif in gnathostome ACTH. Recent studies on cartilaginous fish MC2R orthologs suggest that a single-binding site may be all that is needed for the activation of these receptors (39, 46). Identifying the functional domains within the various gnathostome MC2R orthologs may clarify the ligand selectivity properties of cartilaginous fish and teleost/tetrapod MC2R orthologs.

Finally, while the role for MC2R orthologs in the HPA/HPI axis seems very clear, the role of the MC5R orthologs in the physiology of non-mammalian vertebrates is not resolved. The possibility that MC2R and MC5R may be functioning in the same cells should be considered.

## AUTHOR CONTRIBUTIONS

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# Effect of Novel Melanocortin Type 2 Receptor Antagonists on the Corticosterone Response to ACTH in the Neonatal Rat Adrenal Gland *In Vivo* and *In Vitro*

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Stress-induced increases in neonatal corticosterone demonstrate a unique shift from ACTH independence to ACTH dependence between postnatal day 2 (PD2) and day 8 (PD8) in newborn rats. This shift could be due to the binding of a bioactive, non-immunoreactive plasma ligand to the adrenocortical melanocortin 2 receptor (MC2R) (ACTH receptor). A potent MC2R antagonist would be useful to evaluate this phenomenon in the neonate. Therefore, we investigated the acute corticosterone response to ACTH<sub>(1-39)</sub> injection in rat pups pretreated with newly developed MC2R antagonists (GPS1573 and GPS1574), which have not been tested *in vivo*. The doses used *in vivo* were based on their *in vitro* potency, with GP1573 being more potent than GPS1574. GPS1573 (PD2 and PD8), GPS1574 (PD2 only), or vehicle were injected intraperitoneally (ip) 10 min before baseline sampling. Then, 0.001 mg/kg of ACTH<sub>(1-39)</sub> was injected ip, and subsequent blood samples obtained for the measurement of plasma corticosterone. Pretreatment of PD2 pups with GPS1573 demonstrated augmentation, rather than inhibition, of the corticosterone response to ACTH. In PD8 pups, pretreatment with 0.1 mg/kg GPS1573, but not 4 mg/kg, augmented the corticosterone response to ACTH. Pretreatment with GPS1574 attenuated the plasma corticosterone response to ACTH at 30 min in PD2 pups. The activity of these two compounds *in vivo* do not match their potency *in vitro*, with GPS1573 leading to a small augmentation of the corticosterone response to ACTH *in vivo* while GPS1574 resulted in inhibition.

**Keywords:** ACTH, MC2R, corticosterone, adrenal cortex, antagonist

## INTRODUCTION

Development of the neonatal hypothalamic–pituitary–adrenal (HPA) axis is critical for normal maturation of the lung, closing of the patent ductus arteriosus, and improving vasoconstrictor responses to catecholamines, as well as for stress-induced increases in blood glucose and blood pressure (1–3). In light of the increasing rate of premature births in the United States, it is important to understand the mechanisms of steroidogenesis and HPA axis maturation in the premature and full-term neonate (4). The neonatal rat model of human prematurity serves as a useful tool in studying the development of the HPA axis because the rat is an altricial animal. The full-term, newborn rat is immature compared

to a full-term human neonate (5, 6). The primary glucocorticoid in the neonatal rat, corticosterone, is secreted from the zona fasciculata of the adrenal cortex in response to ACTH binding and activation of the melanocortin 2 receptor (MC2R) (7, 8). The binding of ACTH to MC2R leads to an increase in intracellular cyclic adenosine monophosphate (cAMP), causing activation of protein kinase A (PKA), and subsequent increase in movement of free cholesterol across the mitochondrial membrane into the cytosol (7–10). This transport of free cholesterol, mediated by the steroidogenic acute regulatory protein (StAR), is the rate-limiting step of steroidogenesis (11).

We have previously shown that on postnatal day 2 (PD2), rat pups exposed to hypoxic stress demonstrate an increase in corticosterone without an appreciable increase in immunoassayable ACTH and adrenal cAMP (12, 13). This phenomenon could be due to larger posttranslational products of POMC activating the MC2R receptor (14), which are not necessarily detected in our ACTH immunoassay. Although difficult to study without completely eliminating potential confounders, there are data suggesting that premature infants born at <32 weeks can mount a cortisol response without the large increase in plasma ACTH found in infants born >32 weeks gestational age (15). As stated earlier, the newborn rat is useful as a model of human prematurity (5, 6).

By postnatal day 8 (PD8), pups show an increase in corticosterone with the classic increase in immunoassayable ACTH and adrenal cAMP (12, 13, 16). The ability of the PD2 adrenal gland to respond to stress without a detectable increase in immunoreactive ACTH could be due to a bioactive, non-immunoreactive form of ACTH or another POMC product that can bind to and activate the adrenocortical MC2R. If the corticosterone response to stress in PD2 pups can be blocked by antagonizing the MC2R, it would suggest that a bioactive form of ACTH (not measured by immunoassay) is working through the MC2R in PD2 pups.

GPS1573 (Nle-P-f-R-w-F-K-A-V-G-K-K-R-R NH<sub>2</sub>) and GPS1574 [Nle-(E-f-R-w-F-K)-A-V-G-K-K-R-R NH<sub>2</sub>] are newly described, potent ( $IC_{50} = 66 \pm 23$  and  $260 \pm 1$  nM, respectively), and dose-dependent antagonists of ACTH-stimulated MC2R activity *in vitro* (17). Note that the structures of the two compounds are similar except that GPS1574 has a ring structure. However, they have not been studied *in vivo*. The primary goal of the present study is to investigate the effect of GPS1573 and GPS1574 *in vitro* (adrenal cells) and *in vivo* (neonatal rats) in order to set the stage for its use in evaluating the role of endogenous ACTH in the neonatal adrenal stress response. We hypothesize that these MC2R antagonists are effective in adrenal cells from neonatal rats *in vitro*. Furthermore, we hypothesize that in the neonatal rat, GPS1573 and GPS1574, when given at a 100- to 4000-fold higher dose and 4000- to 8000-fold higher dose, respectively, than exogenous ACTH<sub>(1–39)</sub>, will attenuate the adrenocortical corticosterone response to ACTH.

## MATERIALS AND METHODS

### Animal Treatment and Experimental Protocol

The animal protocol was approved by the Institutional Animal Care and Use Committee of Aurora Health Care. Timed-pregnant

Sprague-Dawley rats at 14–17 gestational days ( $N = 45$ ) were obtained from Harlan Sprague Dawley (Indianapolis, IN, USA), maintained on a standard diet, and had water available *ad libitum* in a controlled environment (0600–1800 hours lights on). Dams were allowed to deliver and care for their pups without interference until experimentation. The MC2R antagonists GPS1573 and GPS1574 were synthesized by Genepep (St. Jean de Védas, France) and reconstituted, as described previously (17).

### Adrenal Cell Preparation and GPS1573 and GPS1574 *In Vitro*

Adrenal cells from rats ( $N = 11$  adults, 46 PD2 pups, and 26 PD8 pups) were dispersed and studied as previously described (18, 19). Briefly, rats were killed by decapitation, and both adrenal glands were removed and cleaned of surrounding fat. Only adult adrenal glands were decapsulated prior to cell dispersion. After a 90-min type I collagenase treatment and washing, viable cells were plated in a 96-well microtiter plate (10,000 cells/well) and pretreated for 1 h with (a) no antagonist (b) 750 nM GPS1573, or (c) 750-nM GPS1574 at 37°C in 10% CO<sub>2</sub> (balance room air). After pretreatment, rat ACTH<sub>(1–39)</sub> (Bachem) was added to cell suspensions at appropriate concentrations and incubated for 1 h. Medium was removed and immediately assayed for corticosterone (20).

### GPS1573 and GPS1574 Studies in the Neonatal Rat *In Vivo*

Rat pups were randomly assigned to experimental groups on the morning of the experiment. Then, rat pups were removed from the dams and placed in a small cage with adequate bedding, where they were allowed free range of motion and room to huddle. A variable control heating pad (Moore Medical, Farmington, CT, USA) was placed beneath the bedding and kept at the lowest setting required to maintain body temperature in normoxic rats at these ages (13, 21). After 10 min, rat pups were removed from the cage and quickly weighed.

In the GPS1573 studies, pups (both sexes;  $N = 252$ ) were studied at PD2 ( $N = 96$ ) and PD8 ( $N = 156$ ) because these ages span the critical time during which the neonatal rat's adrenal response to hypoxia shifts from immunoreactive ACTH independent to ACTH dependent (13, 16). Pups were injected intraperitoneally (ip) with either vehicle (10  $\mu$ l/kg body wt of isotonic saline) or GPS1573 (diluted in isotonic saline) in low or high dose (0.1 or 4.0 mg/kg body wt, respectively). We chose to give GPS1573 ip because (a) it is a small peptide amenable to proper absorption by this route, (b) ACTH injections given ip are effective, and (c) subcutaneous injection was ineffective (data not shown).

In the GPS1574 studies, pups (both sexes,  $N = 42$ ) were only studied at PD2, with the 60-min time point omitted because of drug's limited supply, the data from the 60-min time point with GPS1573 described above, higher necessary dose based on the *in vitro* studies [current experiments and Ref. (17)], and its expense. GPS1574 was given at a dose of 4 or 8 mg/kg ip. Otherwise, the experiments were performed as described for GPS1573.

Ten minutes after GPS1573 or GPS1574 injection, a subset of pups was quickly decapitated and trunk blood was collected (baseline, time 0). Immediately after the baseline collection,

1 µg/kg (0.001 mg/kg) of porcine ACTH (Sigma Chemical, St. Louis, MO, USA) was injected ip, as described (22–24). Subsets of pups were decapitated at 15, 30, or 60 min (GPS1573 only) post-injection. In another group of pups ( $N = 163$ ), the vehicle for ACTH injection (isotonic saline) was injected ip 10 min after GPS1573 or GPS1574 administration, and blood collected as described above. Trunk blood was collected in EDTA tubes (1 pup/sample), processed to plasma, and stored frozen ( $-20^{\circ}\text{C}$ ). Plasma corticosterone was measured by immunoassay, as described previously (MP Biomedicals, Orangeburg, NJ, USA) (25).

## Statistical Analyses

Corticosterone data were analyzed by two-way ANOVA. *Post hoc* analysis was performed by Holm–Sidak multiple range test ( $P < 0.05$ ) (SigmaPlot 11.0). Data are presented as mean  $\pm$  SEM.

## RESULTS

### GPS1573 and GPS1574 *In Vitro*

**Figure 1** shows the corticosterone response to ACTH in dispersed adrenal cells of rat pups (PD2 and PD8) and adults treated with GPS1573 and GPS1574. The cells from adult adrenal glands showed significant inhibition of the corticosterone response to ACTH *in vitro*, with GPS1573 being more potent than GPS1574. A similar response was observed in PD2 pup adrenal cells, as previously described for adult rats (17). In PD8 pup adrenal cells, however, there was equivalent inhibition between GPS1573 and GPS1574, compared to vehicle control.

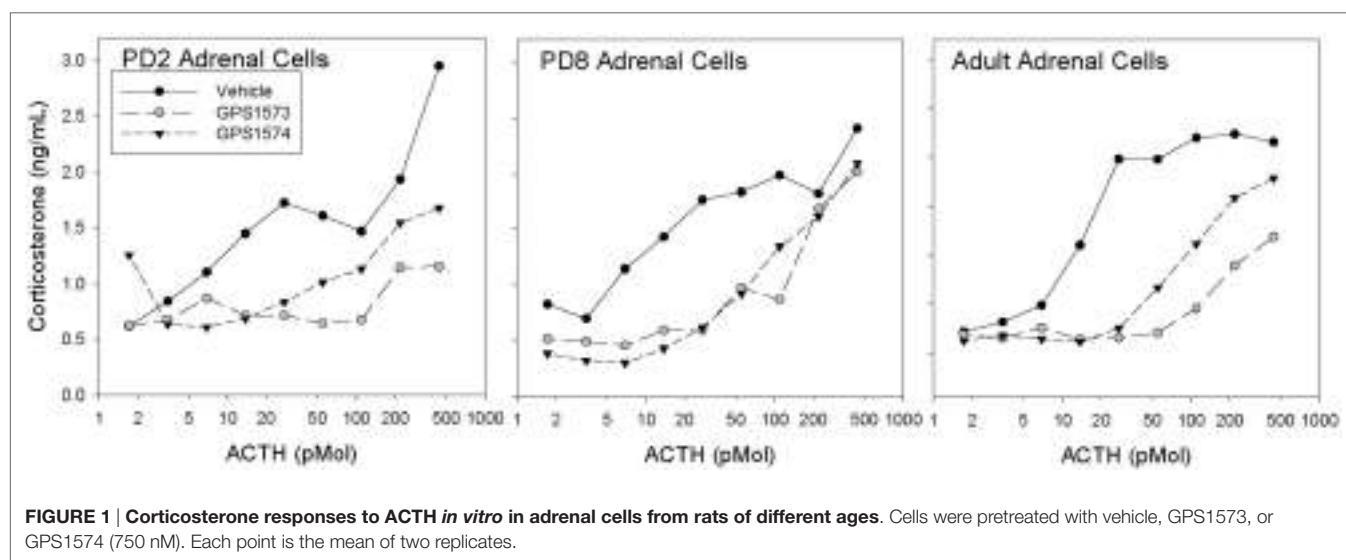
### GPS1573 *In Vivo*

The plasma corticosterone responses to exogenous ACTH in PD2 pups pretreated with either vehicle (for GPS1573) or the low or high dose of GPS1573 are shown in **Figure 2**. Baseline plasma corticosterone responses (10 min after injection of GPS1573) ranged from  $30.4 \pm 3.7$  to  $45.7 \pm 8.7$  ng/ml and were not different from our previously published baseline data without an injection 10 min before sampling (23, 24).

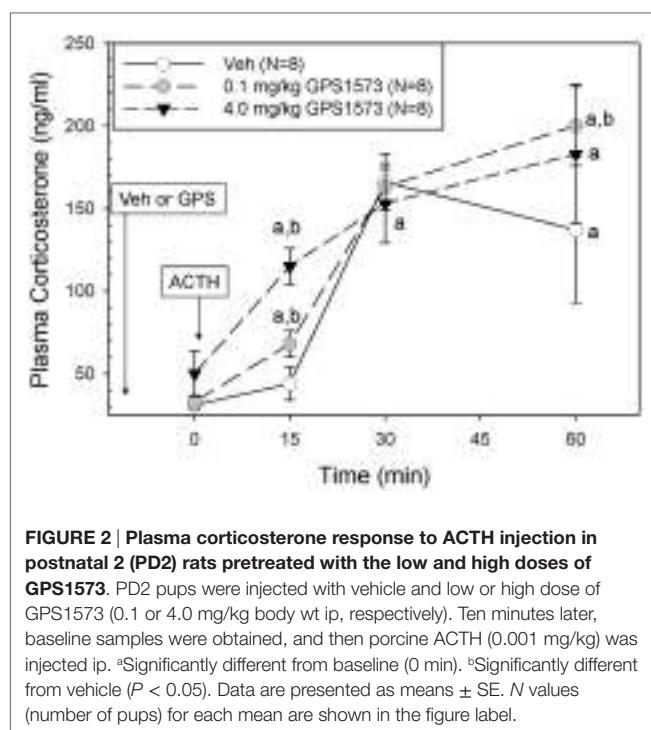
PD2 pups pretreated with vehicle did not show an increase in corticosterone in response to ACTH at 15 min but did have an increase in corticosterone at 30 min ( $166.0 \pm 17.1$  ng/ml) and 60 min ( $137.2 \pm 43.5$  ng/ml). After pretreatment with the low dose of GPS1573, compared to vehicle, the PD2 pups showed a significantly augmented plasma corticosterone response at 15 min ( $67.8 \pm 7.9$  ng/ml) and 60 min ( $200.0 \pm 23.6$  ng/ml). When pretreated with the high dose of GPS1573, PD2 pups demonstrate an even greater augmentation of the corticosterone response at 15 min ( $115.5 \pm 11.0$  ng/ml), when compared to pretreatment with low dose or vehicle.

The plasma corticosterone responses to exogenous ACTH in PD8 pups pretreated with either vehicle (for GPS1573) or the low or high dose of GPS1573 are shown in **Figure 3**. Baseline plasma corticosterone responses (10 min after injection of GPS1573) ranged from  $22.8 \pm 3.7$  to  $25.7 \pm 2.9$  ng/ml and were not different from our previously published baseline data, without an injection 10 min before sampling (23, 24). The plasma corticosterone response to ACTH in PD8 pups was less than PD2 pups. In fact, there was no significant increase in corticosterone in response to ACTH in PD8 pups. Compared to vehicle, PD8 pups pretreated with the low dose of GPS1573 showed an augmentation of the plasma corticosterone response to ACTH at 15 min ( $45.8 \pm 2.6$  ng/ml), 30 min ( $54.5 \pm 3.7$  ng/ml), and 60 min ( $50.4 \pm 6.5$  ng/ml). The corticosterone response to ACTH in PD8 pups pretreated with the high dose of GPS1573 was not different than vehicle at 15 min ( $34.3 \pm 4.5$  ng/ml) and 30 min ( $36.0 \pm 6.0$  ng/ml) but was less than the low dose of GPS1573 at 60 min ( $40.4 \pm 5.9$  ng/ml).

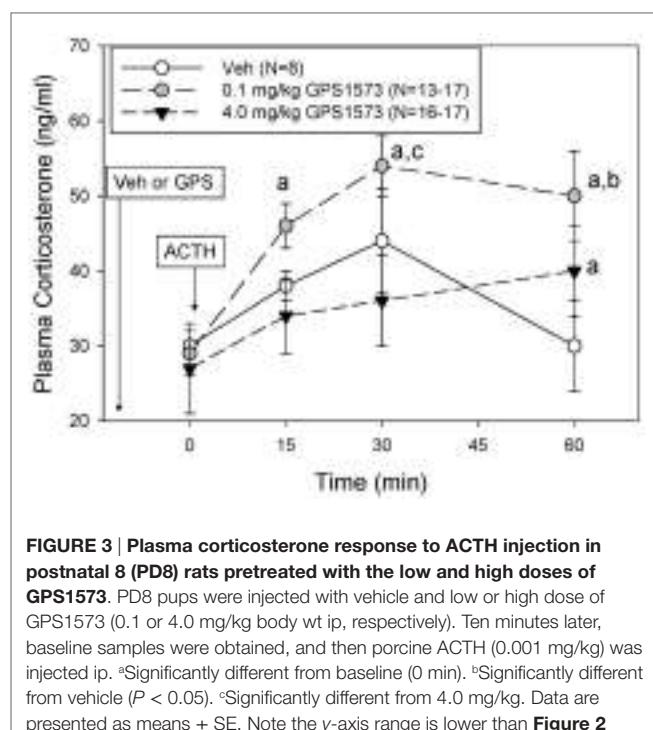
We also evaluated a time control in which pups were pretreated with GPS1573 and then injected with vehicle for ACTH (**Table 1**). For the most part, there were no statistically significant changes in plasma corticosterone. It is important to note that baseline plasma corticosterone concentrations were similar to those in our prior studies without ip injection prior to baseline (23, 24). However, there was a small increase in plasma corticosterone at 15 min in PD2 pups pretreated with the high dose of GPS1573 and at 30 min in PD8 pups pretreated with the low dose of GPS1573.



**FIGURE 1 | Corticosterone responses to ACTH *in vitro* in adrenal cells from rats of different ages.** Cells were pretreated with vehicle, GPS1573, or GPS1574 (750 nM). Each point is the mean of two replicates.



**FIGURE 2 |** Plasma corticosterone response to ACTH injection in postnatal 2 (PD2) rats pretreated with the low and high doses of GPS1573. PD2 pups were injected with vehicle and low or high dose of GPS1573 (0.1 or 4.0 mg/kg body wt ip, respectively). Ten minutes later, baseline samples were obtained, and then porcine ACTH (0.001 mg/kg) was injected ip. <sup>a</sup>Significantly different from baseline (0 min). <sup>b</sup>Significantly different from vehicle ( $P < 0.05$ ). Data are presented as means  $\pm$  SE. N values (number of pups) for each mean are shown in the figure label.



**FIGURE 3 |** Plasma corticosterone response to ACTH injection in postnatal 8 (PD8) rats pretreated with the low and high doses of GPS1573. PD8 pups were injected with vehicle and low or high dose of GPS1573 (0.1 or 4.0 mg/kg body wt ip, respectively). Ten minutes later, baseline samples were obtained, and then porcine ACTH (0.001 mg/kg) was injected ip. <sup>a</sup>Significantly different from baseline (0 min). <sup>b</sup>Significantly different from vehicle ( $P < 0.05$ ). <sup>c</sup>Significantly different from 4.0 mg/kg. Data are presented as means  $\pm$  SE. Note the y-axis range is lower than Figure 2 (PD2 pups). N values (number of pups) for each mean are shown in the figure label.

## GPS1574 In Vivo

Although we originally planned to only evaluate GPS1573 because of its higher potency *in vitro* (17), which was confirmed by our *in vitro* studies, the lack of inhibition with GPS1573 described above led us to do a limited number of new *in vivo* experiments with the apparently less potent GPS1574. We limited the GPS1574 study to PD2 (an age we are most interested in) through 30 min because of the need to use a higher dose of this very expensive drug, which was in limited supply and because the peak response to ACTH injection was at 30 min. As Figure 4 demonstrates, there was no inhibition at 15 min after ACTH administration, but a dose-dependent inhibition at 30 min was seen after ACTH injection. The time control for GPS1574 (saline injection rather than ACTH) did not reveal any changes in corticosterone (data not shown) and had baseline serum corticosterone concentrations similar to our previous studies without injection before baseline sampling.

## DISCUSSION

This study evaluated the corticosterone response to exogenous ACTH injection in neonatal rats pretreated with potent, *in vitro* MC2R antagonists, which have not previously been tested *in vivo* (17). We hypothesized that these compounds would attenuate the adrenocortical MC2R, thereby resulting in a lower corticosterone response to ACTH in PD2 and PD8 rats. If these antagonists were effective *in vivo*, it would allow us to evaluate the possibility that stress-induced increases in corticosterone at PD2 that are independent of increases in immunoreactive plasma ACTH could be explained by binding of a non-immunoreactive corticotrophic ligand to the adrenocortical MC2R (12, 13, 16, 21). We chose to

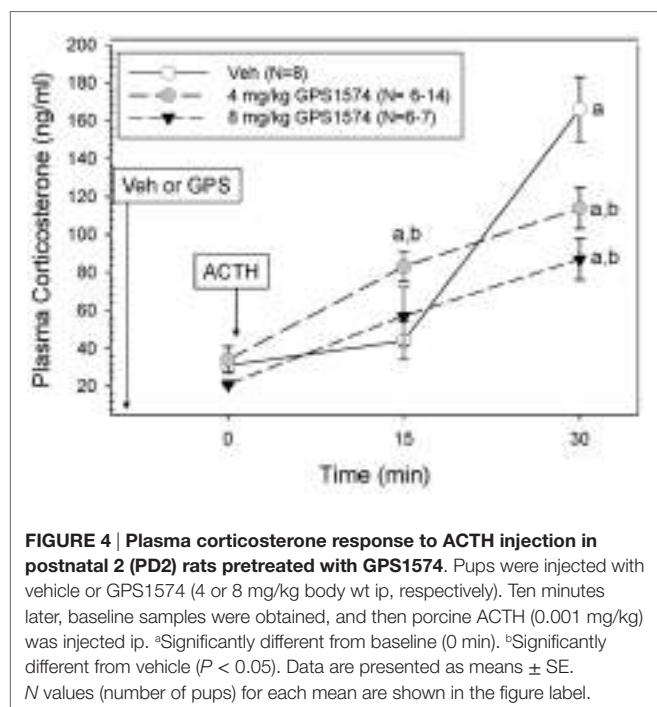
study neonatal rats based on previous studies (23, 24). Additionally, we are currently unable to study these compounds in adult rats because of the very large quantities of the GPS compounds needed, the compounds' expense, and their limited supply.

Although the limited GPS1574 *in vivo* studies were performed after the responses to GPS1573 were ascertained, we will first discuss the GPS1574 data. GPS1574 resulted in an attenuated corticosterone response at 30 min after ACTH injection. We studied GPS1574 at a dose 4000- and 8000-fold higher than the exogenous ACTH dose. We were unable to use an even higher dose because of the limited supply of this compound. With the assumption that these GPS compounds are absorbed in a manner similar to ACTH, we calculated the plasma concentration of the GPS compounds to be at least 10- to 20-fold higher than the peak plasma ACTH concentration after injection. It is possible that these drugs are not as well absorbed as ACTH<sub>(1-39)</sub> and/or that they are metabolized much more readily than ACTH<sub>(1-39)</sub>, perhaps requiring even higher doses ip. These preliminary results show promise for a more comprehensive analysis of this compound at much higher doses.

GPS1573, given *in vivo* at a 4000-fold higher dose of exogenous ACTH, did not antagonize the adrenal response to ACTH at either age. Rather, we demonstrated that pretreatment with GPS1573 augmented the corticosterone response to ACTH stimulation *in vivo*. In PD2 pups, the low dose of GPS1573 significantly augmented the corticosterone response to ACTH at 15 and 60 min compared to vehicle while the high dose demonstrated an even greater augmentation at 15 min compared to

**TABLE 1 |** Plasma corticosterone (nanogram per milliliter) response to vehicle (for ACTH injections) after pretreatment of PD2 and PD8 pups with the low dose (0.1  $\mu$ g/kg) or high dose (4  $\mu$ g/kg) of GPS1573.

Age	Dose	Time (min)			
		Baseline	15	30	60
PD2	Low ( $N = 5$ –6)	25.7 $\pm$ 7.0	49.4 $\pm$ 16.6	37.1 $\pm$ 10.3	42.0 $\pm$ 7.7
	High ( $N = 7$ )	41.0 $\pm$ 9.6	67.4 $\pm$ 6.2 <sup>a</sup>	25.4 $\pm$ 4.9	29.0 $\pm$ 9.4
PD8	Low ( $N = 5$ )	17.0 $\pm$ 4.1	24.6 $\pm$ 3.4	41.2 $\pm$ 4.4 <sup>a,b</sup>	30.3 $\pm$ 11.5
	High ( $N = 11$ –12)	16.4 $\pm$ 2.6	21.0 $\pm$ 3.2	14.5 $\pm$ 3.2	15.7 $\pm$ 2.9

<sup>a</sup>Different from baseline.<sup>b</sup>Different from high dose within PD8.**FIGURE 4 |** Plasma corticosterone response to ACTH injection in postnatal 2 (PD2) rats pretreated with GPS1574. Pups were injected with vehicle or GPS1574 (4 or 8 mg/kg body wt ip, respectively). Ten minutes later, baseline samples were obtained, and then porcine ACTH (0.001 mg/kg) was injected ip. <sup>a</sup>Significantly different from baseline (0 min). <sup>b</sup>Significantly different from vehicle ( $P < 0.05$ ). Data are presented as means  $\pm$  SE. N values (number of pups) for each mean are shown in the figure label.

vehicle and low dose. In PD8 pups, pretreatment with the low dose of GPS1573 showed significant augmentation compared to vehicle at 60 min while pretreatment with the high dose showed no significant increase in corticosterone at any time point. GPS1573 did not consistently act as an agonist *per se*, with only small increases in corticosterone in response to ACTH vehicle (saline) at two time points.

This leads us to conclude that although GPS1573 acts as a competitive antagonist *in vitro* in adrenal cells from PD2, PD8, and adult cells, pretreatment with it *in vivo* results in an augmentation of the response to the natural ligand for the MC2R (ACTH). A similar phenomenon has been shown for nuclear (intracellular) receptors (26).

What could explain the effect demonstrated for a G-protein coupled receptor like the MC2R? First, it could be that the IC<sub>50</sub> of GPS1573 is too high to be effective *in vivo*, despite the fact that we gave it at a 4000-fold higher dose than ACTH. The data from GPS1574 do not corroborate this notion since it has a higher IC<sub>50</sub> *in vitro* but was effective *in vivo*. It is also possible that GPS1573

acts as an antagonist *in vitro* but is a biasing agonist *in vivo*. That is, *in vivo*, rather than blocking the receptor, it actually augments G-protein coupled transduction when ACTH binds to the MC2R (27, 28). This phenomenon could also be attributable to GPS1573 triggering a non-specific sympathetic nervous system-induced increase in the sensitivity of the adrenal cortex to ACTH (22, 29–36). Another possibility is that GPS1573 is inactivated shortly after being injected or that it has a very short half-life *in vivo*.

Potential drawbacks of our study design include the route of administration of GPS1573 and GPS1574. We chose ip administration *a priori*, since these antagonists are small compounds, and we have previously shown that ip ACTH injection is effective in stimulating corticosteronogenesis (22–24). We performed a few pilot studies with subcutaneous injection, which were ineffective (data not shown). However, an alternate route of administration (e.g., intravenous or intramuscular) may be more effective. It is also possible that these compounds would have been more effective *in vivo* in postpubertal rats. Alternate controllers of neonatal steroidogenesis, including postganglionic sympathetic input described above, have been proposed; however, the current study avoids these confounders by injecting ACTH rather than using stress as a stimulus to ACTH (22–24).

In conclusion, it appears that high dose of GPS1574 has potential as a competitive antagonist of ACTH *in vivo*. However, GPS1573 seems to act like a biasing agonist *in vivo* when given before an ACTH injection even though it is not consistently an agonist when given alone. The differences in behavior between these two compounds *in vivo* may be related to the ring structure of GPS1574.

## AUTHOR CONTRIBUTIONS

NN, JB, AG, and HR all contributed substantially to the design and performance of the experiments and assays, writing and editing of the manuscript, approval of the final version, and agreement to account for all aspects of the work.

## ACKNOWLEDGMENTS

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# ACTH Regulation of Adrenal SR-B1

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The adrenal gland is one of the prominent sites for steroid hormone synthesis. Lipoprotein-derived cholesterol esters (CEs) delivered via SR-B1 constitute the dominant source of cholesterol for steroidogenesis, particularly in rodents. Adrenocorticotrophic hormone (ACTH) stimulates steroidogenesis through downstream actions on multiple components involved in steroidogenesis. Both acute and chronic ACTH treatments can modulate SR-B1 function, including its transcription, posttranscriptional stability, phosphorylation and dimerization status, as well as the interaction with other protein partners, all of which result in changes in the ability of SR-B1 to mediate HDL-CE uptake and the supply of cholesterol for conversion to steroids. Here, we provide a review of the recent findings on the regulation of adrenal SR-B1 function by ACTH.

**Keywords:** adrenal, cholesterol, ACTH, SR-B1

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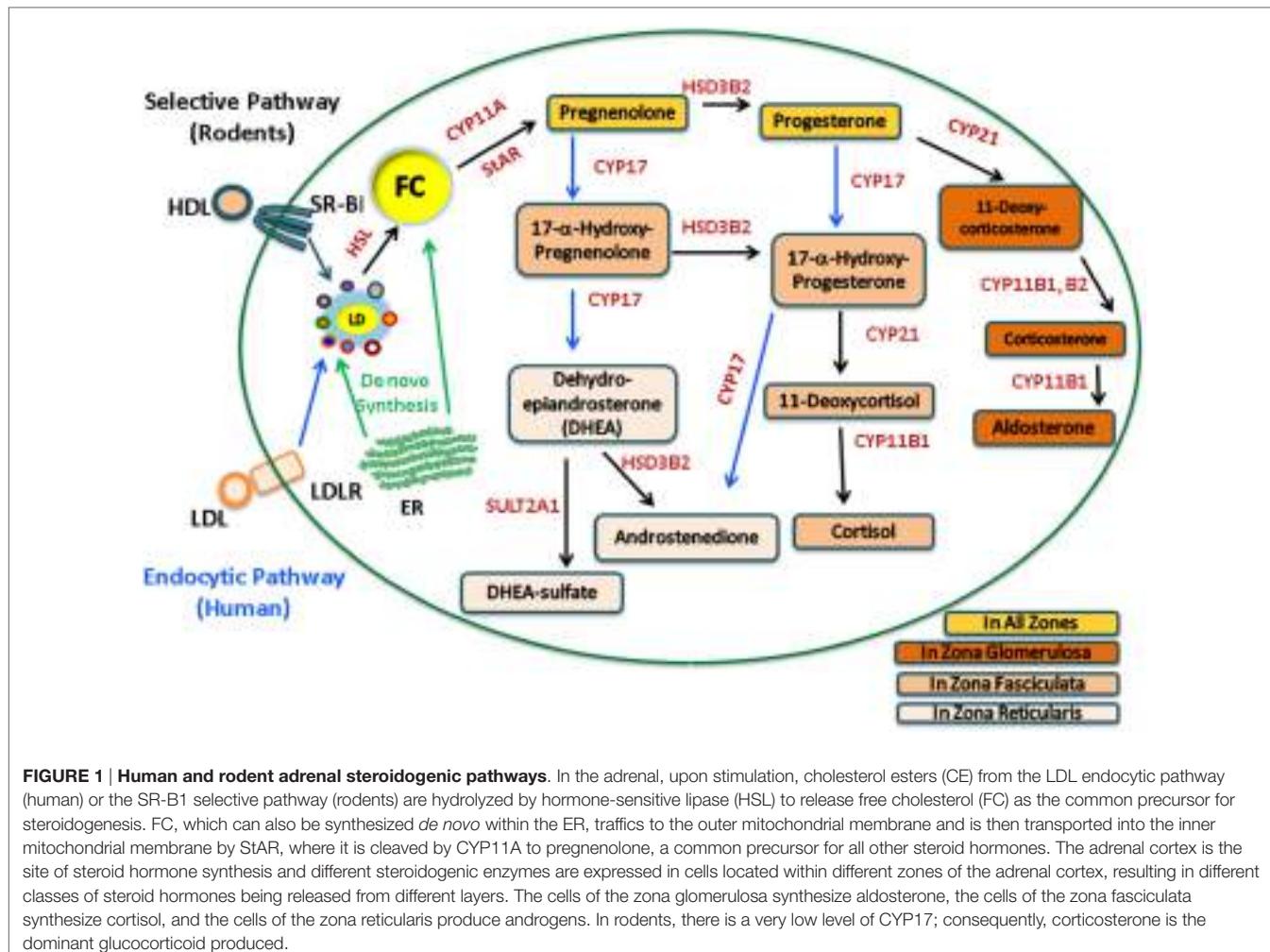
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The adrenal gland, in addition to the gonads, is one of the prominent sites where steroid hormones are synthesized (1–5). Cholesterol is the common precursor for steroidogenesis, which involves the contribution from multiple enzymes and requires the conversion of cholesterol to pregnenolone as the initial step of a multistep process. Pregnenolone is subsequently metabolized to produce various biologically active steroids in a tissue-specific manner. In general, this process is thought to involve five major steps: (1) cholesterol acquisition through *de novo* synthesis and/or uptake from lipoproteins and stored as cholesterol esters (CEs) in lipid droplets (LDs), (2) cholesterol mobilization from CEs that are stored in LDs, (3) trafficking of cholesterol to the cytochrome P450 side-chain cleavage enzyme (P450scc, CYP11A1) at the inner mitochondrial membrane (IMM), following cholesterol trafficking to the outer mitochondrial membrane (OMM), (4) production of pregnenolone by CYP11A1 through cleavage of the cholesterol side-chain, and (5) efflux of pregnenolone from the mitochondria to the endoplasmic reticulum (ER), where enzymes convert it into intermediates that shuttle between mitochondria and ER to produce progestins, estrogens, androgens, glucocorticoids, or mineralocorticoids in a tissue-specific manner (2, 5).

The adrenal gland is a compound endocrine gland composed of two developmentally unrelated tissues, an outer layer of adrenal cortex and an inner layer of adrenal medulla. The adrenal cortex is the site of steroid hormone synthesis and produces three classes of steroid hormones. These are glucocorticoids (cortisol and corticosterone), mineralocorticoids (aldosterone), and androgens [androstenedione and dehydroepiandrosterone (DHEA)]. The cells of the adrenal zona glomerulosa, which is the outermost layer of the adrenal cortex, synthesize aldosterone in response to angiotensin II (1, 2), whereas the cells of the adrenal cortical zona fasciculata-reticularis produce cortisol, corticosterone, or androgens [androstenedione and DHEA/dehydroepiandrosterone sulfate (DHEAS)] in response to adrenocorticotrophic hormone (ACTH) stimulation. Humans synthesize cortisol, but because CYP17 is poorly expressed in the zona fasciculata in rats and mice, consequently, corticosterone is the dominant glucocorticoid produced in rodents. The adrenal steroidogenic pathways are illustrated in **Figure 1**.

All the steroid hormones synthesized within the adrenal cortex utilize cholesterol as the common precursor. For cells that produce polypeptide hormones, large amounts of mature hormones



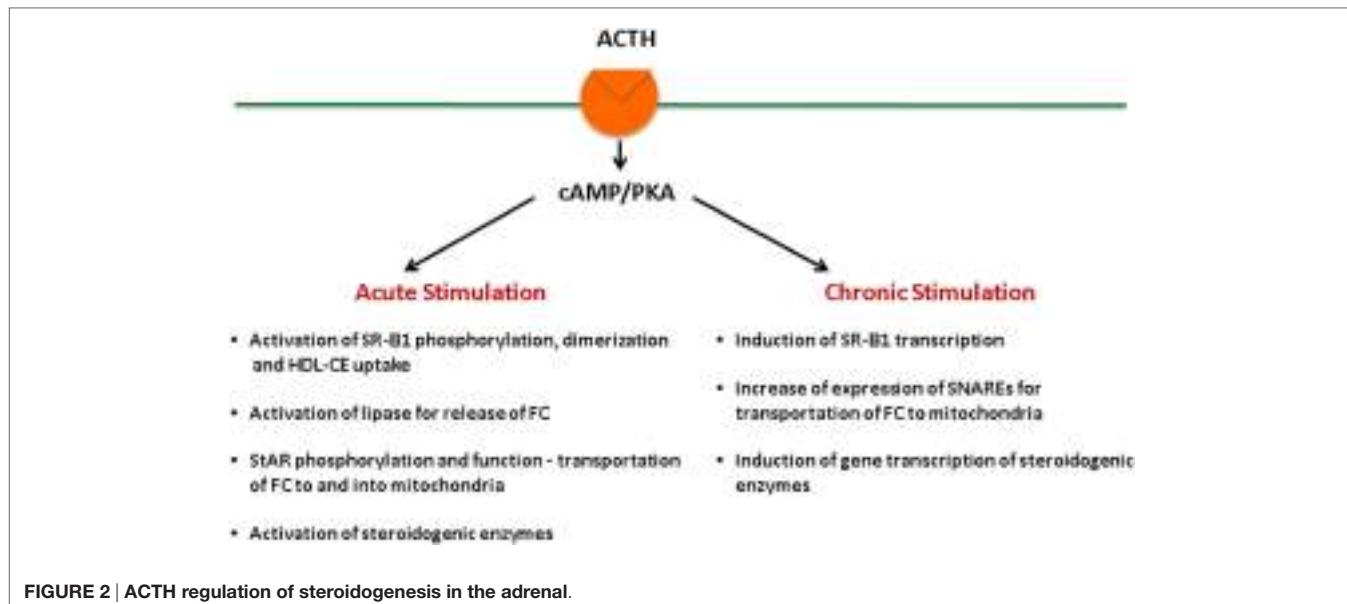
**FIGURE 1 | Human and rodent adrenal steroidogenic pathways.** In the adrenal, upon stimulation, cholesterol esters (CE) from the LDL endocytic pathway (human) or the SR-B1 selective pathway (rodents) are hydrolyzed by hormone-sensitive lipase (HSL) to release free cholesterol (FC) as the common precursor for steroidogenesis. FC, which can also be synthesized *de novo* within the ER, traffics to the outer mitochondrial membrane and is then transported into the inner mitochondrial membrane by StAR, where it is cleaved by CYP11A to pregnenolone, a common precursor for all other steroid hormones. The adrenal cortex is the site of steroid hormone synthesis and different steroidogenic enzymes are expressed in cells located within different zones of the adrenal cortex, resulting in different classes of steroid hormones being released from different layers. The cells of the zona glomerulosa synthesize aldosterone, the cells of the zona fasciculata synthesize cortisol, and the cells of the zona reticularis produce androgens. In rodents, there is a very low level of CYP17; consequently, corticosterone is the dominant glucocorticoid produced.

can be stored ready for rapid release; however, there is very little steroid hormone storage in steroidogenic cells. Therefore, upon stimulation, there is a rapid response from the steroidogenic cells to synthesize new steroids (3, 4), and with this a requirement for a constant supply of the precursor cholesterol to be converted to steroid hormones. The precursor cholesterol for steroidogenesis can be derived from a combination of sources (5–7): (1) *de novo* cellular cholesterol synthesis, (2) the mobilization of CEs stored in LDs, and (3) lipoprotein-derived CEs delivered through endocytic uptake, which is mediated by the LDL receptor or “selective” cellular uptake *via* the scavenger receptor, class B type 1 (SR-B1).

SR-B1 has been shown to be a HDL receptor and can mediate selective uptake of lipoprotein (HDL)-derived CEs both *in vitro* and *in vivo* (8–11). In the selective CE uptake pathway that is mediated by SR-B1, CE-rich lipoproteins bind on the cell surface and deliver the CEs from the hydrophobic core of the lipoproteins to the inside of the cells. The lipoprotein particles remain intact at the cell surface and can be further recycled to deliver more CEs to the cells (12). In contrast, CEs delivered *via* LDL receptor-mediated lipoprotein uptake are hydrolyzed by lysosomal acid lipase, releasing unesterified free cholesterol (FC) from lysosomes

that traffics to the ER and plasma membrane (PM) and is then available to traffic to mitochondria (13, 14). CEs delivered *via* SR-B1 appear to be incorporated directly into LDs (15, 16) and must be hydrolyzed to FC before being used in steroidogenesis. Upon ACTH treatment, adrenal CE stores within LDs are rapidly depleted (17) through the action of hormone-sensitive lipase (HSL), the major neutral cholesteryl ester hydrolase expressed in the adrenal gland (18). This newly released FC from stored LDs is the preferred source of cholesterol. Following LD depletion, lipoprotein-derived CEs delivered *via* SR-B1 become the dominant source of cholesterol for steroidogenesis in rodents (19–21).

Adrenal fasciculata-reticularis cell steroidogenesis is under the regulation of tropic hormone ACTH and is subject to both acute (14, 22–25) and chronic regulation (14, 26–29). ACTH binds to its G protein-coupled receptors, leading to the activation of adenylate cyclase, which generates cAMP and activates cAMP-dependent protein kinase (PKA) (30–33). Stimulation of the cAMP-PKA signaling cascade exerts both acute and chronic effects on the regulation of steroid hormone production (**Figure 2**). On the other hand, angiotensin (AI) stimulates aldosterone biosynthesis in adrenal glomerulosa cells, and its



actions are primarily mediated by the protein kinase C signaling cascade, whereas potassium can also stimulate aldosterone production through  $\text{Ca}^{2+}$ -calmodulin-dependent kinase (34). Both acute and chronic ACTH treatments can modulate SR-B1, including its expression levels as well as its phosphorylation status, dimerization, and the interaction with other protein partners, all of which result in changes of SR-B1 function. Here, we aim at providing a review of the most recent findings relevant to these aspects.

## ACTH STIMULATION REGULATES THE EXPRESSION OF SR-B1

Initial studies established a functional correlation between SR-B1 expression, HDL-CE uptake, and the ability of steroidogenic cells to produce steroid hormones (8, 35–40). Also, in both lower vertebrates (i.e., turtle) and the fruit fly, there is an increase in SR-B1 expression that correlates with the peak cholesterol flux required during their developmental stages (41). Follow-up studies revealed that the bulk of the cholesterol for steroidogenesis is provided from the selective uptake pathway, which is mediated by SR-B1.

Both rodent and human adrenals express exceptionally high levels of SR-B1; indeed, the highest expression level of SR-B1 per gram of tissue has been reported for rodent adrenals (42, 43). *In vivo* treatment of rats and mice and *in vitro* treatment of cultured rodent adrenocortical cells with ACTH increased SR-B1 expression both at the mRNA level and that of SR-B1 protein (8, 44). In one of our recent studies, the level of SR-B1 protein in rat adrenals shows a trend for increased SR-B1 expression as early as 1 h after treatment with ACTH (45). Similar stimulation of SR-B1 expression and regulation by ACTH was also demonstrated for cultured human adrenal cells (8, 43, 46, 47). Indeed, low levels of SR-B1 mRNA expression seen in normal adrenal tissue adjacent

to adenomas causing Cushing's syndrome, where plasma ACTH levels are reduced, is consistent with the notion that the regulation of human SR-B1 is possibly similar to that reported for rodents (43).

Much of the regulation of SR-B1 expression is at the level of transcriptional control. The promoter region of both human and rat SR-B1 contains binding sites for steroidogenic factor-1 (SF-1) (48), which is one of the major transcription factors involved in cAMP regulation of the *SR-B1* gene (49). In addition, the rat promoter has two sterol-responsive elements (SREs) that can bind sterol-responsive element-binding protein (SREBP)-1a and regulate *SR-B1* gene expression in response to altered intracellular sterol levels (50).

Further studies demonstrated that the promoter region of SR-B1 contains sites that can bind both positive and negative regulators. For the expression of SR-B1 in liver and adipose tissue, transcription factors, such as the liver X receptors  $\alpha$  and  $\beta$  (LXR $\alpha$  and LXR $\beta$ ) and the peroxisome proliferator-activated receptor  $\alpha$  (PPAR $\alpha$ ), have been shown to positively regulate the expression of the human *SR-B1* gene in response to oxysterols and fibrates, respectively (51, 52). Other positive regulators include the liver receptor homolog 1 (LRH-1) (53) as well as the estrogen receptors  $\alpha$  and  $\beta$  (ER $\alpha$  and ER $\beta$ ), which bind to three different estrogen-responsive elements on the rat SR-B1 promoter and regulate its activity in response to estrogens (54). The negative regulators of SR-B1 include the nuclear receptor dorsal-sensitive sex adrenal hypoplasia congenital critical region on the X chromosome gene 1 (DAX-1), a protein that plays an important role in adrenal development (49), the Yin Yang 1 (YY1) transcription factor, which represses the activity of the SR-B1 promoter by inhibiting the binding of SREBP-1a (55), and the pregnane X receptor, which represses the human SR-B1 promoter activity in response to the pregnane X receptor agonists rifampicin and lithocholic acid (56). Most of the binding sites for these transcription factors

reside within the 2.2 kb proximal 5'-flanking region of the SR-B1 promoter.

In steroidogenic tissues, SR-B1 expression was shown to be upregulated by ACTH (8, 44, 46, 57–59). As mentioned above, SF-1 was shown to mediate the regulation of SR-B1 gene expression through the cAMP–PKA pathway by binding to the promoter region of SR-B1. The SF-1 binding site in the human SR-B1 promoter (5'-CCAAGGCT-3') resides 77 bp upstream of the transcription start site, and the SF-1 binding site (5'-TCAAGGCC-3') in the rat SR-B1 promoter is located at –645 bp upstream of the translation start site. These two sites share 75% identity (48, 49). Both the human and rat SR-B1 promoters were shown to be active in mouse adrenocortical tumor Y1 cells, and mutagenesis analysis confirmed the involvement of SF-1 in regulating their promoter activity. The SF-1 binding motif in the rat SR-B1 promoter was shown to be involved in both basal and cAMP-induced regulation of SR-B1 gene expression. Further analysis of the functional domains in SF-1 revealed that both amino acids 448–461 and phosphorylation at Ser430 by PKA are involved in regulating the binding to the consensus sequence in the rat SR-B1 promoter. A recent report showed that when glucocorticoid levels are elevated, SR-B1 expression can also be inhibited by feedback regulation by glucocorticoid (60). In corticosterone-insufficient corticotrophin-releasing hormone knockout Crh ( $-/-$ ) mice, there is an increase of SR-B1 mRNA levels in adrenal, and oral administration of corticosterone inhibited SR-B1 gene expression. Further studies reveal that the glucocorticoid receptor (GR) can suppress SR-B1 promoter activity. The region between –201 and –62 of the human SR-B1 promoter was shown to contain putative binding sites for transcriptional repressors that are involved in mediating glucocorticoid regulation of SR-B1 expression. However, examination into the mechanism of suppression suggested that GR suppression of SR-B1 in adrenal cells occurs through an indirect mechanism since no direct binding of GR to the SR-B1 promoter was observed. This was the first report showing that by suppressing SR-B1-mediated HDL cholesterol uptake, steroidogenic tissues maintain steroid hormone homeostasis when the endogenous levels of glucocorticoids are elevated.

Recently, in the search for the cellular and molecular mechanisms involved in the regulation of SR-B1 expression and function in steroidogenic cells, we demonstrated that two microRNAs, miRNA-125a and miRNA-455, can bind to specific sites in the 3' UTR of SR-B1 mRNA and regulate the expression of SR-B1 (61). The expression of miRNA-125a and miRNA-455 is detected in steroidogenic tissue/cells, including adrenal, primary ovarian granulosa cells, and model Leydig cell lines. Both ACTH and cAMP downregulate the expression of miRNA-125a and miRNA-455. When either miRNA-125a or miRNA-455 is overexpressed or inhibited, the amount of SR-B1 protein expressed on the cell surface is decreased or increased, respectively, leading to SR-B1-mediated selective HDL uptake and SR-B1-supported steroid hormone synthesis being inhibited and stimulated, respectively. Therefore, our findings suggest that miRNA-125a and miRNA-455, in response to ACTH stimulation, act as SR-B1 attenuators to negatively regulate SR-B1 expression and SR-B1-mediated selective delivery of lipoprotein cholesterol in steroidogenic cells and, consequently, inhibition of SR-B1-supported steroidogenesis.

## MODULATION OF SR-B1 PROTEIN FUNCTION BY ACTH

SR-B1 facilitates HDL-CE selective uptake in two separate independent steps: binding of the lipid-rich lipoprotein to the extracellular domain (ECD) of SR-B1 and the delivery of the CEs from the hydrophobic core of the lipoprotein to the PM (62, 63). A specialized cell surface structure, termed “microvillar channels,” was reported to be induced by SR-B1 and shown to facilitate selective lipid transfer to inside the cell (64–67). Studies using electron microscopy demonstrated the presence of microvillar membrane domains in rat ovarian luteal, testicular Leydig, and adrenocortical cells. These domains form channels at the PM and various lipoproteins, including HDL, get trapped within the channels. Immunostaining using specific antibodies for SR-B1 revealed that SR-B1 is preferentially localized on these domains (65, 66). SR-B1 was also shown to be able to facilitate the formation of specific lipid rafts, hence change the properties of the PM and, in turn, affect the flux of free cholesterol (67). These lipid rafts were indicated to be necessary for the formation of the membrane microvillar channels, which are considered a trap for HDL particles for enhancing the efficiency of the selective uptake of HDL-CE. Interestingly, expression of SR-B1 and microvilli are under hormonal regulation; ACTH treatment increases, whereas dexamethasone treatment decreases, SR-B1 expression. Furthermore, the steady-state levels of adrenal microvilli are dictated by SR-B1. In response to SR-B1 deficiency, mouse adrenal microvilli become disorganized, and microvillar channels show a disrupted appearance along with substantially reduced binding of HDL particles to the cell surface (64–67).

In addition, experiments utilizing mutational analysis of SR-B1 or chimeras of CD36/SR-B1 have demonstrated that high affinity binding of lipoproteins to the ECD of SR-B1 is important, but not sufficient to mediate efficient lipid uptake (68). However, at the same time the ECD of SR-B1 does influence the efficient transfer of lipid by SR-B1 (69). This dichotomy is highlighted by the findings that some chemicals can increase lipoprotein binding to SR-B1 while actually blocking lipid transfer (70). Further studies show that the dominant characteristic of lipoprotein binding to SR-B1 involves protein–protein interactions between ligand and receptor. Many of the CE donors (HDL, apoA-I/phospholipid bilayer disks, and lipid-free apoA-I) for SR-B1 all share class A amphipathic helices that could be the structural feature to which SR-B1 is binding (68, 69). In addition, SR-B1 occurs as a multimeric complex with itself or other membrane proteins on the cell surface to facilitate lipid transfer, and the ECD of SR-B1 is essential for efficient CE transfer. ACTH has been shown to induce changes in the oligomeric status as well as protein interaction of SR-B1 and hence modulates SR-B1 protein function.

## ACTH Stimulation Modulates the Oligomerization of SR-B1

In response to ACTH stimulation, SR-B1 changes its oligomeric status to facilitate CE uptake (for simplicity, here, we use the term dimerization to include the multiple forms of the SR-B1 protein; i.e., dimers and higher order oligomers). In one of the earliest direct demonstrations of protein–protein interactions

involving SR-B1, SR-B1 was shown to exist as homodimers in PMs isolated from rat adrenals stimulated with 17 $\alpha$ -ethynodiol (70). Subsequently, dimeric and higher order oligomeric forms of SR-B1 were shown to exist in all cells and tissues that display HDL-CE selective uptake activity (65, 71, 72). In normal rat adrenal tissue, SR-B1 exists primarily in a monomeric form with some dimer formation. Upon ACTH stimulation, there is a significant increase in the dimerization/oligomerization of SR-B1 along with increased selective CE uptake. On the other hand, dexamethasone-mediated suppression of ACTH leads to dramatic loss of SR-B1, SR-B1 dimers/oligomers, and HDL-CE selective uptake activity. When combined with the substantial architectural alterations of the cell surface as related to microvillar formation, these findings indicate that SR-B1 dimer/oligomer formation appears to have significant implications for the expression of the functional properties of SR-B1.

Studies in adrenal cells and other steroidogenic tissues have established a strong correlation between the levels of SR-B1 dimers and enhanced HDL-CE selective uptake activity. Co-immunoprecipitation with differentially epitope-tagged SR-B1s further confirmed that SR-B1 can exist as homodimers (71). In addition, in both native steroidogenic cell lines (endogenous) and in a heterologous insect cell overexpressing SR-B1, dimers/oligomers of SR-B1 were seen when cross-linking agents were added to the cell lysates (65). When cellular extracts from SR-B1 transfected HEK-293 cells or ACTH-treated Y1-BS1 cells were analyzed by size-exclusion chromatography and sucrose density centrifugation, a significant portion of SR-B1 eluted at peaks that correlate with the size of dimeric and oligomeric forms of SR-B1. Immunoelectron microscopy was used as an independent means for confirming the homodimerization of SR-B1. For these experiments, differentially epitope-tagged-SR-B1 proteins were co-expressed in HEK-293 cells, and the epitope-tagged proteins were subsequently immunostained and identified using two differently sized gold particles. The observed mixing and clustering of gold particles suggested that the proteins were localized to the same regions of the cell and that many of the gold particles were in extremely close proximity, i.e., within a distance for protein–protein interactions, as detected by fluorescent resonance energy transfer (FRET) technique. Similar results were obtained when Y1-BS1 mouse adrenocortical cells were transfected with differentially epitope-tagged-SR-B1 constructs. Interestingly, transfection of Y1-BS1 cells with SR-B1 in these experiments resulted in substantial architectural changes with the formation of microvillar structures. Gold-labeled secondary antibodies localized SR-B1 to these sites and revealed substantial dimer formation of this protein – shown by close contact between gold particles (71, 72).

Further investigations concentrated on the contribution of the cysteine residues in the ECD of SR-B1 either independently or in cooperation with the C-terminal domain on SR-B1 dimerization. SR-B1 contains a total of eight cysteine (C) residues (C21, C251, C280, C321, C323, C334, C384, and C470) and six of them are located in the ECD. Mutagenesis studies showed that C280, C321, C323, and C334 residues in the ECD are necessary for preserving normal SR-B1 (HDL) binding activity, selective CE uptake, and/or cell surface expression. Interestingly, mutation of any of these four

cysteine residues to serine resulted in a robust induction of SR-B1 dimer formation, but, in contrast to normal SR-B1, these SR-B1 mutants lost their ability to mediate HDL-CE selective uptake. These results indicate that these cysteine residues are most likely essential for optimal HDL binding and selective CE uptake (73).

## ACTH Stimulation Regulates SR-B1 Interaction with Accessory Proteins

Adrenocorticotropic hormone treatment activates the cAMP–PKA signaling cascade, and we have recently shown that the expression of salt-inducible kinase 1 (SIK1), a serine/threonine kinase that belongs to the stress- and energy-sensing AMPK family of kinases, is also rapidly induced in Y1 adrenal cells in response to ACTH via the cAMP–PKA signaling cascade. Previously, it had been suggested that an increased level of SIK1 expression inhibits adrenal steroidogenesis by repressing the cAMP-dependent transcription of steroidogenic proteins, CYP11A1 and STAR, by attenuating CREB transcriptional activity (74). In contrast, we showed that SIK1 stimulates adrenal steroidogenesis by modulating the selective HDL-CE transport activity of SR-B1. Overexpression of SIK1 increases cAMP-stimulated and SR-B1-mediated selective HDL-BODIPY-CE uptake in cell lines without impacting SR-B1 protein levels, whereas knockdown of SIK1 attenuated cAMP-stimulated selective HDL-BODIPY-CE uptake. SIK1 forms a complex with SR-B1 by interacting with its cytoplasmic C-terminal domain, and *in vitro* kinase activity measurements indicate that SIK1 can phosphorylate the C-terminal domain of SR-B1. Among potential phosphorylation sites, SIK1-catalyzed phosphorylation of Ser496 is critical for SIK1 stimulation of the selective CE transport activity of SR-B1. Mutational studies further demonstrated that both the intact catalytic activity of SIK1 and its PKA-catalyzed phosphorylation are essential for SIK1 stimulation of SR-B1 activity. Finally, overexpression of SIK1 caused time-dependent increases in SR-B1-mediated and HDL-supported steroid production in Y1 cells; however, these effects were lost with knockdown of SR-B1. It should be noted that, as opposed to these stimulatory effects of SIK1 on SR-B1 function, we confirmed that SIK1 does suppress CREB activity, which could contribute to the inhibition of steroidogenesis under some conditions. Taken together, these studies establish a role for SIK1 in the positive regulation of selective HDL-CE transport function of SR-B1 and steroidogenesis and suggest a potential mechanism for SIK1 signaling in modulating SR-B1-mediated selective CE uptake and associated steroidogenesis.

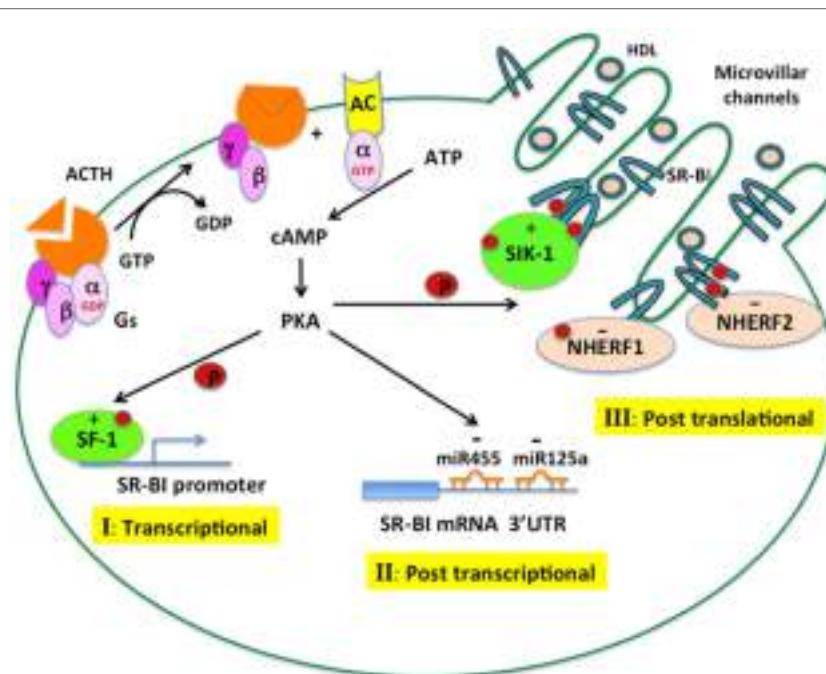
Many studies have also indicated that accessory proteins are crucial for the proper cellular expression of SR-B1 and SR-B1-mediated HDL-CE transport as well as other functions (75–83). For example, it has been shown that PDZK1/NHERF3 regulates hepatic SR-B1 stability and steady-state protein levels. Interestingly, PDZK1/NHERF3 is neither expressed nor essential for SR-B1 abundance or its cellular localization in steroidogenic cells of the adrenal gland, ovary, and testis (77). Recently, we have shown that two other NHERF family members, NHERF1 and NHERF2, negatively regulate the expression and function of SR-B1 in steroidogenic cells of the adrenal and gonads in response to ACTH (84). Specifically, we showed that ACTH

treatment decreases NHERF1 and NHERF2 protein levels in rat adrenals and increases SR-B1 function. Co-immunoprecipitation, colocalization, bimolecular fluorescence complementation, and mutational analysis all indicated that NHERF1 and NHERF2 form complexes with SR-B1 protein and, as a result, inhibit SR-B1-mediated selective CE transport and steroidogenesis. Moreover, we demonstrated that the structural components required for NHERF1/2 to interact with SR-B1 included an intact COOH-terminal PDZ recognition motif (EAKL) in SR-B1 as well as the PDZ1 or PDZ2 domain of NHERF1, the PDZ2 domain of NHERF2, or the MERM domains of NHERF1/2. The *de novo* synthesis of SR-B1 was also inhibited by both NHERF1 and NHERF2 (84). In contrast to NHERF1 and NHERF2, NHERF4 had no effect on selective HDL-CE uptake or steroidogenesis. Altogether, these experiments demonstrated that NHERF1 and NHERF2 bind SR-B1 and negatively regulate SR-B1 expression, selective CE transport, and steroidogenesis (84).

## ACTH AND REGULATION OF SR-B1 UNDER PATHOPHYSIOLOGICAL CONDITIONS

Since ACTH tightly regulates SR-B1 gene transcription, it should be expected that any pathophysiological conditions that affect ACTH levels would also impact SR-B1 gene expression in an identical

manner. However, there is the possibility that altered SR-B1 function may represent an adaptive response to cope with stressful conditions. Indeed, results from studies of mice that underwent chronic psychosocial stress exhibited an exaggerated adrenal corticosterone response along with elevated SR-B1 protein levels (85). On the other hand, since adrenal cholesterol uptake is required for the production of anti-inflammatory glucocorticoids, gene deletion of functional SR-B1 in adrenals results in impaired steroid synthesis (86). Other studies using SR-B1 knockout mice demonstrated that SR-B1 can protect mice against endotoxemia (87). There is an uncontrolled robust inflammatory cytokine response in the SR-B1 deficient animals, and they exhibit higher lethality when challenged with LPS-doses that induce endotoxic shock. Furthermore, these animals also exhibit a dysregulated adrenal glucocorticoid-mediated stress response to fasting. In addition, fasting-induced elevated levels of serum ACTH were the consequence of adrenal glucocorticoid insufficiency in SR-B1 knockout mice (86). Finally, when SR-B1 was only selectively deleted in adrenocortical cells in a tissue-specific manner, the animals had impaired rates of glucocorticoid secretion in response to stress, especially when they were subjected to an endotoxin challenge; these animals with SR-B1 ablated only in adrenocortical cells showed enhanced local and systematic inflammatory response, blunted activation of atrophy genes in skeletal muscle, and have a high incidence of mortality (88). In the setting of acute stress, where the release of corticosterone in



**FIGURE 3 | ACTH regulation of SR-B1 in the adrenal.** ACTH binds to its G protein-coupled receptor, leading to the activation of adenylate cyclase, which generates cAMP and activates cAMP-dependent protein kinase (PKA). The cAMP–PKA signaling cascade can regulate SR-B1 expression and function at different levels. (I) Transcriptional control: PKA increases the phosphorylation of transcription factors, such as SF1, leading to increased promoter activity of SR-B1. (II) Posttranscriptional control: PKA increases the expression of miRNA-125a and miRNA-455, which can bind to the 3' UTR of SR-B1 mRNA and negatively regulate SR-B1 expression. (III) Posttranslational control: PKA induces the oligomerization of SR-B1 and stimulates an interaction with SIK1, leading to phosphorylation of SR-B1, both events resulting in increased SR-B1 protein function. PKA can also increase the interaction of SR-B1 with NHERF1 and NHERF2, which negatively regulate SR-B1 function.

rodents or cortisol in humans peaks rapidly and declines quickly, it is likely that posttranslational regulation of SR-B1, such as protein phosphorylation and dimerization, could be more important in the regulation of steroidogenesis than transcriptional control. In contrast, under chronic stress conditions, mechanisms regulating SR-B1 at the transcriptional level and/or through miRNAs would appear to be more relevant.

Some of the adrenal steroid hormones, i.e., glucocorticoids, display robust daily variations in circulation under the circadian control by ACTH, and their rhythmic activity is considered to play important roles in whole body health and disease (89, 90). Ablation of one of the genes involved in circadian control, BMAL1, results in loss of circadian regulation of glucocorticoids. The BMAL1-deficient animals showed impaired response to ACTH regulation of adrenal function and downregulation of genes involved in cholesterol transport, such as StAR and LDLR, in adrenals (89). There is a well-accepted concept of the adrenal clock, with evidence of differential responses between males and females (91). While studies have shown a circadian variation in the expression of several genes involved in regulating the steroid production, no publications have specifically examined whether SR-B1 expression and/or function displays a circadian rhythm, but this is likely in view of the known regulation of SR-B1 by ACTH.

In summary, through binding to its G protein-coupled receptors, leading to the activation of adenylate cyclase, which generates cAMP and activates PKA, ACTH exerts tight regulation of SR-B1 function in the adrenal at three different levels. As illustrated in

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**Figure 3**, increased cAMP–PKA signaling can increase the phosphorylation of transcription factors, such as SF1, and stimulate promoter activity of SR-B1. ACTH can increase the expression of miRNA-125 and miRNA-455, which can bind to the 3' UTR of SR-B1 and result in suppressed expression and function of SR-B1 protein. Increased cAMP–PKA signaling can induce oligomerization of SR-B1 as well as stimulate phosphorylation of SIK1, which, in turn, increases the phosphorylation of SR-B1 and results in increased function of SR-B1 protein. Meanwhile, ACTH can also induce the expression of NHERF1 and NHERF2, both of which can bind to SR-B1 and negatively regulate SR-B1 function. Altered SR-B1 function may represent an adaptive response to cope with stressful conditions, as demonstrated by ablation of SR-B1 resulting in disturbed anti-inflammatory glucocorticoid homeostasis and impaired steroid synthesis.

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All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# Regulation of StAR by the N-terminal Domain and Coinduction of SIK1 and TIS11b/Znf36l1 in Single Cells

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The cholesterol transfer function of steroidogenic acute regulatory protein (StAR) is uniquely integrated into adrenal cells, with mRNA translation and protein kinase A (PKA) phosphorylation occurring at the mitochondrial outer membrane (OMM). The StAR C-terminal cholesterol-binding domain (CBD) initiates mitochondrial intermembrane contacts to rapidly direct cholesterol to Cyp11a1 in the inner membrane (IMM). The conserved StAR N-terminal regulatory domain (NTD) includes a leader sequence targeting the CBD to OMM complexes that initiate cholesterol transfer. Here, we show how the NTD functions to enhance CBD activity delivers more efficiently from StAR mRNA in adrenal cells, and then how two factors hormonally restrain this process. NTD processing at two conserved sequence sites is selectively affected by StAR PKA phosphorylation. The CBD functions as a receptor to stimulate the OMM/IMM contacts that mediate transfer. The NTD controls the transit time that integrates extramitochondrial StAR effects on cholesterol homeostasis with other mitochondrial functions, including ATP generation, inter-organelle fusion, and the major permeability transition pore in partnership with other OMM proteins. PKA also rapidly induces two additional StAR modulators: salt-inducible kinase 1 (SIK1) and Znf36l1/Tis11b. Induced SIK1 attenuates the activity of CRTC2, a key mediator of StAR transcription and splicing, but only as cAMP levels decline. TIS11b inhibits translation and directs the endonuclease-mediated removal of the 3.5-kb StAR mRNA. Removal of either of these functions individually enhances cAMP-mediated induction of StAR. High-resolution fluorescence *in situ* hybridization (HR-FISH) of StAR RNA reveals asymmetric transcription at the gene locus and slow RNA splicing that delays mRNA formation, potentially to synchronize with cholesterol import. Adrenal cells may retain slow transcription to integrate with intermembrane NTD activation. HR-FISH resolves individual 3.5-kb StAR mRNA molecules via dual hybridization at the 3'- and

**Abbreviations:** 3'EU, oligomer set targeting extended 3'UTR; AMG, aminoglutethimide; CBD, cholesterol-binding domain; CTD, C-terminal domain; CHX, cycloheximide; FISH, fluorescence *in situ* hybridization; HR-FISH, high-resolution fluorescence *in situ* hybridization; IMM, inner mitochondrial membrane; N-SIM, Nikon's structured illumination microscope; NTD, N-terminal domain; OMM, outer mitochondrial membrane; PKA, protein kinase A; p-RNA, primary RNA; sp-RNA, spliced primary RNA; SIK1, salt-inducible kinase 1; StAR, steroidogenic acute regulatory protein.

5'-ends and reveals an unexpectedly high frequency of 1:1 pairing with mitochondria marked by the matrix StAR protein. This pairing may be central to translation-coupled cholesterol transfer. Altogether, our results show that adrenal cells exhibit high-efficiency StAR activity that needs to integrate rapid cholesterol transfer with homeostasis and pulsatile hormonal stimulation. StAR NBD, the extended 3.5-kb mRNA, SIK1, and Tis11b play important roles.

**Keywords:** StAR, Sik1, Tis11b, fluorescence *in situ* hybridization, PCR

## INTRODUCTION

Steroidogenic acute regulatory protein (StAR) functions as a key determinant of steroidogenesis by transferring cholesterol from the outer mitochondrial membrane (OMM) to Cyp11a1 in the inner mitochondrial membrane (IMM) (1–4). Cyp11a1 metabolizes this cholesterol in the adrenal mitochondria very rapidly such that accumulation only occurs when constraints are placed on this turnover. The Cyp11a1 inhibitor aminoglutethimide (AMG) causes the accumulation of 3–5 cholesterol molecules per Cyp11a1 and increased cholesterol–Cyp11a1 complex formation (5). Turnover is driven by NADPH generated from the Krebs cycle (isocitrate dehydrogenase), but highest potency is achieved with succinate dehydrogenase linked to the ATP-dependent NADH/NADPH transhydrogenase (NNT) (6). CYP11a1 not only depends on the shuttling of ferredoxin between the flavoprotein reductase and CYP11a1 (7) but also competes with electron transfer to IMM Cyp11b1 (8).

The role of StAR has been definitively established through transgenic deletion of its gene in mice, which reproduces the pathology of human adrenal lipidemic hyperplasia (ALH) (9, 10). This role extends to testis Leydig cells and multiple cell types in the ovary. Mutations that cause the human disease are concentrated in the cholesterol-binding domain (CBD) rather than the N-terminal domain (NTD) (11). One mutation (R182) resolves cholesterol exchange activity to optimal levels when steroidogenic activity is deficient (12, 13). The NTD retains the net positive charge common to mitochondrial import sequences, but with appreciable helical content and dual cleavage sites that are atypical for mitochondrial target sequences. NTD modulatory activity is suggested by the involvement of the 30–62 sequences in the binding of StAR to VDAC2, which then facilitates both cholesterol transfer and NTD cleavage (14). Deletion of the NTD (N-47 mouse), while clearly establishing cholesterol transfer activity for the CBD alone, equally establishes a major modulatory role for the NTD that is tissue-dependent (15). StAR functions without the NTD to mediate linkage to lipid droplets (16, 17), including in a reconstituted system employing rat adrenal mitochondria (18).

Steroidogenic acute regulatory protein activity under hormonal control is mediated by phosphorylation at S-194 in the CBD, by cAMP and protein kinase A (PKA) in fasciculate cells, and by Ca-dependent kinases in glomerulosa cells (19, 20). StAR activity is inhibited by cholesterol sulfate such that cholesterol sulfatase can enhance activity (21). The large number of cholesterol molecules transferred per each molecule of transiting StAR implicates the controlled generation of OMM/IMM contacts by receptor-like

activity derived from the CBD (1). StAR, or STARD1, was the first member of a family that was identified based on the CBD sequence and structure. Forms D1 and D3 differ in their N-terminal targeting to mitochondria and to late endosomes, respectively; D4, D5, and D6 differ in their carrier specificity for cholesterol derivatives (22). The phosphatidylcholine exchange protein (STARD2) also functions at the mitochondria but with a partnering enzyme, Acot13 (23). Cholesterol transfer into the adrenal cortex mitochondria *in vivo* depends on continuous translation of the 37-kDa StAR pre-protein with concomitant phosphorylation by PKA. Inhibition with cycloheximide (CHX) halts adrenocorticotrophic hormone (ACTH)-stimulated steroidogenesis within 5 min, while accumulating cholesterol in the OMM remains inaccessible to IMM Cyp11a1 (24–26). This intermembrane cholesterol barrier in the adrenal mitochondria caused by CHX treatment is readily breached by mild mitochondrial disruption, including the elevation of  $\text{Ca}^{2+}$ . Such artificial transfer also removes succinate-initiated NNT support for this CHX-sensitive Cyp11a1 activity (6).

The NTD and the timing of the conserved IMM processing link StAR activity to other processes. PBR/TSPO associates with abundant VDAC1 to control OMM/IMM contacts at cholesterol-rich regions (27), including through the formation of a transmembrane complex (28). Participation of the mitochondrial permeability transition pore (MPFP) is a common feature of these processes. PAP7/ACBD3, which partners with TSPO, is a suppressor of SREBP forms, thus representing a link to cholesterol and fatty acid homeostasis (29). GTP enhances the *in vitro* mitochondrial uptake of cholesterol via GTPase activity in partnership with Ca (30). GTPases, such as Mfn2 and Opa1, which mediate the continuous dynamics of inter-mitochondrial fusion and fission, play pivotal roles in cAMP-stimulated cholesterol transfer (31). Mitochondrial fusion with the ER through sigma receptor sites has also been implicated (14). MAPK phosphorylation of StAR (S-232) stimulates StAR activity while slowing N-terminal cleavage (32).

In addition to NTD intervention, StAR activity is restricted at the mRNA level by the salt-inducible kinase (SIK1) (33) and the RNA-binding protein Znf361/Tis11b (34) which are each rapidly induced by cAMP. A critical feature of StAR mRNA is its alternative polyadenylation (1.6- and 3.5-kb StAR mRNA), which introduces an extra 2 kb of 3'UTR, facilitating additional regulation of mRNA stability and translation (34). Here, we have used the high-resolution fluorescence *in situ* hybridization method (HR-FISH) (35, 36) to image primary (p-RNA) and spliced StAR transcripts (sp-RNA) at the StAR gene loci in Y-1 and MA-10

cells (37, 38). Y-1 adrenal cells are distinguished by a proportion of basally active loci that produce cytoplasmic mRNA sufficient to mediate peak activation of steroidogenesis by PKA. Activation of individual cells by Br-cAMP increases StAR transcripts at the gene loci, where we have resolved primary and spliced transcripts before observing increased mRNA in the cytoplasm and specific positioning at the mitochondria.

The need for continuous translation places particular demands on the hormonal stimulation of transcription. StAR transcription depends on multiple promoter sites within 300 bp of the transcription start site, including SF1, CREB, GATA4, and AP1, which are further supplemented by newly synthesized C/EBP and NF4a1/Nurr77 (39, 40). CREB activity, which is essential, is activated by PKA-mediated phosphorylation at S133, which mediates synergistic recruitment of the histone acetyl transferase CBP and the coactivators of the CRTC/TORC family (38, 41–43). PKA deactivates the repressor kinases SIK1 and SIK2, which otherwise maintain CRTC2 in an inactive phosphorylated state. Merely inhibiting SIK is sufficient to stimulate the expression of StAR and most steroidogenic genes in Y-1 cells (33, 41). SIK1 is rapidly induced in adrenal cells *in vivo* and in cultured Y-1 cells by PKA at an early stage of increased StAR expression (41, 42). Importantly, PKA also inhibits activity through S577 phosphorylation, and thus, this increase does not impact StAR until PKA activity declines (38). Br-cAMP also rapidly stimulates Znf361/Tis11b, which binds to specific dual AU-rich elements in the extended 3'UTR. This homodimer complex recruits ribonucleases to selectively degrade the 3.5-kb StAR mRNA. Here, we show that removal of TIS11b enhances the potency of Br-cAMP as a stimulant of StAR expression. Another cAMP-sensitive protein, Akap1, may work with TIS11b to position StAR mRNA at the mitochondria *via* elements in the extended 3'UTR (44).

The work presented here provides evidence that mRNA generation, mitochondrial positioning, translation, and N-terminal targeting of new StAR proteins may be coordinated to match organizational steps involving cholesterol transfer across the mitochondrial membranes, consistent with an integrated CHX-sensitive mechanism.

## MATERIALS AND METHODS

### Cell Culture and DNA Vector Transfection

The MA-10 mouse Leydig tumor cell line (a gift of Dr. Mario Ascoli) was derived from the Leydig tumor M5480P (45). Y-1 mouse adrenocortical tumor cells were expanded from a subclone obtained from Dr. Bernard Schimmer (University of Toronto) with a lower passage number than those available from ATCC. Cells were grown according to previously described method (34). DNA vectors were transfected using TransIt-LT1 (Mirus Bio) and Lipofectamine 2000 (Invitrogen) according to the manufacturers' protocols.

### siRNA Transfection and shRNA

#### Cell Line Generation

ON-TARGETplus SMARTpool siRNA sequences against mouse *TIS11b*, along with non-target siRNA sequences, were purchased

from Dharmacon, Inc. (Lafayette, CO, USA). They were transiently transfected into MA-10 cells using the DharmaFECT 1 reagent according to the manufacturer's protocol. siRNA-transfected samples were prepared according to a previously described method (34). The GeneClip™ U1 Hairpin Cloning system was used to express short hairpin RNAs (shRNAs) in MA-10 and Y-1 cells. Stable cell lines were generated using a positive selection marker, neomycin. The U1 promoter was previously employed with success to knock down the target genes in these cell lines.

### Western Blot Analysis

Protein samples were prepared and assayed according to a previously described method (34). Protein bands were visualized using ECL reagent and Hyperfilm (Amersham Biosciences, Arlington Heights, IL, USA).

### Real-time RT-PCR

Primer design and cDNA synthesis were performed according to previously described methods (34, 37). q-PCR was performed using a BioRad CFX96TM Real-Time PCR Detection System. The q-PCR protocol was carried out as follows: initial denaturation at 95°C for 3 min, followed by 40 cycles of 10 s at 95°C, 30 s at 55°C, and 10 s at 95°C. Fluorescence signals were recorded at each extension step at 55°C. A melting curve analysis was then performed from 65 to 95°C at increments of 0.5°C for 5 s to assess amplicon specificity.

### Northern Blot Analysis

Total RNA was isolated using a QIAGEN RNeasy Mini kit per the manufacturer's instructions. Approximately 10 µg of total RNA was resolved by electrophoresis in a 1% (weight/volume) agarose-formaldehyde-formamide denaturing gel and transferred to a Hybond-N+ membrane (Amersham Biosciences) for approximately 16 h *via* the capillary method. RNA on the membrane was immobilized with a UV Stratalinker 1800 in auto mode (1900 J × 100 for 30 s). The membrane was then prehybridized at 65°C for 1 h in QuickHyb hybridization solution (Stratagene). Hybridization was performed at 65°C for 2 h in QuickHyb with a 0.9-kb mouse StAR cDNA probe that was radiolabeled with [ $\alpha$ -<sup>32</sup>P]dCTP (PerkinElmer, Norwalk, CT, USA; 3000 Ci/mmol) using a Ready-To-Go DNA-labeling kit (Amersham Biosciences). The membrane was exposed overnight and scanned using a phosphorimager (Molecular Dynamics, Sunnyvale, CA, USA) (34).

### High-Resolution Fluorescence *In Situ* Hybridization

The term HR-FISH has been employed to distinguish the use of a probe set consisting of 40 fluorescent 20mers. The RNA probe sets for StAR were generated using the Stellaris probe designer (<http://www.biostech.com/stellarisdesigner/>). Samples were prepared according to a previously described method (37). Freshly prepared p-RNA (Quasar-570, Biosearch Technology), sp-RNA (Quasar-570, Biosearch Technology), and 3" EU (Quasar-570, Biosearch Technology) probe sets and antibodies

for StAR and TIS11B were used. Clean coverslips were placed over the samples to prevent the hybridization solution [10% dextran sulfate (Sigma), 10% deionized formamide (Ambion), 2× SSC] from drying during the incubation. Samples were incubated in a dark humidified chamber at 37°C overnight. After a 30-min wash in wash buffer, samples were incubated in DAPI nuclear stain (wash buffer with 5 ng/ml DAPI) to counterstain the nuclei for 30 min. For combined FISH, an extra wash step was needed for the secondary antibody. Samples were processed according to a previously described method (37).

### Image Acquisition and Analysis

To detect and visualize p-RNA, sp-RNA, and mRNA, we used an Olympus wide-field fluorescence microscope (Model IX81) and the Nikon's structured illumination microscope (N-SIM) to obtain higher resolution images.

#### Olympus IX81

Images were captured by Olympus IX81 motorized inverted microscope equipped with a Hamamatsu Orca2 camera. Images were obtained with 0.2 μm spacing with the 100× oil objective and projected onto a single plane or 3D image. At least 30 Z-sections at 0.2 μm intervals were taken to cover the entire thickness of the cell. The exposure times ranged from 0.2 s (nuclear loci) to 4 s (cytoplasmic mRNA). The exposure time increases not only sensitivity approximately proportionately but also the background. Comparisons are made at the same exposure times. The target size is much smaller than the image due to the dispersion of the fluorescent light from the target. Loci have multiple clustered RNA molecules either p-RNA or sp-RNA. These terms have been used rather than pre-mRNA and mRNA to emphasize the absence of information on RNA processing. We term this fluorescent cluster the RNA locus, which elsewhere has been shown to overlap with the gene (DNA) locus. p-RNA and sp-RNA at the RNA are separate in the images. Captured images were deconvoluted with Slidebook 5.0 software (Intelligent Imaging Innovations, Inc.) for further analysis.

#### Nikon's Structured Illumination Microscope

Super-resolution imaging was performed with an N-SIM microscope system (Nikon) equipped with a SR Apo TIRF 100× 1.49 N.A. oil immersion objective and an iXon3 camera (Andor Technology). 3D-SIM image stacks were acquired with a Z-distance of 0.2 μm, covering the entire thickness of the cell (about 6 μm) using laser power setting 40%. The camera settings were configured as follows: format for capture, no binning; exposure time, 50–200 ms (one frame recommended); readout mode, EM gain 10 MHz 14-bit; gain multiplier, 20–200 ms (max 300); conversion gain. 5.1× 15 raw images per plane were acquired and computationally reconstructed using the reconstruction slice system from NIS-Elements software (Nikon).

### Data Analysis

Statistical significance was determined using Student's *t*-test or ANOVA; *P* < 0.05 was considered statistically significant;

\**P* < 0.05, \*\**P* < 0.01, and \*\*\**P* < 0.001. Data were analyzed using PRISM software (San Diego, CA, USA).

## RESULTS

### N-Terminal Modulation of StAR in Mouse Cell Lines

Steroidogenic acute regulatory protein activity is dependent on co-translational phosphorylation prior to N-terminal processing. This process leads to the inhibition of cholesterol metabolism within 5 min in primary rat adrenal cells or *in vivo* (1). In MA-10 cells, StAR is barely detectable until cells undergo 60 min of stimulation by Br-cAMP. To test whether PKA phosphorylation at S-194 affects this processing, we substantially elevated StAR protein levels *via* the transfection of StAR dUTR prior to the addition of Br-cAMP (Figure 1A). After 30 min of stimulation, extensive new active phosphorylation of p37 was evident, although less than half of this p37 was processed to p30, and scarcely any of the third products, p32, was detectable. In the absence of vector-derived StAR, appreciable amounts of p37, p32, and p30 were generated after 60 min of stimulation by Br-cAMP in proportions similar to those seen with the vector. Again, phosphorylation was appreciable for p30 but was scarcely detectable for p32.

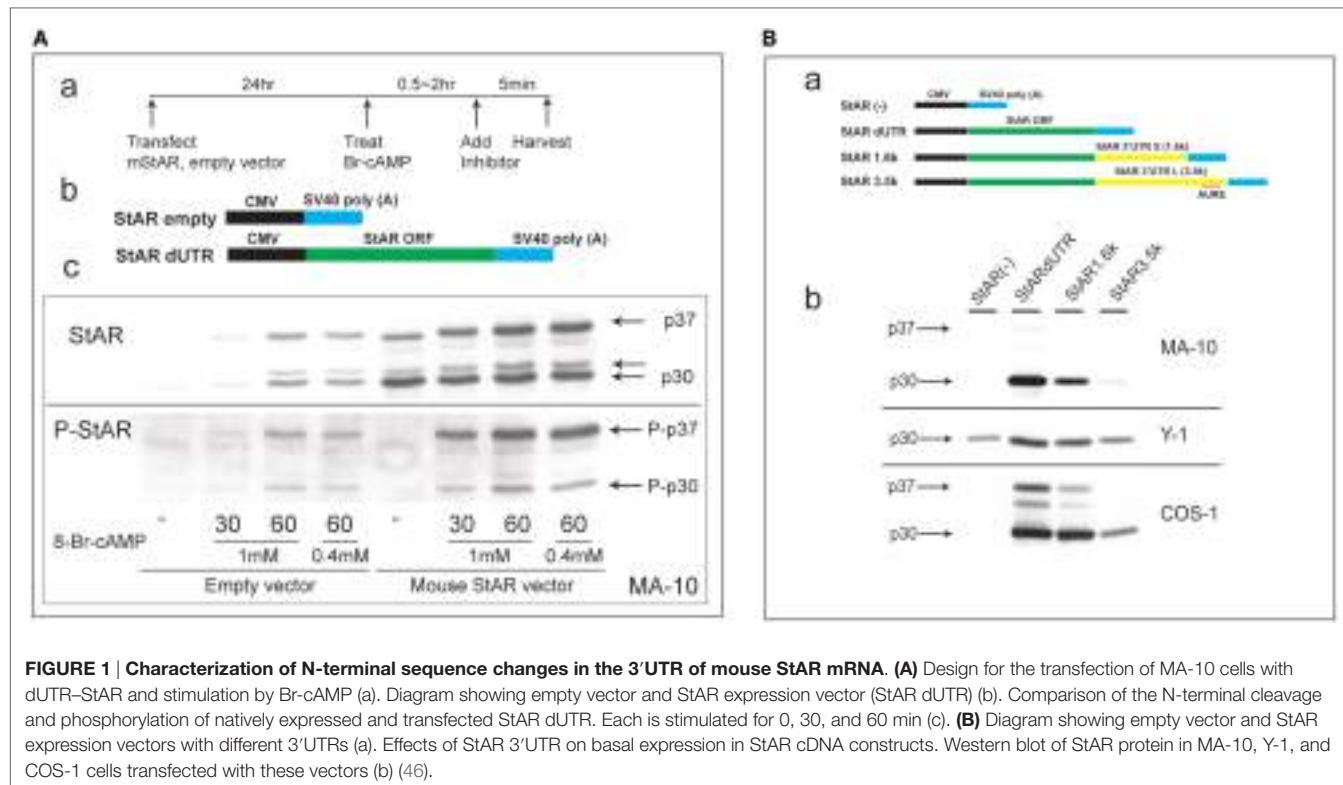
Evidently, transfected StAR is phosphorylated and cleaved at the mitochondria at very similar rates to those observed for endogenous StAR. Mature p30 and, in particular, p32 are phosphorylated at much lower levels, indicating that the PKA targeting of S-194 impacts these cleavage reactions. Phosphorylation occurs outside the mitochondria and therefore before predominant cleavage by the IMM proteases. Either S-194 phosphorylation enhances removal of the processed forms in the matrix or slows their formation.

This N-terminal processing is very consistent across species and adrenal cell lines. The commonly used COS-1 transfection model (monkey kidney line), however, demonstrates a substantial deficiency in the rate of cleavage compared to Y-1 and MA-10 cells (Figure 1B).

### N-Terminal Modulation of StAR in Primary Bovine Adrenal Cells

Bovine adrenal cortex tissue is the best source of information regarding human adrenal mechanisms. The electron transfer activity of purified Cyp11a1 and its partnering ferredoxin was established in bovine adrenal tissue (7). Cultured primary bovine adrenal cortex (BAC) cells, which predominantly express cortisol, are stimulated fourfold by ACTH (47). Inhibition of protein synthesis by CHX has no effect on mitochondrial cholesterol accumulation but prevents increases in both cholesterol-Cyp11a1 complex formation and metabolism (Figure 2A).

N-terminal domain sequences are highly conserved across species (Figure 2B), including the two major cleavage products. These have been characterized by mass spectrometry for bovine StAR and found to have masses of 27,503 and 25,906 Da (48). The major cleavage products in BAC cells match those observed in MA-10 cells and with vector expression in COS-1 cells.



Single and combinatorial mutation of the A (M30) and B (M28) sites in the bovine sequence reveals a complex additive cleavage process; however, mutation in COS-1 cells did not affect StAR activity (without PKA activation). Removal of site A prevents the formation of p30 but results in a concomitant increase in p35, indicating that this cleavage form is an intermediate during the generation of p30 (Figure 2C). Mutation of site B decreases p30 and removes p28 while revealing a major product with a mobility that is intermediate between p28 and p30 (p29). Mutation of B exerts no effect on p35. We envisage p29 as the major precursor to p28. Dual mutation confirms an additive response, further establishing the independence of the two pathways.

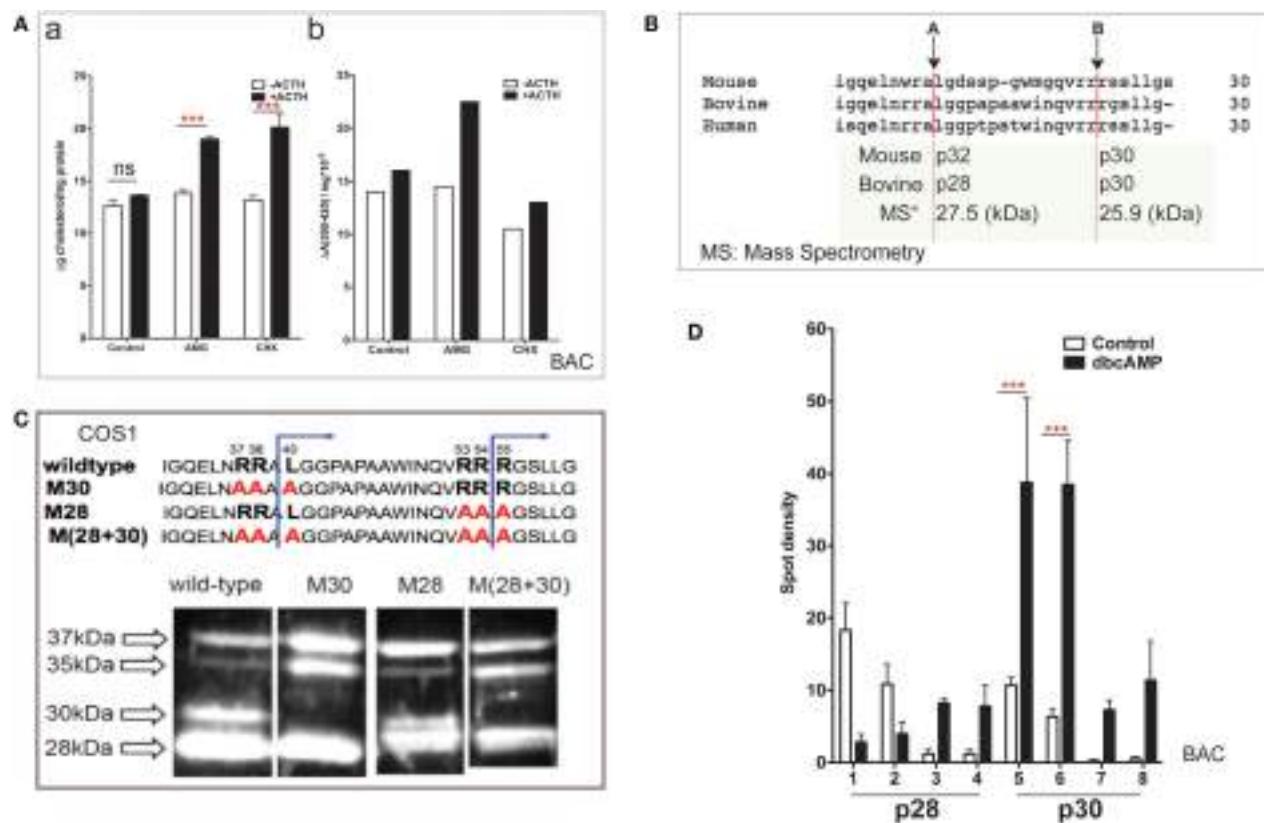
In primary BAC cells, p37 pro-StAR is cleaved into two products with different sizes, lower (28 kDa) and higher (30 kDa), each of which can be divided into four further species in 2D gels based on pI (20). In culture, PKA activation in either glomerulosa or fasciculate BAC appreciably increases the total amount of newly cleaved products within minutes. The low-pI p28 and p30 phosphorylated forms are increased by 5- to 10-fold (Figure 2D). In glomerular cells, the same pattern is observed after stimulation with angiotensin and K+, which both increase intracellular Ca and calmodulin kinase levels. Aldosterone synthesis responds proportionately (dibutyl cA > angiotensin > K+) (20). The close correlation between the appearance of N-terminal cleavage and PKA phosphorylation suggests that these intermediate NTD products contribute to cholesterol transfer. The dual p28 and p30 products resolved by pI indicate further posttranslational modification in addition to

phosphorylation at S-194. S-232 phosphorylation delivered by MAPK represents one possibility (32).

## Y-1 Cells Exhibit Higher Basal StAR Expression and an Acute Response That Is Limited to 15 min

Y-1 adrenal cells, like their primary counterparts and in contrast to MA-10 cells, possess sufficient basal mRNA to mediate maximum steroidogenesis within 15 min without a corresponding increase in mRNA. This basal expression has been observed utilizing a combination of q-PCR and HR-FISH microscopy, described in detail elsewhere (37). The locations for q-PCR target probes in the StAR primary transcript (p-RNA) and spliced transcripts (sp-RNA/mRNA) are shown in Figure 3A. Figure 3B indicates the presence of both p-RNA and sp-RNA/mRNA at a ratio of 1:4 under basal conditions. In both cell types, the ratio of q-PCR at early (E7-S) and late (E7-L) sites in the 3'UTR is close to 1:1, indicating complete transcription of the 3'UTR and formation of the 3.5-kb form at a far greater frequency than the 1.6-kb form (Figure 3C). The much higher p-RNA levels in Y-1 cells compared to MA-10 cells indicate active synthesis under basal conditions. Stimulation by Br-cAMP reveals a fourfold rise in both the early and late introns (1 and 6), but no increase in either exon 7 or spliced transcripts consistent with the approximately 30-min delay in elongation and splicing reported previously for Y-1 and MA-10 cells (37, 38).

We examined changes at the nuclear loci using HR-FISH. The HR-FISH probe locations on the StAR primary transcript



**FIGURE 2 | NTD cleavage in COS-1 cells parallels changes in primary bovine adrenal cells. (A)** Effects of aminoglutethimide (0.5 mM) and cycloheximide (0.2 mM) on cholesterol transfer in ACTH-stimulated cultured bovine adrenal cells (47) are shown in (a) mitochondrial cholesterol and (b) determination of cholesterol-CYP11A1 complex. Data are represented as mean  $\pm$  SEM, \*\*\* $P$  < 0.001. **(B)** Multispecies sequence comparison of StAR NTD that shares conserved MMP cleavage sites A and B. The typically used mouse and bovine identification of products is compared to the average M.W determined by mass spectrometry for bovine products. **(C)** Mutation of conserved cleavage sites (M28, M30) in the NTD of bovine StAR. The blue lines indicate the predicted cleavage sites for the production of the 30- and 28-kDa form of StAR. Expression of CMV bovine StAR vectors in COS-1 cells. Effects of mutations as observed by immunoblotting for StAR (48). **(D)** 2D gel determinations of NTD cleavage products in BAC cells (20). Decreases (p28, #1–2) and increases (p28, #3–4; p30, #5–6) of NTD cleavage products are shown in response to dibutyl cAMP. Data are represented as mean  $\pm$  SEM, \*\*\* $P$  < 0.001.

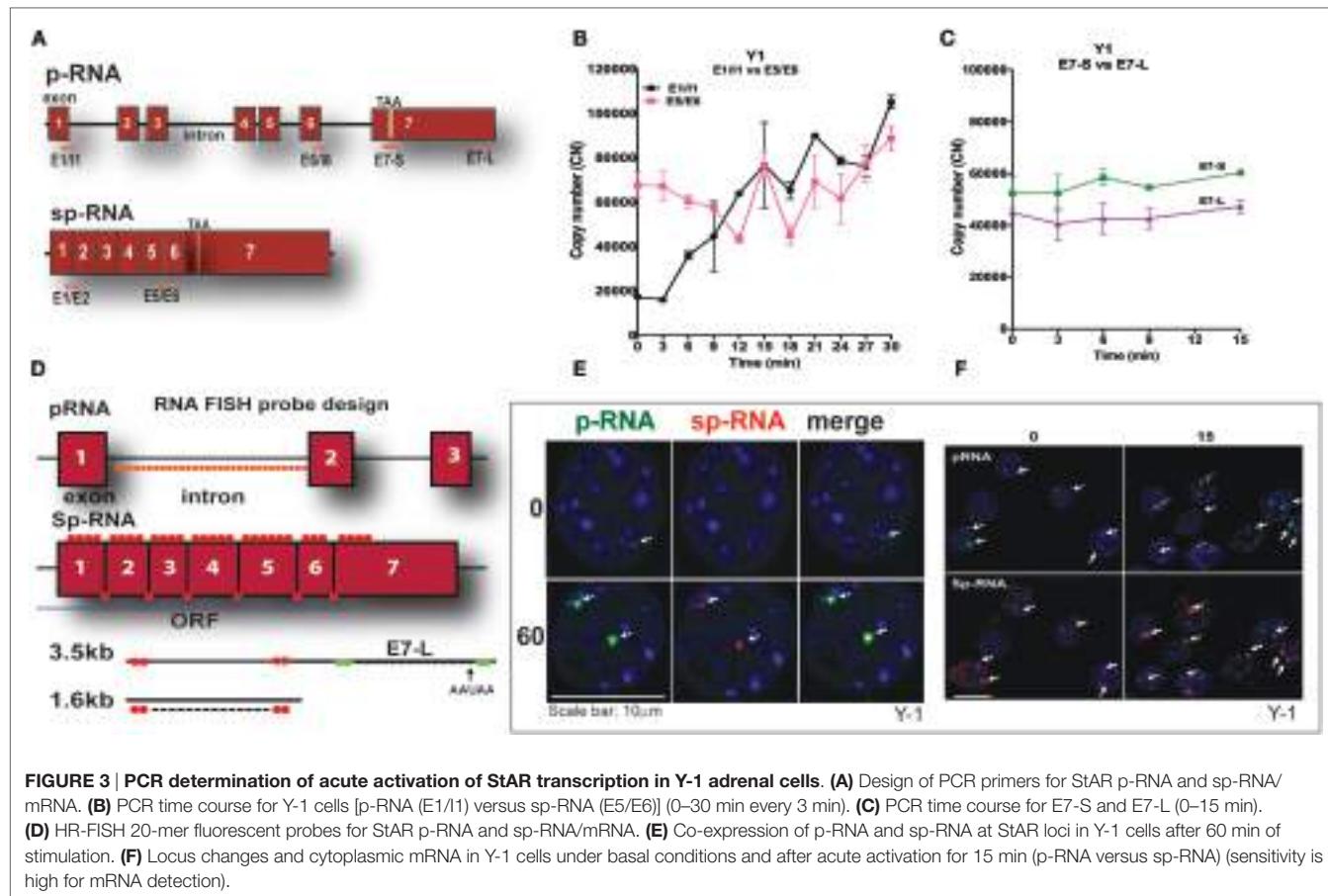
(p-RNA) and spliced form are shown in **Figure 3D**. The dual presence of p-RNA and sp-RNA at the two StAR loci in Y-1 cells after 60-min stimulation with Br-cAMP is shown in **Figure 3E**. Elsewhere, we have shown that spliced and unspliced transcripts can be resolved at these loci with N-SIM ultrahigh-resolution microscopy. Separation in MA-10 cells varies from 100 to 300 nm (38). **Figure 3F** shows that p-RNA and sp-RNA are detectable at low levels in association with the loci in most adrenal cells, although they are undetectable in MA-10 cells. Cytoplasmic mRNA is also appreciable, corresponding to higher levels than those observed in MA-10 cells, even after 60 min of stimulation. There are significant increases for both p-RNA and sp-RNA in approximately half of the loci after 15 min, but no increases in cytoplasmic mRNA.

High-resolution fluorescence *in situ* hybridization confirms the q-PCR basal expression of StAR. Thus, in contrast to MA-10 testis cells, HR-FISH imaging of Y-1 cells reveals p-RNA and sp-RNA at the loci, although at much lower levels than those observed after 60 min of stimulation. Variable levels of expression can be seen at the loci in each cell, representing

different numbers of transcripts. Some cells have only one active locus, and some have no active loci, corresponding to the asymmetric activation of StAR loci and the asynchronous responses of individual cells as previously reported for MA-10 cells (37, 38). Importantly, mRNA is visible in the cytoplasm under basal conditions with this greater experimental sensitivity. Following a 15-min stimulation by Br-cAMP that induces maximum steroidogenesis in Y-1 cells (1), the intensity of p-RNA expression at the loci increases. However, the increase in sp-RNA is modest, consistent with the q-PCR results (**Figure 3B**), indicating no increase in cytoplasmic mRNA, despite maximal steroidogenesis.

## Y-1 Cells Share Undergo Early Onset Slow Splicing and Delayed Rapid Splicing Phases

q-PCR analyses of Br-cAMP stimulation through 120 min (**Figure 4A**) indicate that both early and extended sites on the 3'UTR only increase after 30 min, although with comparable



copy numbers for the E5 and E6 spliced RNA. In **Figure 4B**, the images of StAR loci in multiple cells reveal that as stimulation progresses, more loci per cell become active for both p-RNA and sp-RNA, again mirroring the q-PCR results. The intensities of p-RNA and sp-RNA at the loci also increase as stimulation progresses. In **Figure 4C**, the images of typical nuclei indicate that levels of p-RNA and sp-RNA are not comparable until 60 min of stimulation. This is consistent with the delay in splicing identified from the q-PCR analyses.

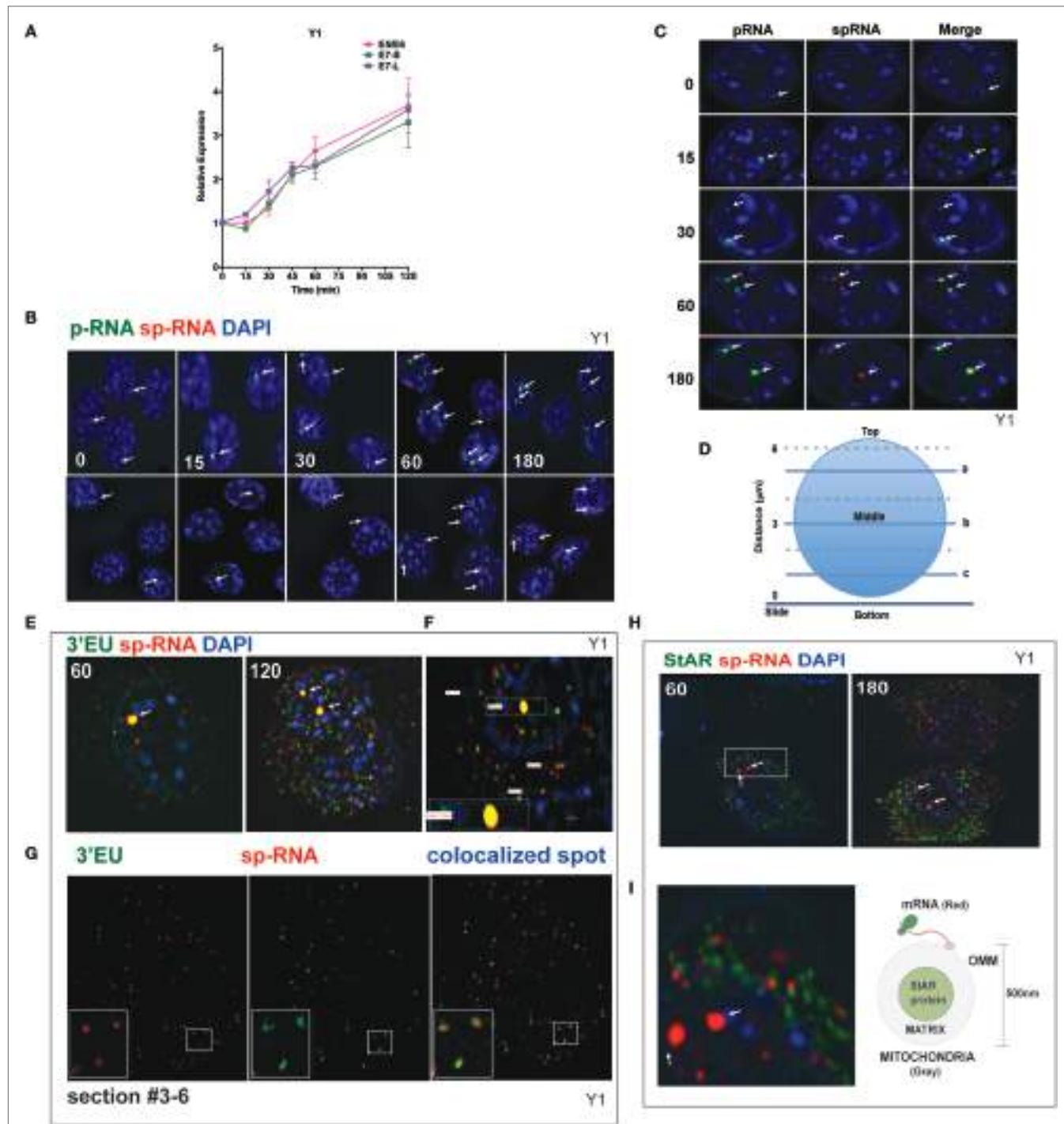
**Figure 4D** diagrams different optical slices taken at various cellular depths. The StAR loci are always positioned close to the nuclear midline, whereas the cytoplasmic mRNA is largely distributed at lower levels. To determine the proportions and distribution of the 1.6- and 3.5-kb StAR mRNA forms, we used both sp-RNA probes and an additional set, targeting an 800-bp sequence at the 3' end of the 3.5-kb mRNA UTR (3'EU probes). **Figure 4E** indicates that cytoplasmic 3.5-kb mRNA exhibits dual labeling. These probes extensively overlap at the loci, which appear strongly yellow. **Figure 4F** shows the measurement of the nucleus, locus, and individual messages. The length of the diameter of Y-1 cell is approximately 5–10  $\mu$ m. The length of a diffraction-limited mRNA dot is about 0.2  $\mu$ m. The juxtaposition of the two probes on the 3.5-kb mRNA is evident for approximately 60% of mRNA particles (**Figure 4G**). The size distribution is consistent with a single mRNA species (37). However, equal numbers of mRNAs

are singly hybridized either by sp-RNA or 3'EU. Single 3'EU hybridization should represent incomplete binding to the 3.5-kb mRNA, similar to single sp-RNA hybridization. Detection of the 1.6-kb mRNA form is evidenced by the enrichment of such binding in lower Z-slices.

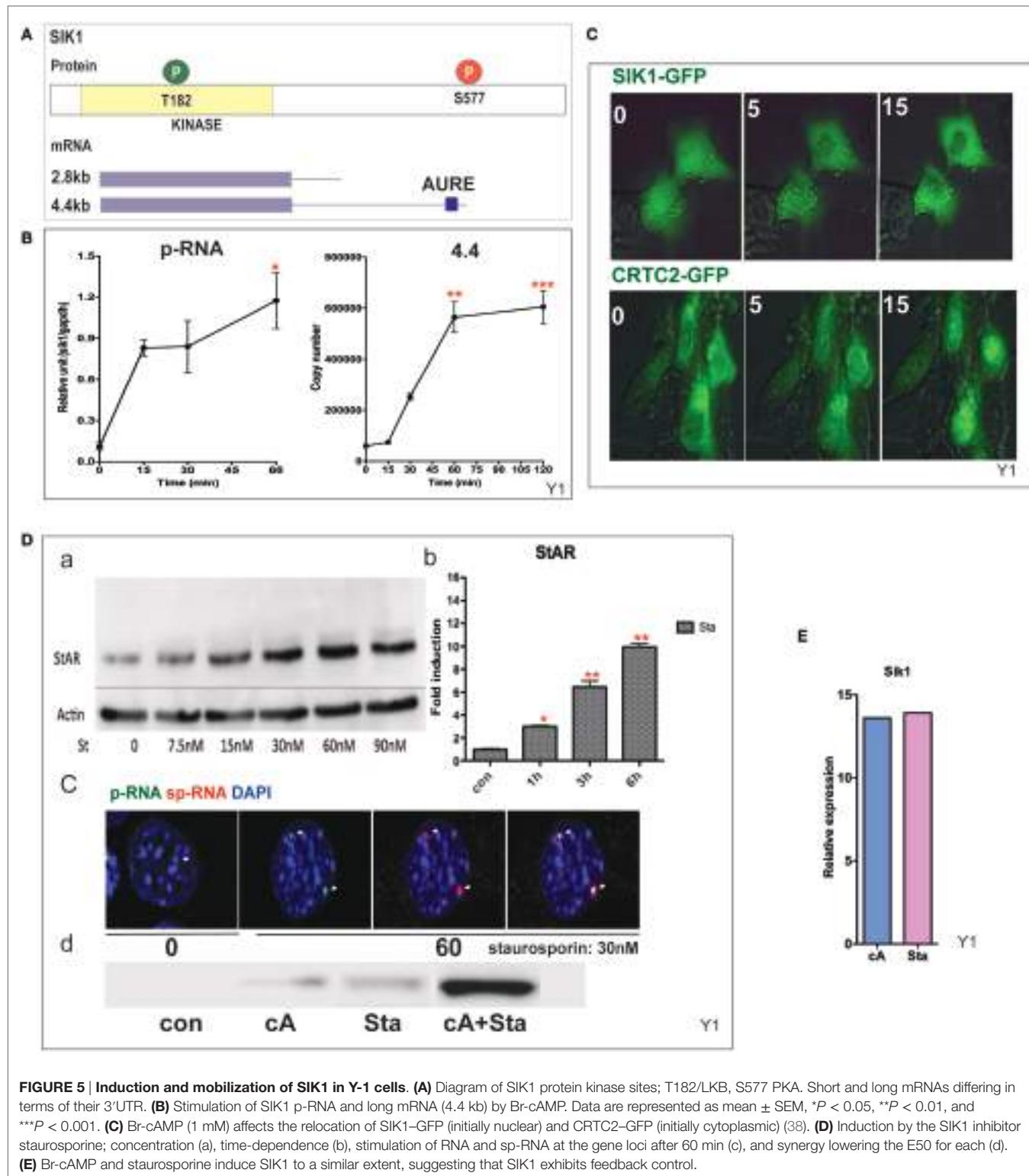
The mitochondria uniformly express the StAR protein in the inner matrix (38). Interestingly, after 60 min of stimulation, approximately half of StAR sp-RNA hybridization, which is predominantly represented by the 3.5-kb mRNA form, pairs with StAR proteins (**Figure 4H**). StAR mRNA is expected to position on polyribosomes attached to the OMM (**Figure 4I**). Adrenal mitochondria have a diameter of approximately 0.5  $\mu$ m (15).

### SIK1 Is Induced Rapidly via Expression of the 4.4-kb mRNA but Prior to the Increase in Protein Levels

CRTC2 activation, which is essential for StAR transcription, is suppressed by SIK1 and SIK2 in adrenal cells. Each shares a conserved mechanism of control, but SIK2 is predominantly constitutive and located in the cytoplasm, whereas SIK1 is highly inducible by PKA. SIK1 is regulated through dual phosphorylation at T182 by LKB1 in the catalytic domain and by PKA at SIK577 in the C-terminal region (43) (**Figure 5A**). After Br-cAMP stimulation, SIK1 p-RNA increases to a steady



**FIGURE 4 | Spatiotemporal regulation of StAR mRNA in Y-1 cells. (A)** Time course of 0–120 min for the stimulation of StAR RNA as determined by q-PCR to detect the 3'UTR (E7-S, E7-L) and translated sequence (E5/E6). **(B)** p-RNA and sp-RNA at StAR loci in multiple cells; sensitivity is sufficient to detect RNA concentrated at loci but not cytoplasmic mRNA. Lower expression of mRNA requires a higher sensitivity setting for the microscope. **(C)** Loci in representative nuclei (0–180 min). p-RNA, sp-RNA, and merged. **(D)** Nuclear positioning for Z-sections a, b, and c. The positioning of sp-RNA versus 3'EU HR-FISH probes. StAR loci are found near the nuclear midline; cytoplasmic StAR mRNA is primarily observed between slice b and the adherent plasma membrane (37). **(E)** Z-projections of HR-FISH for StAR RNA at high sensitivity (N-SIM microscope) after stimulation for 60 and 120 min (dual addition of 3'EU and sp-RNA). StAR loci (yellow, due to probe overlap), sp-RNA (red), and 3'EU (green) visualize cytoplasmic mRNA. DAPI (blue) visualizes nuclear DNA. **(F)** Measurements of the nucleus, locus, and individual message. Scale bar, 1  $\mu$ m. **(G)** N-SIM mRNA images after 120 min in the lower portion of the cell (slice c) with dual labeling by sp-RNA and 3'EU. Enlargement resolution of dual labeling with StAR mRNA and sp-RNA or 3'EU. **(H)** HR-FISH of StAR mRNA (sp-RNA) and immunochemistry of StAR proteins after 60 and 180 min of stimulation employing N-SIM microscopy. The StAR protein is present in the matrix of all Y-1 mitochondria (38). **(I)** Enlarged region after 60 min of stimulation showing the pairing of StAR mRNA and mitochondrial matrix-localized StAR proteins. Diagram of the spatial relationship between matrix proteins and OMM-associated StAR mRNA.



**FIGURE 5 | Induction and mobilization of SIK1 in Y-1 cells. (A)** Diagram of SIK1 protein kinase sites; T182/LKB, S577 PKA. Short and long mRNAs differing in terms of their 3'UTR. **(B)** Stimulation of SIK1 p-RNA and long mRNA (4.4 kb) by Br-cAMP. Data are represented as mean  $\pm$  SEM, \* $P$  < 0.05, \*\* $P$  < 0.01, and \*\*\* $P$  < 0.001. **(C)** Br-cAMP (1 mM) affects the relocation of SIK1-GFP (initially nuclear) and CRTC2-GFP (initially cytoplasmic) (38). **(D)** Induction by the SIK1 inhibitor staurosporine; concentration (a), time-dependence (b), stimulation of RNA and sp-RNA at the gene loci after 60 min (c), and synergy lowering the E50 for each (d). **(E)** Br-cAMP and staurosporine induce SIK1 to a similar extent, suggesting that SIK1 exhibits feedback control.

state within 15 min, while the 4.4-kb mRNA form that contains the extended 3'UTR exhibits a delay of 15 min but reaches a steady state after 60 min (**Figure 5B**). The q-PCR primers for the translated sequence, which quantify both mRNA forms, indicate that mRNA transcription responses follow the same time course

(not shown). This 4.4-kb form, like the 3.5-kb StAR mRNA form, contains AU-rich elements. The SIK2 form with high basal expression responds minimally to Br-cAMP [Ref. (38, 49), p. 502]. After the inhibition of SIK2 when PKA is activated, CRTC2 is dephosphorylated by cytoplasmic phosphatases and

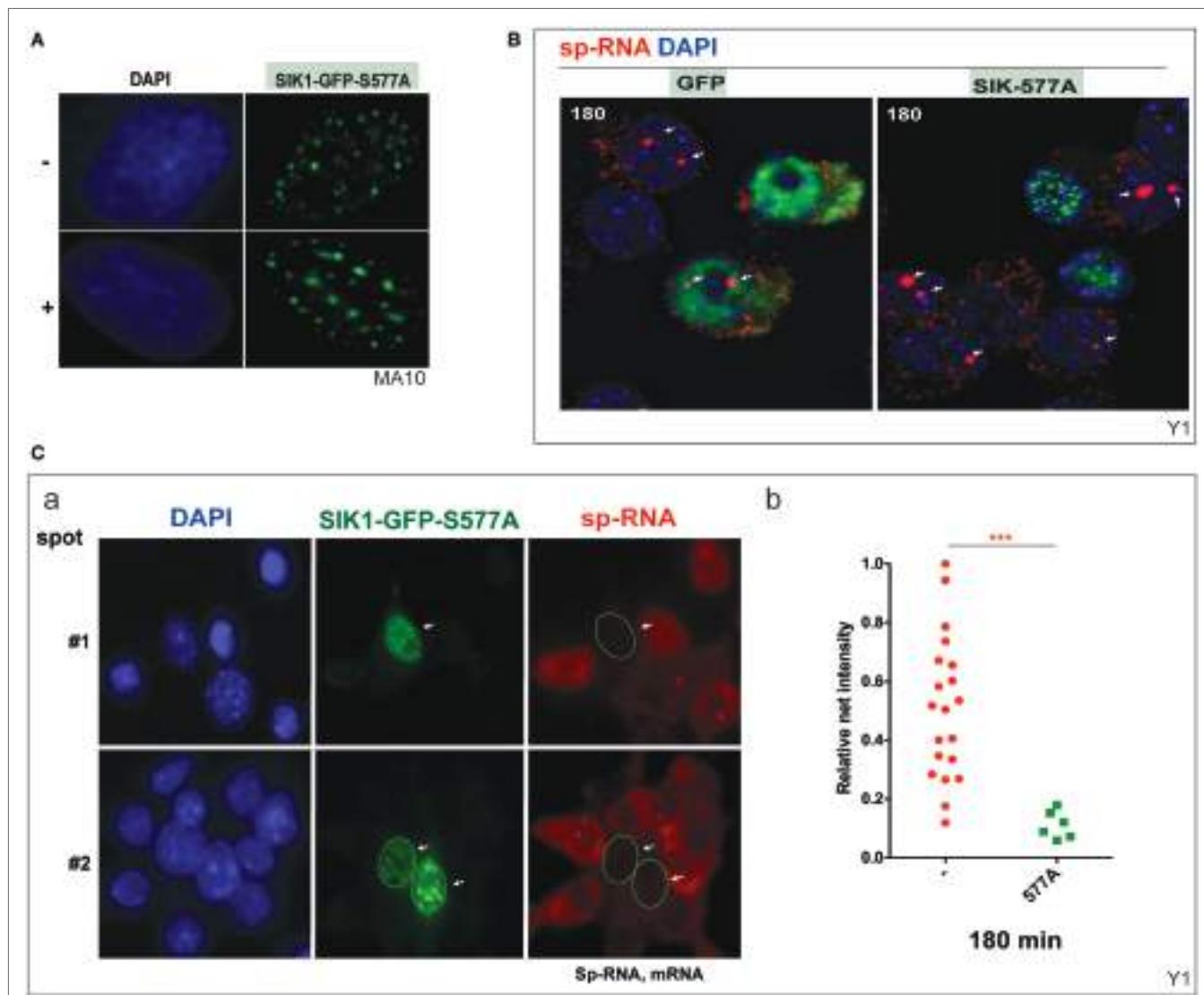
then migrates into the nucleus (38). This relocation is stimulated by ACTH within the same time frame *in vivo* (49). The two proteins complete their exchange within 15 min (**Figure 5C**). As noted earlier, some StAR loci require between 15 and 60 min for initiation. We found no evidence for significant asynchrony in CRTC2 or SIK1 transfer, indicating that the availability of CRTC2 and SIK1 is not a cause of asynchrony.

Staurosporine, although a general kinase inhibitor, potently inhibits SIK forms and induces StAR by 10-fold within 6 h (EC<sub>50</sub> = 15 nM). Br-cAMP and staurosporine demonstrate effective synergy, lowering the EC<sub>50</sub> for each (**Figure 5D**). Br-cAMP and staurosporine induce SIK1 to a similar extent, suggesting that SIK1 exhibits feedback control (**Figure 5E**). This inhibition of the SIK forms does not affect CREB phosphorylation. The slower

staurosporine induction of StAR transcription compared to Br-cAMP should reflect additional PKA contributions, including CREB S133 phosphorylation.

## SIK1 Completely Blocks StAR Transcription in Y-1 Cells When PKA Sites Are Blocked by Mutations

Mutation at SIK1-S577 prevents the inhibition of SIK1 and also induces the complete relocation of SIK1 to the nuclear speckles and prevents PKA-mediated export to the cytoplasm (**Figure 6A**). We have previously shown that nuclear SIK-S577A does not slow the initial transfer of CRTC2-GFP to the nucleus but eventually limits the extent of transfer. Surprisingly, CRTC2



**FIGURE 6 | Inhibition of basal and induced expression of StAR by PKA-resistant SIK1-S577A-GFP. (A)** Nuclear location of SIK1-S577A-GFP with and without Br-cAMP. **(B)** Suppression of Br-cAMP induction (180 min) of StAR RNA at the loci or in the cytoplasm in cells that transfected with SIK1-S577A-GFP versus GFP. **(C)** Quantitative expression in transfected Y-1 cells versus neighboring untransfected cells (a, image #1 and image #2). Associated mRNA intensity in 20 transfected cells versus 6 adjacent untransfected cells (b).

and SIK1-S577A co-localize in these nuclear speckles before CRTC2 is eventually phosphorylated by SIK1 and reverts to its cytoplasmic localization (38).

We show that the stimulation of Y-1 cells by Br-cAMP for 180 min is completely prevented in cells that express SIK1-S577-GFP, but not the control GFP vector (Figure 6B). StAR expression was undetectable in Y-1 cells expressing SIK1-S577A-GFP, but expression was normal in cells lacking the inhibitory vector or expressing the GFP control vector. We have quantitated the impact on acute expression and expression after 180 min of stimulation (Figure 6C).

### StAR Is Selectively Targeted by Znf36l1/Tis11b in the 3'UTR of the 3.5-kb mRNA

The Zn-finger protein Znf36l1/Tis11b binds as a dimer to the extended 3'UTR of the 3.5-kb form of StAR through a TATTATT sequence that forms a homodimer with Tis11b (Figure 7A). Based on this element, TIS11b targets a variety of regulatory proteins, including VEGF (50) (Figure 7B). This target sequence is conserved in the extended region of the StAR 3'UTR at equivalent positions surrounding the polyadenylation site for both mice and humans (Figure 7C). CMV-StAR expression vectors differing only in their 3'UTR sequence were co-expressed with a TIS11b expression vector in MA-10 cells. StAR protein levels are most extensively decreased with the full 3'UTR sequence. Similar results were obtained with Y-1 cells. Co-expression of TIS11b specifically decreases expression of the extended 3'UTR that contains the TIS11b recognition element. This loss of the StAR protein is matched by the loss of StAR mRNA (Figure 7D).

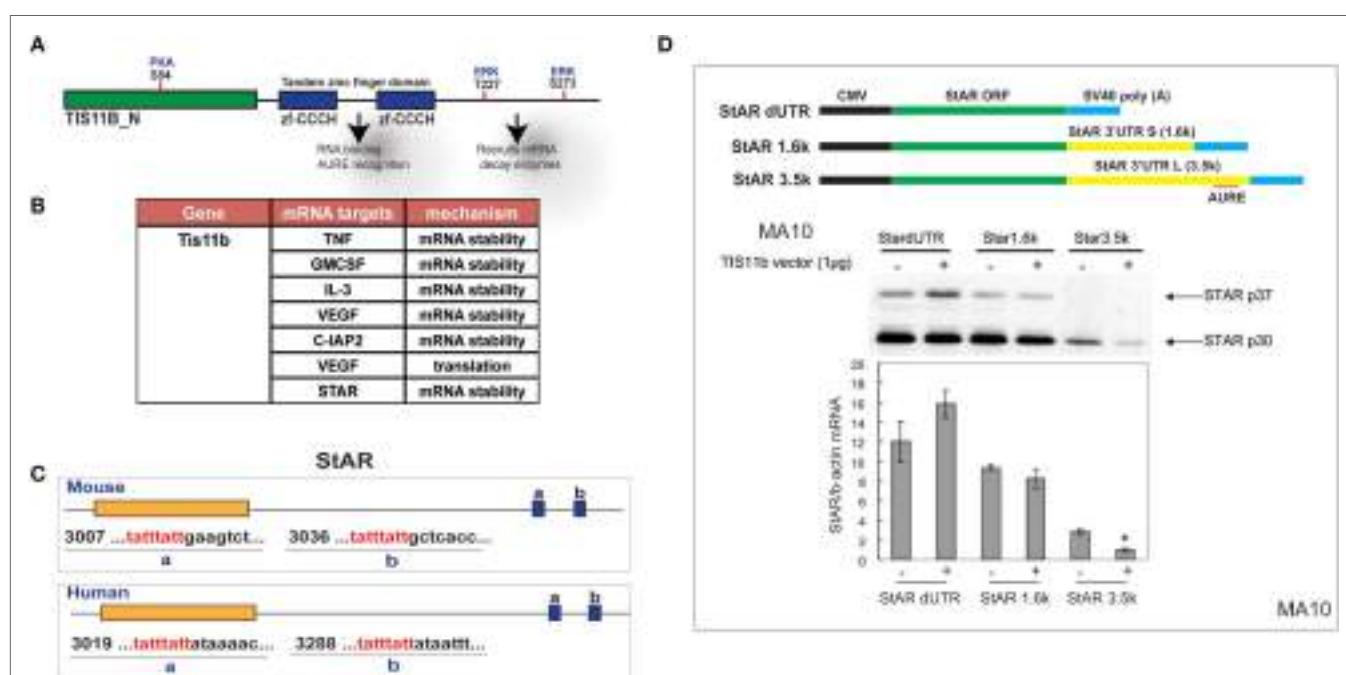
### StAR 3.5-kb mRNA Is Suppressed by the Expression of Tis11b in Y-1 and MA-10 Cells. Deletion of TIS11b via Long-term shRNA Expression

Tis11b is highly induced by Br-cAMP in MA-10 and Y-1 cells (Figure 8A). We tested the effects of TIS11b on StAR expression in MA-10 and Y-1 cells via the transient introduction of siRNA and shRNA and the generation of cell lines expressing a CMV-promoted shRNA vector. Tis11b levels were reduced by the transfection of siRNA, 24 h prior to the addition of Br-cAMP (Figure 8B). The near complete suppression of TIS11b selectively increases StAR 3.5-kb mRNA levels by approximately 50% without altering 1.6-kb mRNA levels (Figure 8C).

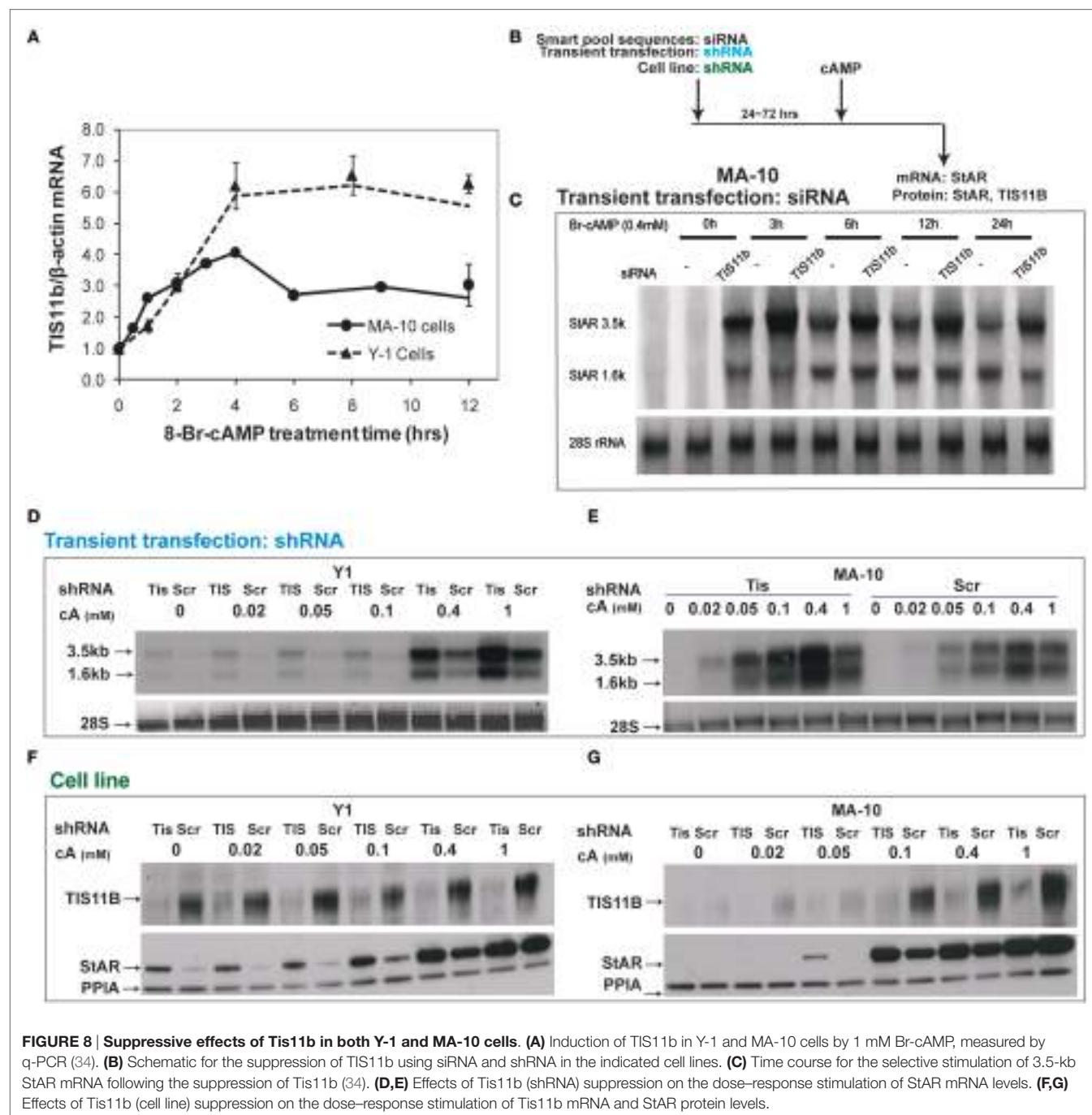
Tis11b was equivalently suppressed in Y-1 and MA-10 cells following the permanent expression of shRNA (versus scrambled shRNA) targeting the Tis11b sequence. This process differs from direct siRNA in that it requires significant cell amplification following the selection process. Passage number affects the PKA response characteristics. TIS11b suppression equally affected both the 3.5- and 1.6-kb forms at all doses of Br-cAMP in both Y-1 and MA-10 cells (Figures 8D,E). The effects of Tis11b suppression correspond to a 5- to 10-fold increase in the EC50 for Br-cAMP in each line (Figures 8F,G).

### Nuclear versus Cytoplasmic Distribution of TIS11b

Separation of the nuclei and cytoplasm in each cell line revealed that appreciable amounts of TIS11b were present



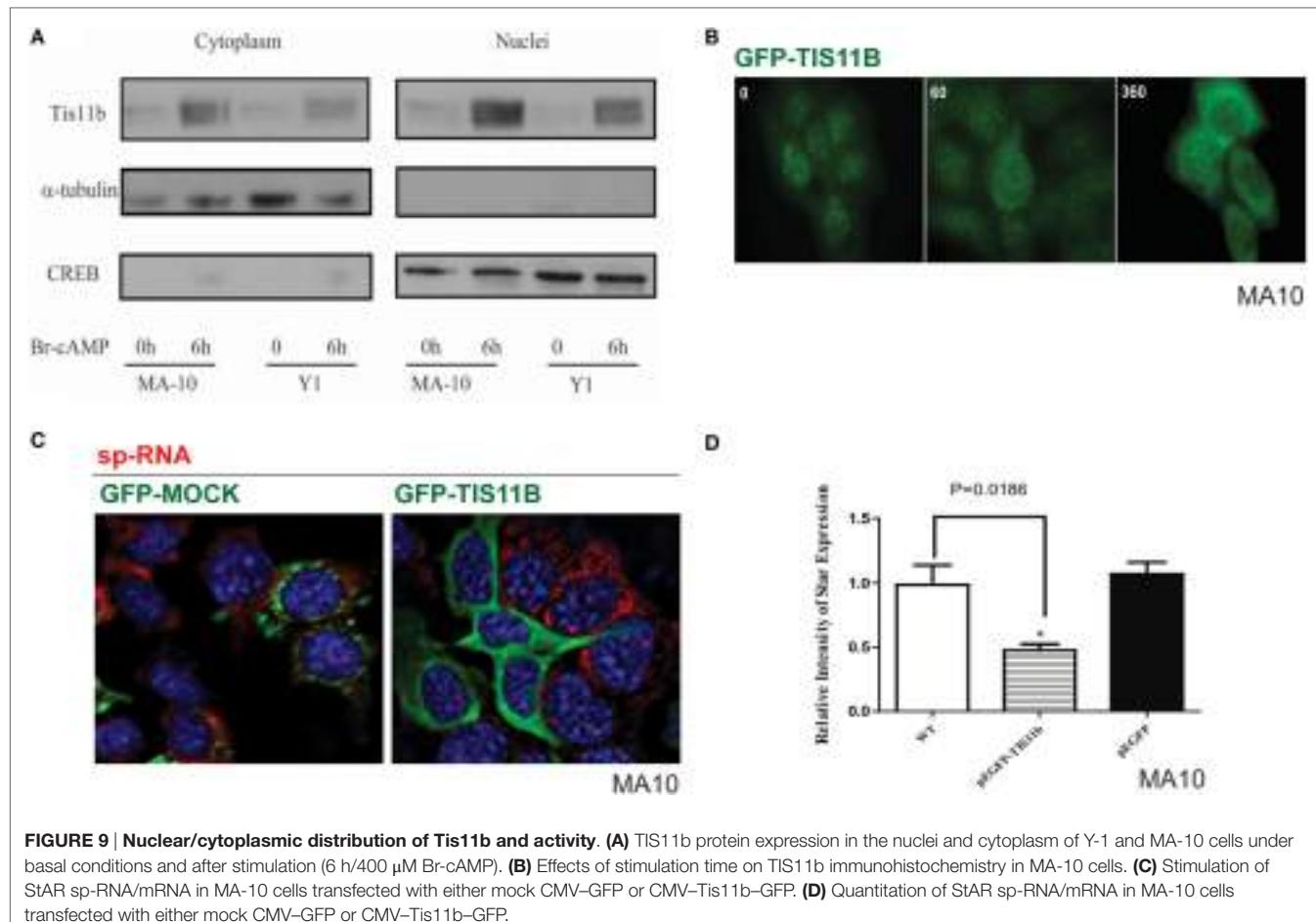
**FIGURE 7 | Selective targeting of extended StAR 3'UTR by transfected TIS11b/Znf36l1.** (A) The structure of TIS11b/Znf36l1 with RNA binding Zn-finger domains and kinase sites. (B) Other genes exhibiting RNA target sites (TATTATT) (51). (C) Tis11b targeted sequences are conserved in mouse and human StAR 3'UTRs, specifically the terminal cleavage/polyadenylation site. (D) The impact of TIS11b on StAR mRNA and protein expression according to terms of the 3'UTR length: CMV-StAR vectors; StAR protein expression (immunoblot) and mRNA (q-PCR relative to actin) with and without Tis11b transfection (34).



**FIGURE 8 | Suppressive effects of Tis11b in both Y-1 and MA-10 cells. (A)** Induction of TIS11b in Y-1 and MA-10 cells by 1 mM Br-cAMP, measured by q-PCR (34). **(B)** Schematic for the suppression of TIS11b using siRNA and shRNA in the indicated cell lines. **(C)** Time course for the selective stimulation of 3.5-kb StAR mRNA following the suppression of Tis11b (34). **(D,E)** Effects of Tis11b (shRNA) suppression on the dose-response stimulation of StAR mRNA levels. **(F,G)** Effects of Tis11b (cell line) suppression on the dose-response stimulation of Tis11b mRNA and StAR protein levels.

in both fractions and that expression in both the nuclear and cytoplasmic fractions was increased by the application of Br-cAMP (Figure 9A). Examination of MA-10 cells by immunohistochemistry showed that TIS11b was predominantly present in nuclear speckles under basal conditions but then increased in the cytoplasm following stimulation by Br-cAMP with a half-life of approximately 60 min and peak cytoplasmic levels after 3–6 h. We also created a TIS11b-GFP fusion protein. Unlike native Tis11b, the fusion protein does not exhibit net transfer into the nucleus, even under basal

conditions (not shown) (Figure 9B). Analysis of StAR sp-RNA in the nuclei and cytoplasm of cells expressing Tis11b-GFP indicated suppression at both the StAR loci and in the cytoplasm. Expression is maintained in cells expressing the GFP control (Figure 9C). Figure 9D illustrates the quantitation of mRNA in cells that individually express either or neither of the GFP vectors. The efficacy of Tis11b-GFP is evident. Finally, we show the modulation of StAR control of cholesterol availability via the N-terminal StAR regulatory domain, SIK1, and TIS11b (Figure 10).



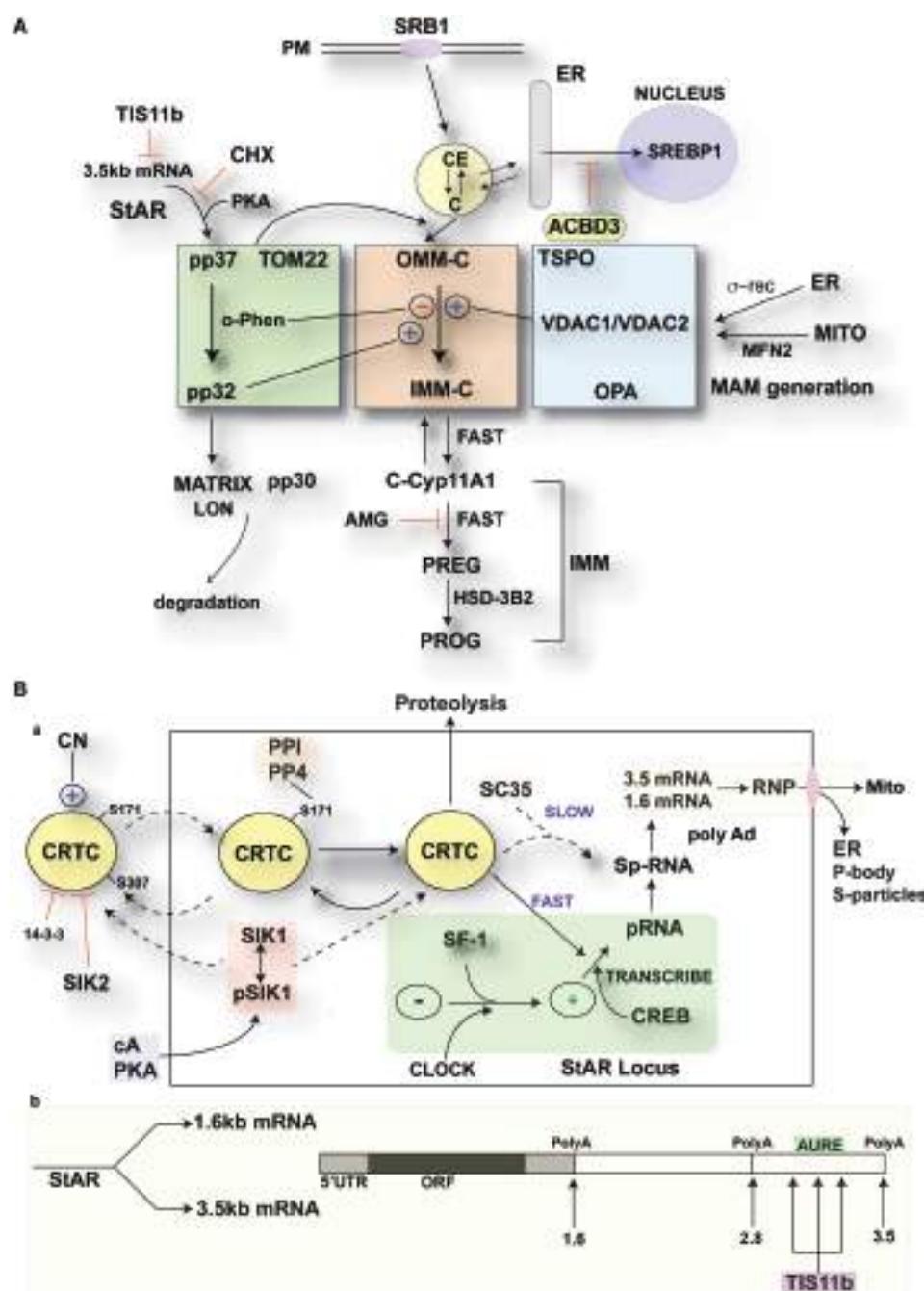
**FIGURE 9 | Nuclear/cytoplasmic distribution of Tis11b and activity. (A)** Tis11b protein expression in the nuclei and cytoplasm of Y-1 and MA-10 cells under basal conditions and after stimulation (6 h/400 μM Br-cAMP). **(B)** Effects of stimulation time on Tis11b immunohistochemistry in MA-10 cells. **(C)** Stimulation of StAR sp-RNA/mRNA in MA-10 cells transfected with either mock CMV-GFP or CMV-Tis11b-GFP. **(D)** Quantitation of StAR sp-RNA/mRNA in MA-10 cells transfected with either mock CMV-GFP or CMV-Tis11b-GFP.

## DISCUSSION

Previous work to elucidate the mechanism of mitochondrial cholesterol transfer has largely focused on the COS-1 model, in which StAR CBD is transfected with a functioning mitochondrial Cyp11a1 system (11, 52). In this model, the StAR CBD is sufficient to obtain maximum cholesterol transfer, including when this domain anchored to the OMM. NT sequences that result in rapid CBD import attenuate activity, consistent with primary CBD activity on the OMM and low activity derived from transit to the IMM. However, these definitive findings raise questions concerning the much more potent activity observed in adrenal cells. In COS-1 cells, p37 StAR import and processing are similar to Y-1 cells (Figure 1B) but are far slower. Therefore, p37 is resident for longer periods of time at the OMM, shifting greater weight onto the OMM steps. Even in MA-10 cells, CYP11a1 levels are modest compared to primary adrenal cells, thus demanding less of the StAR transfer process. In Y-1 cells, more rapid StAR import delivers results in much more rapid cholesterol transfer with very low levels of StAR expression, including a notably lower proportion of OMM p37. This paper addresses the role of the NTD in increasing the potency of StAR and decreasing the inadvertent side effects of cytoplasmic StAR (53). The rapid removal of StAR and glucocorticoids is a component of this NTP

activity, which is an important feature of ACTH physiology (54). Here, we provide an update regarding two acute cAMP-mediated responses in adrenal cells that antagonize the StAR mechanism.

Under physiological conditions, ACTH stimulates cholesterol transfer activity in adrenal cells, not only at low StAR mRNA levels but also through a coordinated translation/phosphorylation mechanism (47). CHX inhibition results in cholesterol reaching the OMM, but with a distribution that provides minimal access to IMM Cyp11a1 (Figure 2A). This initial dependence on StAR CBD activity in the OMM suggests that a first step involves the redistribution of cholesterol to sites where transfer occurs to the IMM, albeit at a suboptimal rate for adrenal cells. Inter-mitochondrial membrane and ER membrane fusion processes appear to be important for this process (31, 52) as well as proteins, such as TSPO, the sigma receptor, and various VDAC forms (28). A relationship with the IMM Ca-sensitive permeability complex, which is regulated by TSPO/VDAC1, appears likely (27). Membrane cross-linking experiments have identified various intermembrane complexes (28), although typically under conditions where mitochondrial integrity has not been a point of focus. Here, we note the importance of this integrity because contacts and cholesterol transfer readily occur even when perturbations result in the retention of Krebs cycle activity. We have previously suggested that low levels of succinate depend on



**FIGURE 10 | Modulation of StAR control of cholesterol availability via the N-terminal StAR regulatory domain, SIK1, and TIS11b.** (A) Modulation of the cholesterol transfer activity of the C-terminal StAR domain by the N-terminal regulatory domain. Three functional compartments are distinguishable: green: StAR transfer from OMM cholesterol-binding activity and transfer of pp37 from the OMM to the IMM, where proteolytic processing occurs with possible supplemental activity in adrenal cells. Brown: cholesterol transfer at sites of membrane contact, possibly directed by mPTP modulation. Blue: factors modulating the partnership between StAR and cholesterol, including VDACs, TSPO, GTPases (OPA1, MFN2), and the activation effects of membrane fusion (ER, inter-into). (B) (a) schematic detailing the activities of SIK1 and CRTC2 in mediating the effects of cAMP and PKA on StAR mRNA expression in comparison to Tis11b and (b) modulation of StAR mRNA availability to the mitochondria by SIK1/CRTC2 (transcription and splicing) and Znf361/Tis11b (regulation of 3.5-kb StAR mRNA location/3'UTR processing).

mitochondrial integrity and ATP generation to support NADPH generation by NNT (8).

The deletion of 47 amino acids from the StAR NTD largely restores corticosterone synthesis to StAR-ko mice, but only with a

compensating rise in cytoplasmic cholesterol. This increase alone should enhance mitochondrial uptake. In contrast, N-47 StAR is completely ineffective in Leydig cells where no such compensation occurs (15). The presence of cytoplasmic StAR that exerts

effective cholesterol transfer activity (55) redirects cholesterol to other sites and activities (53). The C-terminal domain (CTD) alone works with sufficient ACTH to overstimulate SR-B1/HSL to activate homeostasis directed by the SREBP and LXR forms (56).

The HR-FISH approach presented here shows individual StAR 3.5-kb mRNA molecules paired with single mitochondria (**Figure 4I**). StAR-mediated cholesterol transfer into adrenal mitochondria is a co-translational process as evidenced by the rapid inhibition induced by CHX. Close access of StAR mRNA to the mitochondria is necessary. StAR mRNA 5'-3' dual hybridization, size analyses, and counts per cell in terms of q-PCR copy numbers indicate that nearly all StAR mRNA was imaged in the 3.5-kb form. NTD, targeting the import process, determines the key spatiotemporal aspects of this process. Rigorous MS analyses of the intermediate StAR forms revealed two conserved cleavage sites. Metalloprotease cleavage enzymes (MMPs) on the inside of the IMM generate intermembrane StAR with either BAC or rodent sequences. We propose that the first cleavage (A) is an activation step (p30/p32 likely refers to same cleavage site), while the second cleavage inactivates and perhaps moves the StAR core to LON-directed clearance sites in the matrix (53). Site A cleavage exposes a hydrophobic sequence in an intermediate form that has been recognized as being at least dually modified in 2D gels (1, 32). Interaction of the p30/p32 forms with VDAC2 has been linked to the cholesterol transfer process (14). Potent inhibition of this MMP cleavage in Y-1 cells by o-phenanthroline selectively intervenes in this process without affecting mitochondrial Cyp11a1 activity (1).

High-resolution fluorescence *in situ* hybridization analyses of StAR expression at the gene loci established that slow splicing of StAR p-RNA attenuates mRNA during acute stimulation and ultradian oscillations. Prolonged effects of stress or diurnal changes are necessary to increase StAR mRNA.

cAMP also rapidly induces SIK1, which decreases the transcription of StAR. The ready entry of SIK1 into the nucleus and the susceptibility of CRTC2 to direct inhibition there through S-171 phosphorylation represent a rapid mechanism to minimize StAR p-RNA accumulation (41, 57). SIK1 induction through a 4.4-kb labile mRNA that is induced by CREB/CRTC2 activation also introduces rapid feedback inhibition. SIK1 becomes active as cAMP and PKA activity decline, thus releasing the inhibitory dephosph-S577 form. SIK inhibition alone is nearly as effective as cAMP in producing both transcription and splicing at the StAR loci (**Figure 5D**). We expect that the high levels of SIK1 during the rapid cAMP decline after the application of pulsatile stimuli will contribute to the non-genomic decline of StAR p-RNA (54).

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TIS11b exclusively targets the terminal segment of the 3.5-kb mRNA through the formation of homodimers at conserved AU-rich sites in the extended 3'UTR. This control mechanism, likely targeting the mitochondria (44), provide an explanation for the near-exclusive initial formation of the 3.5-kb mRNA, although this form is less stable, and the 1.6-kb form is an equivalent source of protein (46). Tis11b may target nuclear StAR p-RNA and 3.5-kb mRNA prior to conversion to the 1.6-kb mRNA form. Interestingly, Tis11b mRNA is also expressed with a long 3'UTR that contains the homodimer recognition element, thus allowing self-regulation. Tis11b, the SIK forms, and CRTC2 can function coordinately through the shared and competitive binding of their phosphorylated forms to cytoplasmic 14–3–3.

## Highlights for StAR Regulation in Adrenal Cells

Adrenocorticotrophic hormone stimulation of cholesterol metabolism requires continuous translation and phosphorylation of p37 StAR. The CBD generates substantial activity through the redistribution of cholesterol at the OMM aided by other OMM proteins, including TSPO and VDAC1. Peak glucocorticoid formation is achieved with low basal levels of the 3.5-kb mRNA form, which individually pairs with single mitochondria. Cholesterol transfer may be enhanced by conserved site A NTD cleavage by IMM metalloproteases and terminated by site B cleavage. StAR 3.5-kb mRNA formation is slowed by a pause in elongation, slow splicing, and the intervention of TIS11b through sites at the end of the 3'UTR. Dephosphorylated CRTC assumes control of transcription and splicing at StAR gene loci, where p-RNA and sp-RNA have been spatially separated by HR-FISH. The inhibition of SIK forms alone replicates the PKA activation of StAR expression in adrenal cells. cAMP-mediated induction of SIK1 by ACTH attenuates StAR through migration into nuclear sites shared with CRTC2, once ACTH levels and cAMP decline during physiological pulses.

## AUTHOR CONTRIBUTIONS

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# Adrenal Mitochondria and Steroidogenesis: From Individual Proteins to Functional Protein Assemblies

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The adrenal cortex is critical for physiological function as the central site of glucocorticoid and mineralocorticoid synthesis. It possesses a great degree of specialized compartmentalization at multiple hierarchical levels, ranging from the tissue down to the molecular levels. In this paper, we discuss this functionalization, beginning with the tissue zonation of the adrenal cortex and how this impacts steroidogenic output. We then discuss the cellular biology of steroidogenesis, placing special emphasis on the mitochondria. Mitochondria are classically known as the “powerhouses of the cell” for their central role in respiratory adenosine triphosphate synthesis, and attention is given to mitochondrial electron transport, in both the context of mitochondrial respiration and mitochondrial steroid metabolism. Building on work demonstrating functional assembly of large protein complexes in respiration, we further review research demonstrating a role for multimeric protein complexes in mitochondrial cholesterol transport, steroidogenesis, and mitochondria–endoplasmic reticulum contact. We aim to highlight with this review the shift in steroidogenic cell biology from a focus on the actions of individual proteins in isolation to the actions of protein assemblies working together to execute cellular functions.

**Keywords:** cholesterol transport, translocator protein, steroidogenic acute regulatory protein, voltage-dependent anion channels, cytochrome P450 enzyme system, mitochondria, endoplasmic reticulum

## INTRODUCTION

The cortex of the adrenal is the principal site of synthesis of vertebrate glucocorticoid and mineralocorticoid steroid hormones (1). These hormones are two of the five classes of steroid hormones which are indispensable for mammalian development and physiology, the remaining including the estrogens, progestins, and androgens (2). The steroidogenic capacity of the adrenal gland is highly compartmentalized, performed by specialized cells, organelles, and proteins. The biosynthesis of steroids has been authoritatively reviewed (3), and this manuscript looks to focus attention on the compartmentalization of steroidogenesis of the adrenal mitochondria. To this end, several topics will be covered, including adrenal tissue zonation, mitochondrial organellar organization, and macromolecular protein complexes, all contributing to the regulation and optimization of adrenal

endocrine signaling. Special attention will be devoted to our increasing understanding of multiprotein assemblies in mitochondrial function.

## ADRENAL GLAND ZONATION

The adrenal gland is composed of two anatomically and functionally distinct compartments: the cortex and the medulla. The adrenal medulla is innervated with chromaffin cells and plays a key role in catecholamine synthesis and the sympathetic stress response (4), while the cortex contains the steroidogenic cells responsible for the adrenal's contribution to the endocrine system (5). The adrenal cortex is further compartmentalized, in humans functionally and morphologically divided into three concentric layers: the zona glomerulosa, the zona fasciculata, and the zona reticularis (**Figure 1**).

The zona glomerulosa, lying under the adrenal capsule (**Figure 1**), functions as part of the renin–angiotensin–aldosterone endocrine axis and contributes to organismal electrolyte balance (6). In response to the peptide angiotensin-II, or elevated plasma potassium, the cells of the zona glomerulosa secrete aldosterone. Aldosterone, such as all steroids, is synthesized from cholesterol *via* a multienzyme pathway particular to each steroidogenic tissue, resulting in successive modifications to the sterol backbone (**Figure 2**). Aldosterone, a mineralocorticoid, in turn promotes sodium and water retention, as well as potassium excretion by the kidney (7). Ultrastructurally, glomerulosa cells are characteristically contain numerous mitochondria with lamelli form cristae and some lipid droplets in the cytoplasm (8). The zona fasciculata, the next layer of the adrenal cortex (**Figure 1**), is responsible for organismal glucocorticoid production – cortisol in humans, corticosterone in rodents (**Figure 2**) (3). The cells of the zona fasciculata participate in the hypothalamic–pituitary–adrenal endocrine signaling axis and respond to pituitary

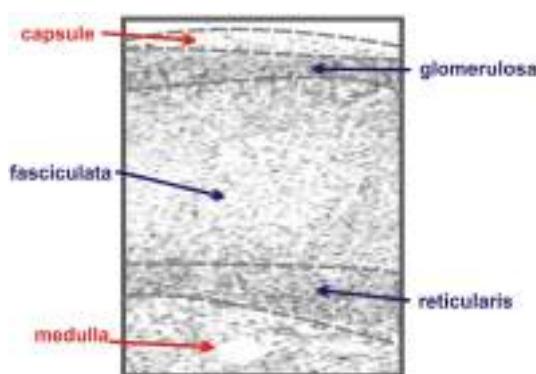
adrenocorticotrophic hormone (ACTH) signaling through the ACTH receptor (M2CR) and its accessory protein, the melanocortin 2 receptor accessory protein (MRAP). The fasciculata cells are organized in cord-like bundles – the fascicles – surrounded by fenestrated capillaries (8). Ultrastructurally, these cells also contain numerous mitochondria, although their cristae take a more tubulovesicular form. Fasciculata cells, consistent with their prolific capacity to synthesize glucocorticoids, contain prominent smooth endoplasmic reticulum (ER) and large numbers of lipid droplets (9). The layer of the cortex abutting the medulla in humans, the zona reticularis, is not part of currently well-defined endocrine axis, but does secrete significant amounts of the androgen dehydroepiandrosterone (DHEA; **Figure 2**) (10). The cells of the zona reticularis resemble those of the fasciculata ultrastructurally, although contain relatively fewer lipid droplets with comparatively greater numbers of lysosomes (9).

Developmentally, the adrenal cortex arises from the adrenocortical primordium, itself derived from the urogenital ridge, a specialized region of the embryonic coelomic epithelium that also serves as the developmental precursor of the kidneys and hematopoietic progenitors (11). Cells in the adrenocortical primordium express the transcription factor genes Wilms tumor suppressor-1 (WT1), GATA-binding protein 4 (GATA4), and steroidogenicfactor-1 (SF1/NR5A1) (8, 12, 13). As development proceeds, adrenal progenitor cells in the migrate dorsomedially from the adrenocortical primordium into subjacent mesenchyme, concurrently upregulating expression of SF1, and downregulating expression of WT1 and GATA4 (13, 14). The developing adrenal gland is subsequently innervated by sympathoblasts from the neural crest, the precursors of the chromaffin cells of the medulla (15), and finally enveloped by capsule cells derived from the surrounding mesenchyme.

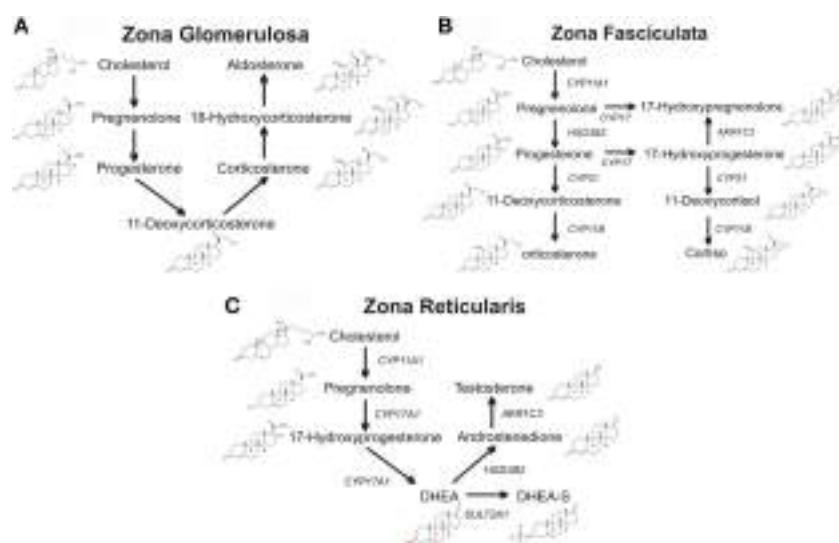
## CELLULAR COMPARTMENTALIZATION AND MITOCHONDRIAL RESPIRATION

Eukaryotic cells are characteristically compartmentalized, containing numerous membrane-bounded organelles, each with specialized functions. These organelles achieve their specialization through non-uniform segregation of molecules, whether they are nucleic acids, proteins, lipids, or carbohydrates. The mitochondria are famously known as the “powerhouse of the cell” for their respiratory capacity and synthesis of adenosine triphosphate (ATP). Although mitochondrial energetics have traditionally served an ancillary role in steroidogenic research (16), the recent finding that mitochondrial function directly impacts neuroendocrine, metabolic, inflammatory, and transcriptional responses to acute psychological stress (17) prompts a brief review. We will introduce individual proteins involved and use the mitochondrial respiratory chain serves an example of higher order functional protein assemblies.

The mitochondria generate ATP by oxidizing hydrogens derived from carbohydrates (through the tricyclic acid cycle) and fats (through fatty acid  $\beta$ -oxidation). Electrons from nicotinamide adenine dinucleotide (NADH) are donated to the iron–sulfur (Fe–S) clusters of mitochondrial complex I (NADH



**FIGURE 1 | Schematic of adrenal zonation.** The different functional zones of the human adrenal gland are depicted with the outermost capsule layer overlying the mineralocorticoid-synthesizing glomerulosa layer. The fasciculata layer lies under the granulosa layer and is responsible for the synthesis of the glucocorticoid cortisol. The final layer of the cortex, the reticularis, synthesizes the androgen dehydroepiandrosterone (DHEA), while the innermost layer of the schematic, the medulla, is composed of chromaffin cells, responsible for the production of the catecholamine epinephrine.



**FIGURE 2 | Schematics of adrenal steroidogenic pathways.** The metabolism of cholesterol to pregnenolone by the mitochondrial CYP11A1 is common to all three zones of the human adrenal. **(A)** The mitochondrial/microsomal enzyme HSD3B converts pregnenolone to progesterone, which is metabolized to 11-deoxycorticosterone by the microsomal CYP21. The final reactions of aldosterone synthesis are catalyzed by the mitochondrial CYP11B2, which converts 11-deoxycorticosterone to corticosterone, which is hydroxylated at C18 to form 18-hydroxycorticosterone which is then finally converted to aldosterone. **(B)** In the zona fasciculata, the microsomal CYP17 and the mitochondrial/microsomal HSD3B can generate 17-hydroxyprogesterone, progesterone, and 17-hydroxyprogesterone. The microsomal CYP21 preferentially metabolizes 17-hydroxyprogesterone to 11-deoxycortisol, which is finally metabolized to the glucocorticoid cortisol by the microsomal CYP11B2. CYP21 can also metabolize progesterone to 11-deoxycorticosterone, which CYP11B2 converts to the glucocorticoid corticosterone, although this pathway is secondary in humans (although the principal pathway in rodents). **(C)** In the zona reticularis, CYP17 hydroxylates pregnenolone to 17-hydroxypregnolone, and then DHEA. DHEA is the major steroid product of the reticularis, with sulfated DHEA (DHEA-S), androstenedione, and testosterone serving as only minor steroidogenic products.

dehydrogenase), a multimeric inner mitochondrial membrane (IMM) protein complex. From Complex I, the electrons are sequentially shuttled to ubiquinone (coenzyme Q/CoQ), giving rise to ubiquinol ( $\text{CoQH}_2$ ). Ubiquinol transfers its electrons to a cytochrome-containing IMM protein complex, Complex III (ubiquinol/cytochrome *c* oxidoreductase), which further shuttles the electrons to cytochrome *c*. From cytochrome *c*, the electrons flow to the cytochrome-containing Complex IV (cytochrome *c* oxidase, COX), the terminal IMM protein complex of the mitochondrial respiratory chain, which uses the electrons to reduce  $\text{O}_2$  to yield  $\text{H}_2\text{O}$ . The free energy of electron movement through the ETC is used to pump protons ( $\text{H}^+$ ) out of the mitochondrial matrix into the mitochondrial intermembrane space (IMS), creating a capacitance across the IMM. This potential energy is utilized to drive ATP synthesis by the IMM Complex V (ATP synthase), which condenses  $\text{ADP} + \text{P}_i$  to form ATP while pumping protons back into the matrix. Matrix ATP is then exchanged with cytosolic ADP by the adenine nucleotide translocator (ANT/SLC25), which works in conjunction with the outer mitochondrial membrane (OMM) voltage-dependent anion channel (VDAC) to form an energy-transducing mitochondrial contact site (18).

An interesting development in mitochondrial respiratory electron transport and one that, as discussed below, offers insight into mitochondrial steroidogenesis, began at the beginning of the twenty-first century with the proposal that the mitochondrial respiratory chain was organized in supramolecular assemblies termed “respirasomes” (19). This paradigm shift changed the

view of mitochondrial electron transport from one of randomly organized respiratory chain complexes – in which components associated *via* random diffusion (20) – to one of respiratory chain supercomplexes locally transferring electrons between restricted components (21). Numerous studies utilizing blue native gel electrophoresis, preserving the fidelity of weakly associated members of protein complexes (22), have repeatedly shown that the oxidative phosphorylation complexes associate in supercomplexes of the three proton-translocating units: Complex I, Complex III, and Complex IV (19, 23, 24). Moreover, these supercomplexes have been demonstrated to be functional *in vitro*, further supporting a role for complex molecular assemblies in cellular function (25).

The conceptual justification of such supercomplexes derives from theoretical work indicating that spatial proximity of chemical reactions fosters efficiency, providing fitness advantages for evolutionary selection. This concept was originally proposed by Welch in the 1970s (26) as the concept of the “metabolon,” and subsequently popularized by Srere (27), among others. This is not a novel concept for steroidogenic research, as Lieberman and Prasad utilized the metabolon construct in their work on steroidogenic metabolism (28). The crux of the metabolon is that groups of enzymes and/or proteins within metabolic pathways physically associate. This association, as noted above, would facilitate metabolite channeling, increasing the regulation, control, and speed of metabolic pathways. The metabolon appeals to common sense and biological evolutionary arguments, as increased efficiency is a requirement of biological survival

in hostile environments. However, the metabolon has been difficult to demonstrate experimentally. Biochemical pull-down experiments have been reticent to demonstrate large biological metabolic complexes (29), and it has been argued that metabolic protein complex association is weak to easily facilitate regulation. The development of non-denaturing molecular assays, such as the blue native PAGE described above, as well as high-resolution fluorescent microscopy, has supported research into transient, but functionally critical, aspects of protein–protein interactions. Indeed, increasing evidence of such interactions in cellular metabolism is receiving attention, ranging from descriptions of cytoplasmic purinosomes – regulating purine biosynthesis (30, 31) – to mitochondrial fatty acid translocation machinery – contributing to mitochondrial fatty acid beta-oxidation and energetics (32, 33). The following sections describe the molecular components of mitochondrial steroidogenic machinery, first introducing individual proteins before transitioning description of a model of steroidogenesis functionally incorporating these proteins into larger protein assemblies, much akin to what has been observed for mitochondrial respiration.

## STEROIDOGENIC CELLS, PROTEINS, AND MITOCHONDRIA

Cholesterol serves as the metabolic precursor of all adrenal steroids, and as such, the adrenal cortex can be viewed as a highly specialized lipid processing organ. Cholesterol possesses fascinating structural characteristics, making it one of the most studied and versatile molecules in biological systems. Steroid hormones, in turn, are oxygenated forms of cholesterol, a characteristic they share with bile acids and oxysterols (2), and one which changes their chemical properties from highly hydrophobic to modestly hydrophilic. This change in chemical properties underlies the shift in their biological role from structural (as cholesterol plays in lipid membranes) to informational (as steroids play in biological signaling).

Any discussion of the steroidogenic mitochondria of the adrenal gland must revolve around the mitochondrial steroidogenic enzymes. These steroidogenic enzymes fall into two broad families: the cytochrome P450 (CYP) and hydroxysteroid dehydrogenase/ketosteroid reductase (HSD/KSR) enzymes. The CYP enzymes represent the majority of the steroidogenic enzymes and will receive the greatest attention here as they are the most abundant steroidogenic enzymes of mitochondria. A highly diverse superfamily of enzymes, the CYPs are characterized by a single heme prosthetic group and the ability to absorb light at 450 nm when reduced with carbon monoxide. Enzymatically, P450 enzymes exhibit an extraordinary ability to insert oxygen into non-activated carbon–hydrogen bonds at the same time exhibiting high structural selectivity. They accomplish this feat through their ability to activate molecular oxygen, although the iron atom of their heme tetrapyrrole prosthetic group with the aid of an ancillary redox partner (34).

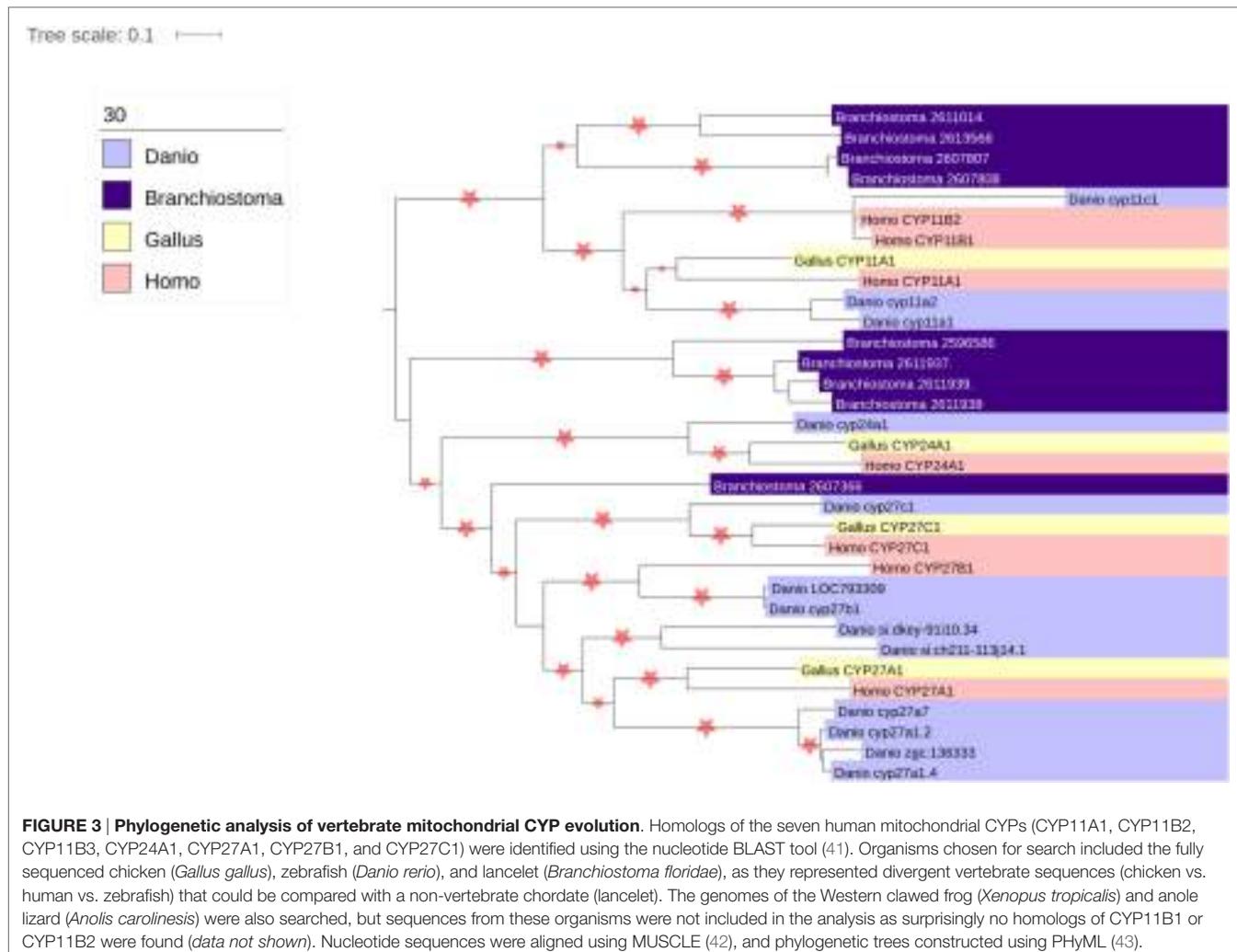
Thousands of CYPs have been identified, in all domains of life, suggesting that this class of protein has ancient roots. There are 57 CYP genes encoding CYP enzymes in humans, subdivided into 18 clades. The majority of the CYP enzymes are localized to the ER,

but one clade is localized to the mitochondria. While ER-localized CYPs obtain their electrons from a single P450 oxidoreductase, the mitochondrial CYPs utilize an electron transport chain containing the ferredoxin reductase (FDXR) and ferredoxin proteins (34). There are seven mitochondrial CYPs in humans, namely CYP11A1, CYP11B1, CYP11B2, CYP24A1, CYP27A1, CYP27B1, and CYP27C1. Of these, CYP11A1, CYP11B1, and CYP11B2 are involved in steroidogenesis, metabolizing cholesterol and steroids. Interestingly, CYP24A1 and the three CYP27 isozymes are all involved in cholesterol metabolism, playing roles in bile acid, oxysterol, and vitamin D biosynthesis (35, 36), suggestive that the ancestor of the mitochondrial clade of enzymes was involved in sterol metabolism. Interestingly, however, phylogenomic analysis of the CYP enzymes has repeatedly shown that the CYP11 family appears with, or shortly before, the emergence of vertebrates (37, 38) (Figure 3). Indeed, it has been postulated that the advent of steroidogenesis and steroid hormone receptor signaling and the increased developmental complexity of vertebrates are intimately linked (39). Moreover, evolution of the mitochondrial CYP11 family is itself of interest within the vertebrate lineage, for while homologs of the CYP11 family, responsible for the first step of synthesis for all steroids, are observed throughout vertebrates from fish to mammals (37, 38) (Figure 3), the CYP11B family, responsible for glucocorticoid and mineralocorticoid synthesis, is underrepresented throughout this subphylum (Figure 3). Thus, while the molecular details of adrenal mitochondrial steroid biosynthesis are well understood, as described below, the evolution of this system remains an important and poorly understood area of research (7, 40).

## CHOLESTEROL SIDE CHAIN CLEAVAGE: CYP11A1

CYP11A1 is absolutely essential for the synthesis of all vertebrate steroids, which are all characterized by the CYP11A1 reaction, namely cleavage of the cholesterol aliphatic side chain. The proposed reaction mechanism of CYP11A1 involves three sequential modifications of cholesterol. In the first step, CYP11A1 hydroxylates cholesterol at carbon 22 of the aliphatic tail; in the second step, cholesterol is hydroxylated at the carbon 20 of the aliphatic tail; finally, in the third step, oxidative scission of the C20–22 bond of the subsequent 20,22-dihydroxycholesterol yields the steroid pregnenolone and the reactive aldehyde, isocaproaldehyde (44). This reaction mechanism, originally proposed in the 1970s based on purified enzyme catalysis of hydroxycholesterol (45), has found support in the publishing of x-ray crystal structures of bovine and human CYP11A1 (46, 47), which indicate that the heme prosthetic group lies proximally to the 20' and 22' carbons of cholesterol. Recent high-resolution temporal enzymatic work further indicates that transient cholesterol hydroperoxy serves as reaction intermediates (48, 49), further supporting the sequential oxidative cleavage model of this enzyme.

The cellular expression CYP11A1 is hormonally regulated in the steroidogenic tissues of the adrenal and gonads, with circulating pituitary hormones stimulating intracellular cAMP production, which in turn promotes CYP11A1 expression (50). A number of paracrine and endocrine factors affect the expression



of CYP11A1. Pituitary hormones, such as ACTH or angiotensin-II, stimulate CYP11A1 expression through a cAMP-dependent mechanism (51, 52), and the human CYP11A1 promoter contains two cAMP-responsive regions (53). Additional factors that stimulate cAMP in adrenocortical cells, such as activin (54), are also able to stimulate CYP11A1 expression. In contrast to cAMP, intracellular signaling pathways activated by  $\text{Ca}^{2+}$  and protein kinase C (PKC) can suppress CYP11A1 transactivation (55). In addition to the cAMP-responsive sites (CRSs) in the CYP11A1 promoter, the transcription factor SF-1 also contains an activating site (56, 57), which modulates the basal and cAMP-stimulated levels of CYP11A1 expression through association with the transcription factors p300 and CREB-binding protein (CBP) (58).

## GLUCOCORTICOID AND MINERALOCORTICOID SYNTHESIS: CYP11B1 AND CYP11B2

The final steps in the synthesis of glucocorticoids and mineralocorticoids are catalyzed by two closely related mitochondrial

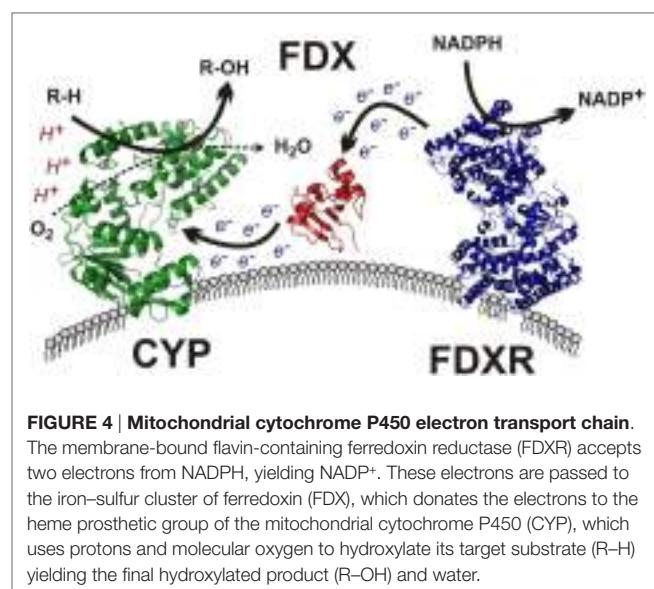
enzymes: CYP11B1 and CYP11B2 (59). Similar to CYP11A1 and the other mitochondrial CYPs, CYP11B1 and CYP11B2 are associated with the IMM and are expressed with leader peptides targeting them to the mitochondrial matrix (60). CYP11B1 is the more abundantly expressed of the two proteins and is expressed predominantly in the zona fasciculata, and to a lesser extent in the zona reticularis, but not in the zona glomerulosa (61). CYP11B1 catalyzes the  $11\beta$ -hydroxylation of 11-deoxycorticosterone and 11-deoxycortisol yielding corticosterone and cortisol, respectively (Figure 2) (44). CYP11B1 also has the capacity to hydroxylate C18 of 11-deoxycorticosterone or corticosterone to form 18-hydroxycorticosterone (62); however, it cannot catalyze the oxidation of the 18-hydroxy group to form aldosterone. This last reaction is catalyzed by CYP11B2, which is able to catalyze the sequential  $11\beta$ -hydroxylation of 11-deoxycorticosterone, the hydroxylation of C18 and the subsequent oxidation of C18 to yield the C18 aldehyde group of aldosterone (Figure 2) (63–65). CYP11B2 inefficiently catalyzes oxidation of corticosterone; this finding has lent support to the zonation theory of mineralocorticoid and glucocorticoid synthesis, as the products of CYP11B1 would not be sequentially catalyzed to aldosterone (65).

Although it is unclear whether the CYP11B clade of mitochondrial CYPs emerged with the advent of vertebrates or mammals, within the mammalian lineage, the CYP11B clade exhibits significant diversity. While humans possess the two enzymes discussed above, cattle and pigs possess a single enzyme, CYP11B (66, 67), which is able to catalyze all of the reactions of CYP11B1 and CYP11B2. Conversely, within the rodent lineage, rats (but not mice) possess three CYP11B genes – CYP11B1, CYP11B2, and CYP11B3 – with CYP11B1 and B2 exhibiting homologous activity to their human orthologs, and CYP11B3 possessing the ability to convert deoxycorticosterone to 18-deoxycorticosterone, but lacking the 18 activity necessary to synthesize aldosterone (68).

CYP11B1 and CYP11B2 are located on human chromosome 8q21–22 (59, 69). Consistent with a tandem duplication event, the two human genes are closely linked, separated by 40 kb, exhibiting similar intron/exon structure, and 90 and 95% identity in the coding and non-coding sequences, respectively (59, 70). CYP11B1 expression is induced by ACTH via cAMP (71, 72), through a mechanism relying on cAMP response element (CRE) and activating transcription factor (ATF) sequences in the CYP11B1 promoter (73). Orphan nuclear receptors play a critical role in CYP11B1 genetics, with SF1/NR5A1 and LRH-1/NR5A2 both contributing to the relative expression of CYP11B1 and regulating the comparative expression of CYP11B1 vs. CYP11B2 (73, 74). CYP11B2 transcription in granulosa cells is induced by potassium and by angiotensin-II, in both cases stimulating calcium ( $\text{Ca}^{2+}$ ) influx and stimulation of PKC and calmodulin-dependent protein kinase kinase (CAMKK) intracellular signaling (75, 76). CYP11B2 expression requires the action of the transcription factors NURR1 and NGF1B, but interestingly contrasts with CYP11B1 in its relationship with the transcription factor SF-1 (77, 78). Transcriptional regulation of CYP11B2 is also influenced by the activity of chicken ovalbumin upstream promoter transcription factor I (COUP-TF1), which itself is coactivated by the small ubiquitin-related modifier-1 (SUMO-1) conjugase and ligase Ubc9 and PIAS1 (79).

## ELECTRON TRANSFER TO CYP11A1: FERREDOXIN REDUCTASE AND FERREDOXIN

The mitochondrial clade of CYP enzymes uses two sequential electron-transfer donors – ferredoxin (FDX) and FDXR – as intermediates in electron donation from NADPH (Figure 4). These are ancient proteins, with orthologs expressed in all domains of life and involved in numerous processes outside of steroidogenesis (80). FDXR is a 54.5-kDa flavoprotein affixed to the IMM that reduces NADPH and contains of two domains (81): (1) a NADPH-binding domain and (2) a flavin adenine dinucleotide-binding domain, with electron transfer occurring between the two. This cleft possesses a number of basic residues, residues essential for interaction with acidic residues on its electron donor partner, FDX (82). FDXR is broadly expressed in numerous tissues, but is comparatively abundant in steroidogenic tissues (83), where its abundance contributes to the catalytic activity of the mitochondrial CYPs (84). High SF-1 expression



**FIGURE 4 | Mitochondrial cytochrome P450 electron transport chain.**

The membrane-bound flavin-containing ferredoxin reductase (FDXR) accepts two electrons from NADPH, yielding NADP<sup>+</sup>. These electrons are passed to the iron–sulfur cluster of ferredoxin (FDX), which donates the electrons to the heme prosthetic group of the mitochondrial cytochrome P450 (CYP), which uses protons and molecular oxygen to hydroxylate its target substrate (R–H) yielding the final hydroxylated product (R–OH) and water.

in steroidogenic tissue likely contributes this preponderance of FDXR, as SF-1-binding sites are present in the FDXR promoter and SF-1 overexpression in adrenal cell models drives FDXR expression (85).

The electron donor partner of FDXR, FDX, is a 14-kDa mitochondrial matrix-localized protein containing a Fe–S cluster tethered by four cysteine residues (86). Two ferredoxins exist in humans, with FDX1 supporting steroidogenesis and FDX2 participating in heme and Fe/S cluster protein synthesis (87). After obtaining electrons from FDXR, FDX1 subsequently transfers its electrons to mitochondrial P450 enzymes, including CYP11A1, CYP11B1, and CYP11B2, among others (Figure 4). FDX has been described as a mobile, indiscriminate, diffusible electron shuttle (3), much as cytochrome *c* and ubiquinol have been described previously (20).

## IDENTIFICATION OF STEROIDOGENIC PROTEIN COMPLEXES

Research on CYP enzymes has contributed to the emerging picture of CYPs operating in functional complexes with other CYPs (88) as well as with their cognate electron donor partners (89). As noted in the previous section, the mitochondrial CYPs and the FDX and FDXR redox partners have been considered to interact randomly in the mitochondrial inner membrane (3). However, examination of native protein complexes in steroidogenic mitochondria from tumor Leydig cells using BN-PAGE and mass spectroscopy suggested that CYP11A1 and FDXR physically associate (90). In addition, these natively isolated CYP11A1 complexes were functionally active, cleaving the aliphatic tail of a fluorescent cholesterol reporter. Taken together, these findings support a model for CYP11A1–FDXR electron transport occurring in a physically associated metabolon, much akin to the electron transport of the respirasomes discussed above (24). This CYP11A1 metabolon model integrates with work on the

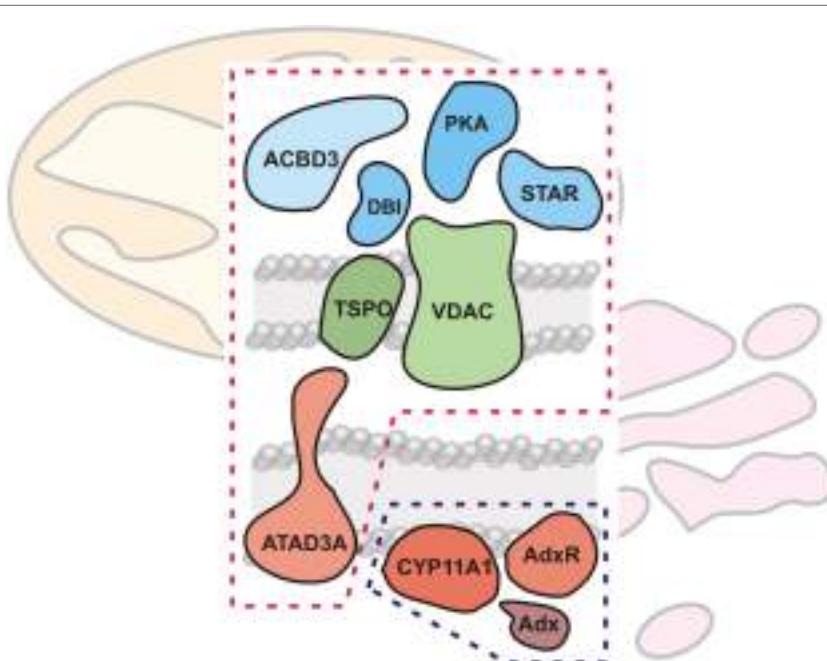
steroidogenic transduceosome, a multiprotein complex traversing the OMM and IMM of the steroidogenic mitochondria integrating the movement of cholesterol with intracellular signaling to CYP11A1 (90, 91) (**Figure 5**). The steroidogenic transduceosome and metabolon complexes contain a number of cytoplasmic and mitochondrial components (**Figure 5**): cytoplasmic proteins include the steroidogenic acute regulatory (StAR) protein, the protein kinase cAMP-dependent type I regulatory subunit alpha (PRKAR1A), the diazepam-binding inhibitor (DBI), and the acyl-CoA-binding domain containing 3 (ACBD3); mitochondrial proteins include the VDAC, the translocator protein (TSPO), ATPase family, AAA domain containing 3A (ATAD3A), CYP family 11 subfamily A member 1 (CYP11A1), and FDXR. In addition, functional partners of the transduceosome have been identified, including mitochondria-associated members of the 14-3-3 adaptor protein family (92, 93) as well as kinase signalers such as extracellular signal-regulated kinase (ERK) 1/2 (94); the physical association and temporal interaction of these proteins with the transduceosome remain an active area of research. Because the focus of this review is upon mitochondrial contributions to steroidogenesis, the mitochondrial components of the transduceosome and metabolon are focused upon below.

## VOLTAGE-DEPENDENT ANION CHANNEL

Voltage-dependent anion channel is the most abundant protein of the mitochondrial outer membrane and is widely accepted as the

principal route and control of metabolic flux between the cytosol and mitochondria (95). VDAC is a 32-kDa beta barrel protein and has been implicated in numerous cellular processes, ranging from cellular energetics to apoptosis (96). Three VDAC isoforms are expressed in the human genome, with VDAC1 located on chromosome 5q31, VDAC2 located on chromosome 10q22, and VDAC3 located on chromosome 8p11 (97). The relative abundance of the different VDAC isoforms vary by tissue, by VDAC1 is the predominant form, followed by VDAC2, with VDAC3 expression low in comparison. Although functional redundancy is believed to exist between the isoforms, significant differences in roles of the VDAC proteins has been slowly teased out through biochemical and genetic investigations (98). Interestingly, mice null for *Vdac1* and *Vdac3* are viable and display little overt phenotypical changes; however, on this particular murine genetic background, *Vdac2*<sup>-/-</sup> mice were embryonically lethal (99, 100). Moreover, although the VDAC proteins have been recurrently implicated in apoptosis, mitochondria from *Vdac1*<sup>-/-</sup> and *Vdac3*<sup>-/-</sup> null mice, as well as cell lines null for all three isozymes fail to show changes in mitochondrial permeability transition and Bcl-2 family member-driven cell death compared with wild-type mitochondria.

Voltage-dependent anion channel is found at contact sites between the OMM and the IMM (101) where it may complex with energetics-related proteins, such as hexokinase, ANT, and creatine kinase, or with apoptotic proteins of the Bcl-2 family (18), and as noted above, and discussed below in greater detail, appears



**FIGURE 5 | Mitochondrial cholesterol transport and metabolism machinery.** The mitochondrial cholesterol import and metabolism machinery are shown, demarcated by red (transduceosome) and blue (metabolon) dashed lines, respectively. The transduceosome contains cytoplasmic (StAR, ACBD3, DBI, and PRKARI; colored blue), OMM (VDAC and TSPO; colored green), and IMM (ATAD3A; colored red) components which assemble in response to hormonal stimulation and transduce the resultant cAMP signal to the mitochondria for cholesterol import. It is important to note, however, that molecular details of cholesterol import are still lacking. Once cholesterol is imported into the mitochondria, the IMM metabolon (CYP11A1, FDX, and FDXR; colored red) metabolizes cholesterol to pregnenolone, the precursor to all other steroids, including adrenal glucocorticoids and mineralocorticoids.

to play a central role in facilitating mitochondrial steroidogenic cholesterol transport through interactions with several key proteins. Moreover, VDAC also appears to play a significant, albeit poorly understood, role in cellular cholesterol homeostasis (102). Structural characterization of VDAC indicates that it is a cholesterol-binding protein; whether this cholesterol binding is a non-specific artifact of VDAC hydrophobicity, or whether this cholesterol binding plays a physiological role in steroidogenesis or other processes remains to be determined. It is unlikely that VDAC itself participates as a cholesterol channel, as the center of its ring-like structure is hydrophilic, suitable for anion transport but unsuitable for hydrophobic molecule transport (103, 104). After nearly half a century of work on this ubiquitous protein, much remains to be understood, especially in the context of mitochondrial cholesterol metabolism.

## TRANSLOCATOR PROTEIN, 18 kDa

In the 1970s and 1980s, studies of benzodiazepine drug binding to sites outside of the central nervous system led to the identification of a peripheral benzodiazepine-binding site, generally expressed throughout the body, but concentrated in the steroidogenic cells of the adrenal and gonad (105–107). Isolation of this benzodiazepine-binding site led to the identification of an 18-kDa integral OMM protein, originally named the peripheral benzodiazepine receptor (PBR) (106). Subsequent research demonstrated that benzodiazepine and other chemically distinct PBR ligands were able to stimulate steroid biosynthesis by mobilizing mitochondrial cholesterol transport in isolated mitochondria, cell cultures, as well as in humans (108–111), and based on work implying the involvement of this protein in mitochondrial cholesterol and heme tetrapyrrole import, the PBR was renamed the TSPO (112).

Biochemical evidence and recent structural determinations of the TSPO protein indicate that TSPO is predominantly  $\alpha$ -helical, containing five helices clustered into a barrel-like shape (113–115). Although antioxidant properties of TSPO have been demonstrated (115), no classical redox enzymatic or prosthetic groups, such as transition metals or active thiols, have been observed, and functional analysis of the protein has been difficult owing to its hydrophobicity (116). TSPO does contain an evolutionarily conserved C-terminal cholesterol recognition amino acid consensus (CRAC) motif (117, 118), which has been shown to facilitate cholesterol binding through a conserved tyrosine residue (119). This cholesterol-binding activity for TSPO has been implicated in steroidogenesis, as small molecules targeting the CRAC motif inhibit steroid production in cell and animal models (120, 121), and a naturally occurring human polymorphism in the protein proximal to the C-terminal CRAC motif (A147T) reduces cellular steroid production (122).

Hormonal stimulation of steroidogenesis in a steroidogenic cell model resulted in increased TSPO polymerization in correlation with increased ligand-binding affinity and steroid production (123, 124). Much like VDAC discussed above, TSPO has been identified as concentrated at OMM–IMM contact sites (125), and in addition to homooligomerization, TSPO has been consistently shown to physically associate with VDAC (126–128). Moreover, VDAC–TSPO interaction affects binding of TSPO ligands (127),

suggestive of a functional relationship between the two proteins. The TSPO–VDAC platform appears to serve as an OMM base for the steroidogenic transduceosome cholesterol-transfer machinery (90, 91), a complex which appears to predominantly contain the polymerized form of TSPO (90). However, the mechanistic details of TSPO involvement in this complex are unclear at this time. Recent genetic mouse models in which TSPO had been deleted tissue specifically and globally have yielded conflicting results regarding steroidogenesis, ranging from no effect on steroidogenesis to severe compromise of ACTH-stimulated production of corticosterone (129–131). The complex effects of genetic background and selection for compensatory changes likely play a role in the experimental variability, however, especially in light of skewed embryonic Mendelian ratios in TSPO null mice (131).

## STEROIDOGENIC ACUTE REGULATORY PROTEIN

Pharmacological inhibition of protein synthesis by compounds, such as cycloheximide, was long known as an inhibitor of steroidogenesis (132), suggesting that rapid protein synthesis was a necessary driver of steroid biosynthesis. The StAR protein was originally identified as a labile protein factor rapidly induced in response to hormonal stimulation of steroidogenic cells in correlation with increased steroid production (133, 134). Moreover, a number of studies have demonstrated that StAR is a direct target of hormonally stimulated cellular kinase signaling pathways, including the protein kinase A (PKA) and ERK kinase pathways (94, 135, 136). The necessity of StAR for steroidogenesis derived from work in humans showing that a broad spectrum of mutations in StAR contribute to congenital adrenal lipoid hyperplasia, a condition characterized by the inability to synthesize steroids, resulting in impaired sexual development and adrenal dysfunction leading to infant death unless treated with glucocorticoid supplementation (137).

Steroidogenic acute regulatory protein is located on chromosome 8p11 and is expressed as a 37-kDa mitochondrial pre-protein containing a mitochondrial targeting leader sequence, and subsequently imported into mitochondria, where the presequence is cleaved by Lon proteases in the matrix to a 30-kDa mature protein (although it is interesting to note that theoretical calculation of StAR protein molecular mass indicate 30 and 25 kDa masses for the pre- and mature proteins, although the reason for this discrepancy is unclear at this time). Surprisingly, StAR was found to stimulate steroid production in cell model systems without its leader sequence and import into the mitochondria (138). This led to elegant work on StAR action involving its molecular tether to the OMM, IMS, and IMM which indicated that StAR acts on the OMM and that mitochondrial import to the matrix inactivates StAR activity (139). Interestingly, however, *in vivo* work has demonstrated that the StAR mitochondrial presequence has biological necessity, as mouse models in which full-length StAR has been replaced by StAR that lacks the mitochondrial targeting sequence stochastically exhibit the CAH phenotype of steroidogenic failure (140). Moreover, CAH-causing mutations have been found in the leader sequence of human StAR-mutation patients (141), collectively arguing that the StAR relationship

with its leader peptide is more complex than previously thought. The finding that StAR physically interacts with VDAC1 (142) and can be found in the OMM transduceosome complex when proteins are crosslinked after steroidogenic stimulation (91) supports a model in which StAR serves as a “key” acting to “start” the mitochondrial cholesterol import machinery. However, the precise molecular details of this model remain to be determined.

## ATPase FAMILY, AAA DOMAIN CONTAINING 3A

The ATAD3A protein belongs to the AAA<sup>+</sup> family of ATPases, a broadly conserved family of ATPases implicated in various cellular processes (1, 3–5). ATAD3A is characterized by two N-terminal coiled-coil domains: a central transmembrane helix and a conserved C-terminal AAA<sup>+</sup>-type ATPase domain (143). Several studies have localized ATAD3A to the mitochondria (143, 144), where it appears to be involved in mitochondrial membrane dynamics. Trypsin digestion assays have been used to study the membrane topology of ATAD3A, suggesting that the C-terminal ATPase domain is localized in the mitochondrial matrix, the transmembrane segment traverses the IMM, and the N-terminal coiled-coils anchor the protein to the OMM. ATAD3A appears capable of homooligomerization, and as noted above, appears to be a partner protein in the mitochondrial transduceosome of steroidogenic cells, critical for cholesterol import into mitochondria and steroidogenesis (90, 145).

## MITOCHONDRIA-ASSOCIATED MEMBRANES: SITES OF ER-MITOCHONDRIAL MEMBRANE INTERACTION

The ER is a complex cellular organelle, formed by an interconnected network of cisternae (146), is distributed across the cell and is involved in numerous processes, including lipid and protein synthesis. The ER is well known in steroidogenic research as the site of action of numerous steroidogenic CYP and HSD/KSR enzymes (3), but recently, the ER has attracted recent interest in mitochondrial cholesterol metabolism as a possible source of cholesterol (147). The ER and mitochondria are considered cholesterol poor organelles, especially in contrast to the high levels present in plasma membranes and endosomes. However, the ER appears to be a staging platform for cellular cholesterol homeostasis, as endogenous cellular cholesterol is synthesized in the ER, and cholesterol taken up by cells from circulating lipoproteins makes its way to the ER before incorporation into lipid droplets. In addition, the ER houses the sterol regulatory element-binding protein (SREBP) sensory machinery, which senses ER sterol levels and subsequently regulates transcription of genes involved in cholesterol and fatty acid synthesis and uptake (148). Although lipid droplets have been considered the classic source of steroidogenic cholesterol, the intimate relationship between lipid droplets, the ER and mitochondria, suggest a complex relationship in mitochondrial cholesterol delivery for steroidogenesis (149).

Almost 30 years ago, Vance demonstrated phospholipid synthesis in cellular fractions enriched in mitochondrial and ER markers (150). Electron microscopic investigations of mitochondria-ER association have consistently revealed the existence of specific regions of close apposition between the ER membranes and the OMM, with these regions representing between 5 and 20% of the mitochondrial surface (151–153). These mitochondria-associated membrane (MAM) sites have become recognized as possessing their own particular makeup, characterized by a number of resident proteins (154). Interestingly, several of these proteins have been demonstrated to participate in mitochondrial cholesterol transport and steroid biosynthesis. VDAC itself has been demonstrated to be present in MAMs (155), and, interestingly, StAR appears to interact with VDAC2 in steroidogenic cell model MAMs, an interaction necessary for its steroidogenic activity and mitochondrial import (156). In addition to VDAC, which is predominantly localized to mitochondria, several highly enriched resident MAM proteins have been demonstrated to play a key role in mitochondrial cholesterol transport. The first of these proteins, the sigma-1 receptor (SIGMAR1), was found to coimmunoprecipitate with VDAC2 in a steroidogenic cell model as well as disrupt mitochondrial cholesterol metabolism when its expression was reduced by short interfering RNA (siRNA) (157). Interestingly, SIGMAR1 appears to promote the compartmentalization of cholesterol in ER membranes (158), although depletion of cholesterol promoted mitochondrial-ER association in *in vitro* membrane association assays and cell models (159). A second resident MAM protein, acyl-CoA synthetase 4 (ACSL4), an enzyme involved in cellular arachidonic acid metabolism, participates in mitochondrial arachidonic acid movement (160). Of note, silencing the expression of ACSL4 inhibits steroidogenesis in a cell model, overexpression of ACSL4 promotes steroidogenesis (161), and cAMP signaling promotes increased mitochondrial colocalization of ACSL4 (162), collectively suggestive of a MAM relationship to mitochondrial arachidonic acid and cholesterol import. Finally, ATAD3A, in addition to forming a physical link between the IMM and OMM, may be involved in linking the mitochondria to the ER at MAMs. A long isoform of ATAD3A was found to be present in the MAMs of steroidogenic cells (145), and this work, in conjunction with the VDAC-StAR-MAM work cited above (156), suggesting that the transduceosome complex may serve to link not only the membranes of the mitochondria to the CYP metabolon but also the metabolon to cholesterol reserves in the ER and beyond.

## CONCLUSION

Steroidogenesis begins with the mobilization and movement of cholesterol from intracellular stores into mitochondria. Control of steroidogenic output is organized at two levels: substrate availability and targeting, and enzyme expression and localization. Past and recent studies in hormone-inducible steroidogenic cells showed that cholesterol trafficking and targeting into mitochondria is rate limiting and driven by intracellular protein networks, referred to as the transduceosome, which amplifies the cAMP signal at the OMM, and the steroidogenic metabolon. This mitochondrial metabolon prevents unwanted crosstalk of the substrate

cholesterol with other pathways, optimizing substrate concentration and targeting to CYP11A1. Deciphering the organization and regulation of intracellular protein assemblies that interact with the steroidogenic machinery will provide insight into the intracellular events involved in normal and disease states, facilitating diagnosis and treatment. These studies suggest a shift in focus in steroidogenic cell biology from the actions of individual proteins in isolation to the actions of protein assemblies working together to execute specialized cellular functions, in this case adrenal steroid formation.

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## AUTHOR CONTRIBUTIONS

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# ACTH Action on StAR Biology

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Adrenocorticotropin hormone (ACTH) produced by the anterior pituitary stimulates glucocorticoid synthesis by the adrenal cortex. The first step in glucocorticoid synthesis is the delivery of cholesterol to the mitochondrial matrix where the first enzymatic reaction in the steroid hormone biosynthetic pathway occurs. A key response of adrenal cells to ACTH is activation of the cAMP-protein kinase A (PKA) signaling pathway. PKA activation results in an acute increase in expression and function of the *Steroidogenic Acute Regulatory protein* (StAR). StAR plays an essential role in steroidogenesis- it controls the hormone-dependent movement of cholesterol across the mitochondrial membranes. Currently StAR's mechanism of action remains a major unanswered question in the field. However, some insight may be gained from understanding the mechanism(s) controlling the PKA-dependent phosphorylation of StAR at S194/195 (mouse/human StAR), a modification that is required for function. This mini-review provides a background on StAR's biology with a focus on StAR phosphorylation. The model for StAR translation and phosphorylation at the outer mitochondrial membrane, the location for StAR function, is presented to highlight a unifying theme emerging from diverse studies.

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## STAR (STARD1) DISCOVERY AND FUNCTION

The study of steroid hormone production has origins in work on adrenocorticotropin hormone (ACTH)-stimulated adrenal steroid synthesis (Macchi and Hechter, 1954a,b,c; Stone and Hechter, 1954). ACTH binds to its cognate 7-transmembrane G-protein coupled receptor, the melanocortin 2 receptor (encoded by the MC2R gene) that is located at the plasma membrane of adrenal fasciculata cells. ACTH binding to MC2R results in activation of multiple signal transduction pathways with the cAMP-dependent protein kinase A (cAMP-PKA) pathway being central to hormone-dependent activation of adrenal glucocorticoid and androgen synthesis (reviewed in Gallo-Payet and Payet, 2003; Spat et al., 2016). All steroid hormones are produced from cholesterol and the steroidogenic enzymes in cortisol synthesis, the major adrenal steroid produced in response to ACTH, have been reviewed in detail (Miller and Auchus, 2011). This mini-review focuses on the rapid or acute response to tropic hormonal stimulation- the movement of cholesterol into mitochondria. The first enzymatic reaction is the conversion of cholesterol to pregnenolone by the cytochrome P450 side chain cleavage enzyme (P450scc). P450scc is located in the mitochondrial matrix and work from the 1950s–1970s laid a solid foundation that cholesterol transport into mitochondria for access to P450scc was a key control point in steroidogenesis (Ferguson, 1962, 1963). The prevailing model at that time was that *de novo* synthesis of a protein factor upon hormonal stimulation was necessary for the cholesterol transfer. Furthermore, this factor needed to fulfill the following criteria: be newly synthesized upon hormonal stimulation in a time- and dose-dependent manner, be localized at the mitochondria, and have a short-half (reviewed in

Clark and Stocco, 2014; Stocco et al., 2016). Here, I will highlight the studies which demonstrated that the Steroidogenic Acute Regulatory protein (StAR) fulfills the criteria for the acute regulator of steroidogenesis.

The first characterization of StAR was as phosphoproteins (pp with MW in kDa) pp37, pp32, and pp30 that appeared in rat adrenal after ACTH stimulation (Krueger and Orme-Johnson, 1983; Pon and Orme-Johnson, 1986; Pon et al., 1986; Alberta et al., 1989; Epstein and Orme-Johnson, 1991). Both *in vivo* and cell culture approaches provided strong correlative data that ACTH-cAMP-PKA induction of these proteins coincided with steroid production. In addition, the proteins were shown to be associated with mitochondria, and the pp32 and pp30 forms were processed forms of pp37 (Alberta et al., 1989; Epstein and Orme-Johnson, 1991; Krueger and Orme-Johnson, 1983; Pon and Orme-Johnson, 1986; Pon et al., 1986). Similar hormonal responsive protein(s) were characterized in the MA-10 mouse Leydig tumor cells, and ultimately the StAR protein was purified and cDNA cloned from this cell line (Clark et al., 1994). The deduced amino acid sequence encodes a protein with estimated molecular weight of 31.6 kDa with the amino-terminal region containing a classical mitochondrial targeting sequence (Clark et al., 1994). Expression of the cDNA in steroidogenic cells or *in vitro* in the presence of isolated mitochondria followed by Western blot analysis confirmed the cDNA encoded the pp37 protein previously characterized (Clark et al., 1994; King et al., 1995; Lin et al., 1995). The cDNA encoded a functional protein based on assays that measured increased steroid production in COS-1 cells or steroidogenic cells after transient expression of the cDNA. As anticipated, the 37 kDa StAR protein was imported and processed by mitochondria to generate the 30 kDa StAR protein (King et al., 1995). However, steroidogenesis ceases with removal of tropic hormone stimuli yet the 30 kDa form of StAR localized in the mitochondrial matrix is present with an estimated half-life of 4–5 h (Stocco and Sodeman, 1991; Granot et al., 2003). Thus, the requirement for a labile, short half-life criteria for the acute regulator of steroidogenesis required subsequent structure-function studies. Database searches using the cDNA and protein sequences revealed that StAR represented a novel protein (Clark et al., 1994). Shortly after the initial reports on StAR appeared, a conserved protein domain named the START domain (for steroidogenic acute regulatory protein (StAR)-related lipid-transfer domain), was identified using Web-based resources for predicting putative functional domains based on primary sequence data (Ponting and Aravind, 1999). Members of the START domain protein superfamily share a 210 amino acid region that folds into an  $\alpha/\beta$  helix-grip fold structure containing a long hydrophobic cleft for lipid binding (reviewed in Stocco, 2001; Clark, 2012). The START domain within the StAR protein spans amino acids 65–285, which encodes the processed 30 kDa form, and binds cholesterol. Key studies showed that only the START domain is required for StAR's function: (1) addition of the 30 kDa StAR protein to isolated mitochondria promotes cholesterol transfer and pregnenolone production; and (2) expression of a cDNA encoding only the START domain, e.g., lacking the N-terminal mitochondrial targeting sequence (N62StAR), is capable of stimulating steroid production in

steroidogenic cells or heterologous COS-1 cells (Arakane et al., 1996; Wang et al., 1998). Furthermore, mutations in the human STAR gene (*STARD1*) were identified in patients with congenital lipoid adrenal hyperplasia (lipoid CAH), a disorder marked by a lack of adrenal and gonadal steroidogenesis due to the inability to move cholesterol into the mitochondria [(Lin et al., 1995; Bose et al., 2000); reviewed in (Miller, 2014)]. The finding that *STAR* mutations are the genetic basis for lipoid CAH was key to establishing the essential role for StAR in ACTH-stimulated steroidogenesis as well as gonadotropin-stimulated steroidogenesis (Lin et al., 1995; Caron et al., 1997). A common mutation found in the *STAR* gene is a nonsense mutation, Q258X, which results in truncation of the last 28 amino acids (Nakae et al., 1997; Kim et al., 2011). Expression of C-terminal truncated forms of StAR in COS-1 cells confirmed that the loss of C-terminal helix, within the START domain, results in an inactive protein (Arakane et al., 1996; Wang et al., 1998).

The finding that N-terminal truncated StAR (N62StAR) was functional in cell-based assays, indicated that mitochondrial import was not required for function. However, the targeting of StAR to mitochondria may be important for efficient cholesterol transfer and steroid production *in vivo*. A mouse model of lipoid CAH was generated by “knocking out” StAR expression and, as anticipated, the animals lacked steroid production and significant amounts of cholesterol accumulated in the adrenals and gonads (Caron et al., 1997). Expression of a full-length *STAR* transgene in the StAR knockout mice restored adrenal and gonadal steroidogenesis while expression of the N62*STAR* transgene only partially restored steroidogenesis in a tissue- and gender-specific manner (Sasaki et al., 2008). The mice with the N62StAR transgene retained modest lipid accumulation in the adrenal and gonads. These data support that StAR is capable of functioning without the N-terminal mitochondrial targeting sequence, but highlight the importance of correct and efficient subcellular localization of the protein for full function *in vivo*. In the absence of mitochondrial import, N62StAR still associates with mitochondria outer membrane (Arakane et al., 1996; Wang et al., 1998).

## STAR PHOSPHORYLATION AND FUNCTION

Early studies proposed an association between StAR phosphorylation and function (Pon et al., 1986; Chaudhary and Stocco, 1991; Clark et al., 1995; Hartigan et al., 1995). A PKA-dependent phosphorylation at S194 (mouse) or S195 (human) was validated as an important post-translational modification required for StAR's function in cell-based and *in vitro* assays (Arakane et al., 1996; Jo et al., 2005; Baker et al., 2007). StAR-S194/195 nomenclature reflects the deletion of 3 nucleotides in exon 2 that eliminates a residue which lies within the cleavable mitochondrial signal sequence in mouse StAR. A key study confirmed the relevance for StAR-S194 phosphorylation *in vivo*; StAR knock-out mice were used to demonstrate that re-expression of wild-type StAR but not StAR-S194A restored steroidogenesis in the adrenal and testis

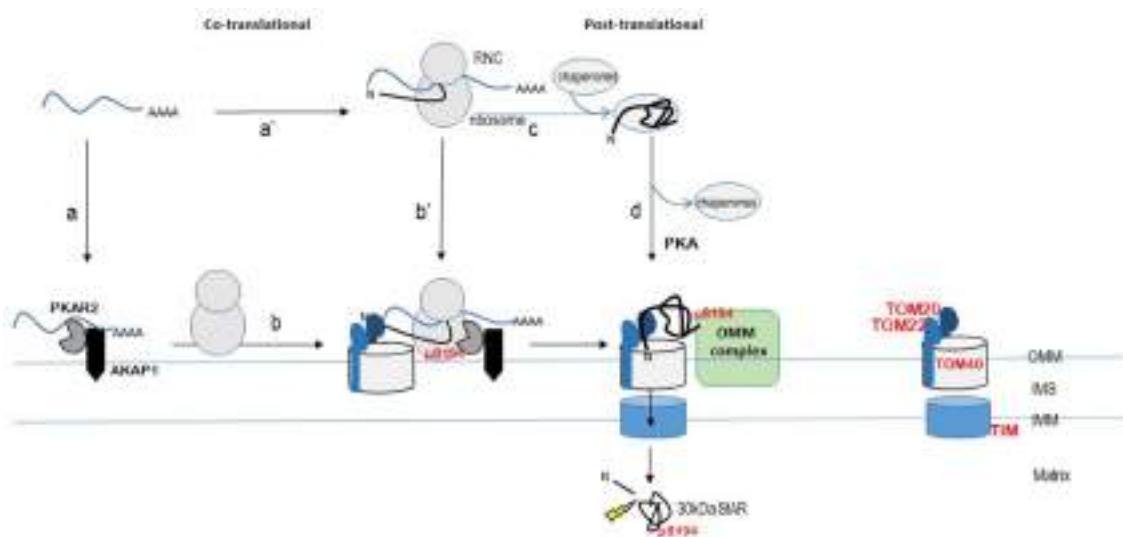
(Sasaki et al., 2014). The newborn StAR-S194A transgenic male mice had lipid accumulation in the adrenal and testis, and circulating hormone levels (high ACTH and low corticosterone and testosterone) similar to StAR knock-out mice. Thus, StAR phosphorylation at S194/195 is critical for function *in vivo*.

The molecular mechanism of action for StAR-mediated cholesterol transport across the mitochondrial membranes is not fully defined, therefore it remains a challenge to define the role of S194/195 phosphorylation in StAR's function. The current model for StAR-mediated cholesterol transport asserts that protein-protein interactions between StAR and OMM protein(s) triggers cholesterol transfer (Bose et al., 1999; Mathieu et al., 2002; Baker et al., 2007; Roostaei et al., 2008; Rajapaksha et al., 2013). Structural changes in the START domain induced by cholesterol binding and by interaction with the OMM as well as the kinetics of cholesterol transfer across the mitochondrial membranes have been reviewed elsewhere (Miller, 2007). Precursor StAR processing to the mitochondrial 30 kDa form is reported to be very efficient, stoichiometric, and dependent upon cholesterol-induced structural changes (Artemenko et al., 2001; Rajapaksha et al., 2013). Import of StAR is proposed to be the "off switch" for cholesterol transfer due to loss of StAR interaction with an OMM protein complex (Bose et al., 1999; Miller, 2007). However, the transit time at the OMM contributes to StAR function with slower mitochondrial import associated with greater activity (Bose et al., 2002). Does StAR phosphorylation affect cholesterol binding or mitochondrial import? Purified hStAR START domain containing the S195A mutation was shown to have the same cholesterol binding kinetics as wild-type StAR START domain, indicating that phosphorylation is not required for cholesterol binding (Baker et al., 2007). StAR-S194A/S195A or non-phosphorylated forms of wild-type StAR are efficiently processed to the 30 kDa mitochondrial matrix form both in cell culture and *in vivo* studies, thus phosphorylation doesn't appear necessary for StAR import (Arakane et al., 1997; Jo et al., 2005; Sasaki et al., 2014; Clark and Hudson, 2015). The region that slows StAR import is a protease-resistant domain that spans amino acids 63–188 (Bose et al., 1999, 2002), therefore, it is unlikely that S194/195 phosphorylation alters StAR import rates, although this has not been directly tested.

While StAR phosphorylation doesn't appear to be required for mitochondrial import, understanding the import mechanism may provide insight into StAR-OMM interactions that are required for function. TOM20 and TOM22 are the receptor components of the Translocase of the outer membrane (TOM) complex that function with the pore-forming TOM40 complex to control protein import into mitochondria (Becker et al., 2012; Harbauer Angelika et al., 2014). Classically, post-translational protein import via a TOM20/22-TOM40 pathway is associated with proteins that are synthesized in the cytoplasm with a cleavable amino-terminal amphipathic helix that serves as a mitochondrial matrix targeting sequence, such as StAR (reviewed in Harbauer Angelika et al., 2014, **Figure 1**). The proteins are in complex with chaperones to maintain an unfolded state for import while the amino terminal sequence binds TOM20/22 receptors (Harbauer Angelika et al., 2014). However, a co-translational mechanism for import of matrix-localized proteins

with amino-terminal cleavable signal sequences was proposed over 40 years ago (Kellems et al., 1974) and recent data in yeast support this model. mRNAs co-purify with isolated mitochondria in wild-type yeast strains but not TOM20 deficient strains following cycloheximide (CHX) treatment (Eliyahu et al., 2010). These data indicate that blocking translation and stabilizing polysomes by CHX enhances ribosome-mRNA interactions with mitochondria in a TOM20-dependent manner. StAR has been shown to co-purify with TOM22 and siRNA knock-down of TOM22 in MA-10 cells results in diminished cholesterol metabolism by isolated mitochondria (Prasad et al., 2015, 2016). Since the StAR import mechanism is incompletely described, I use TOM20/22 to reflect both receptors work together for preprotein import in the classical mitochondrial import model. However, it may be that TOM20 and TOM22 can serve redundant functions for StAR import while TOM22 is required for StAR function (Rajapaksha et al., 2013, 2016).

Localized translation and post-translational modification of StAR at the mitochondria is an attractive model for the acute control of steroidogenesis (Dyson et al., 2008; Eliyahu et al., 2010; Lesnik et al., 2015). This model is supported by data that show StAR mRNA was associated with mitochondrial A-kinase anchoring protein 121 (AKAP121) and type II PKA in MA-10 mouse Leydig cells (Dyson et al., 2008, 2009). Independent studies have also demonstrated a PKA-dependent co-localization of AKAP121, acyl-CoA, and upstream ERK1/2 kinases with StAR at the mitochondria (reviewed in Poderoso et al., 2009; Paz et al., 2016). Thus, the co-localization of PKA and AKAP proteins at the mitochondria is emerging as a consistent theme. siRNA-mediated silencing of AKAP121 in MA-10 Leydig cells resulted in loss of PKA (PKAR2) association with mitochondria, independent of Bt<sub>2</sub>cAMP treatment, indicating that an AKAP121-PKAR2 complex is constitutively poised at the OMM (Dyson et al., 2008). Immunofluorescence-based detection of AKAP1 in H295R human adrenocortical cell line showed co-localization with TOM20, indicating localization with mitochondria in an adrenal cell line (Grozdanov and Stocco, 2012). Furthermore, a portion of StAR 3'UTR mRNA was shown to directly bind to AKAP1 and in H295R cells and StAR mRNA was recovered following AKAP1 immunoprecipitation (Grozdanov and Stocco, 2012). The StAR mRNA-AKAP1 interaction was detected only after Bt<sub>2</sub>cAMP treatment of H295R cells suggesting that newly transcribed StAR mRNA associates with AKAP1. Our earlier work demonstrated that StAR mRNA levels and endogenous protein levels are greatly diminished in the PKA deficient mouse Kin-8 adrenocortical cells following Bt<sub>2</sub>cAMP treatment compared to the PKA responsive Y1 adrenocortical cells (Clark et al., 1997; Clark and Hudson, 2015). Yet despite the lower StAR mRNA levels, the mRNA was associated with actively transcribing ribosomes and StAR protein synthesis rates were the same in both cell lines, indicating translation is intact in the absence of PKA signaling (Clark and Hudson, 2015; Clark et al., 1997). Conversely, in MA-10 cells that lack AKAP1 via siRNA-mediated silencing, Bt<sub>2</sub>cAMP treatment increased StAR steady-state mRNA levels but StAR protein levels were diminished. Together the data support that newly transcribed StAR mRNA associates with AKAP1 in adrenocortical cells (Kin8



**FIGURE 1 | Model for localized StAR translation and phosphorylation at mitochondria.** The TOM20/22-40 complex is an established pathway for import of matrix-localized proteins that contain an N-terminal cleavable signal peptide. StAR is modeled within this import pathway, although the import pathway for StAR remains to be determined. The ribosome nascent chain complex (RNC) is shown as StAR mRNA bound by the ribosome and protein depicted as black line with the N-terminus denoted (N). The START domain is depicted by the “folded” black line. StAR translation is modeled on both free ribosomes (steps c and d) and mitochondria-associated ribosomes (steps a to b or a' to b') as described in the text. Cleavage of the N-terminal signal sequence by matrix metalloproteases (indicated by the thunderbolt) produces the 30 kDa StAR protein (START domain) in the matrix. The import of StAR is the “off switch” for cholesterol transfer due to loss of interaction with an OMM protein complex. The OMM protein complex (indicated here as a green rectangle) that facilitates the StAR-dependent cholesterol transfer from the OMM to the matrix remains to be determined. Proposed OMM components of this complex include StAR, AKAP, PKA, VDAC1/2, TOM22, PCP, TSPO, and ACBD3. The OMM protein that interacts specifically with phosphoStAR to trigger complex formation for cholesterol transfer across the mitochondrial membranes remains to be identified. TIM (Translocase of the inner membrane) complex; IMS, intermembrane space; OMM, outer mitochondrial membrane; IMM, inner mitochondrial membrane. Concepts of this model have been presented by others in Dyson et al. (2008), Poderoso et al. (2009), Grozdanov and Stocco (2012), Aghazadeh et al. (2015), Prasad et al. (2015), Lee et al. (2016b), Midzak and Papadopoulos (2016), and Paz et al. (2016).

and H295R cells) and that the AKAP1-StAR mRNA association contributes to translation efficiency. The model that AKAP1 localizes PKAR2 at the OMM provides a platform for localized phosphorylation of StAR (Grozdanov and Stocco, 2012; Merrill and Strack, 2014, **Figure 1**). Recently StAR mRNA association with mitochondria in MA-10 cells was demonstrated using high resolution fluorescence *in situ* hybridization (Lee et al., 2016a). Key findings from this work indicate that StAR mRNA-mitochondrial interactions are dynamic, and suggest that when StAR transcription is maximally activated, newly transcribed and processed StAR mRNA is delivered to the cytoplasm and mitochondria for efficient translation.

The prevailing concept is that StAR phosphorylation influences key interactions with OMM proteins that promote cholesterol transfer. The challenge is that several StAR-interacting complexes at the OMM have been proposed yet the identity of the functional StAR cholesterol transfer complex remains to be validated (Bose et al., 2008; Rone et al., 2012; Issop et al., 2015). Early work characterized a OMM protein complex that included 18 kDa translocator protein (TSPO) and voltage-dependent anion channels 1 (VDAC1) (Liu et al., 2006). The working model was that upon hormonal stimulation StAR is expressed and associates with acyl-coenzyme A binding domain containing 3 protein (ACBD3), previously referred to as TSPO-associated protein

(PAP7), leading to a StAR-PKA-ACBD3-TSPO-VDAC1 complex at the OMM (reviewed in Papadopoulos et al., 2015; Midzak and Papadopoulos, 2016). *In vitro* studies using mitochondria isolated from sheep adrenal incubated with radiolabeled, *in vitro* synthesized StAR protein identified VDAC1 and phosphate carrier protein (PCP, SLC25A3) as components of a StAR-containing complex (Bose et al., 2008). VDAC1, VDAC2, TOM22, and StAR have been shown to be part of large multiprotein complexes purified from mitochondria or mitochondria-associated endoplasmic reticulum (ER) membrane (MAM) regions isolated from rat testes and MA-10 mouse Leydig tumor cells (Prasad et al., 2015). The functional significance for these proposed complexes is based on data that demonstrated siRNA-mediated silencing and/or pharmacological inhibition of TSPO, VDAC1, VDAC2, or ACBD3 decreased or blocked cAMP-PKA-mediated steroid production in steroidogenic cells or StAR-mediated steroid production in COS-1 cells. However, mouse models lacking either TSPO or VDAC1 expression are viable and fertile (Weeber et al., 2002; Sileikyte et al., 2014; Tu et al., 2014), although VDAC1<sup>-/-</sup> mice present with mitochondrial alterations in a strain-dependent manner. Loss of VDAC2 results in embryonic lethality (Anflous et al., 2001; Craigen and Graham, 2008). These studies indicate that TSPO and VDAC1 are not obligatory for steroidogenesis, and suggest a redundancy of function for cholesterol transfer

may occur in the absence of these factors. The question is whether StAR phosphorylation drives interaction with any of these OMM proteins. siRNA-mediated silencing of VDAC1 or PCP protein expression in COS-1 cells resulted in decreased vector-driven StAR protein levels with a corresponding decrease in phosphoStAR levels and steroidogenic response (Bose et al., 2008). The 30 kDa StAR was detected by Western blot analysis indicating that mitochondrial import of StAR was intact in VDAC1 null cells. However, 30 kDa phosphoStAR was greatly diminished indicating that in the absence of VDAC1, import of phosphorylated StAR into mitochondria was blocked. Addition of cysteine protease inhibitors to the VDAC1 deficient cells restored StAR phosphorylation and phosphoStAR mitochondrial import and steroid production to levels observed in control cells (Bose et al., 2008). One interpretation of these data is that VDAC1 may help stabilize precursor StAR allowing for StAR phosphorylation and enhanced cholesterol transport function. In a separate study, COS-1 cells with siRNA-mediated loss of VDAC2 expression retained vector-driven StAR synthesis and phosphorylation but there was no steroidogenic response (Prasad et al., 2015). In the absence of VDAC2, as with VDAC1, no mitochondrial import of phosphoStAR was detected (unphosphorylated StAR was not measured) (Prasad et al., 2015). Furthermore, a StAR-VDAC2 complex was immunoprecipitated from COS-1 cells overexpressing VDAC2 and N62StAR, providing the first evidence for a possible direct interaction between StAR and an OMM protein (Prasad et al., 2015). These data suggest that synthesis of phosphorylated precursor StAR is not sufficient for function, and support a role for specific phosphoStAR-OMM interactions mediating cholesterol transfer. The authors propose that StAR is synthesized at MAM regions and interacts with VDAC2 for subsequent mitochondrial import and StAR processing (Prasad et al., 2015). Perhaps the loss of phosphoStAR import in the absence of either VDAC1 or VDAC2 reflects a loss of StAR's N-terminal interaction with the TOM20/22-TOM40 complex, leading to loss of StAR structure which is necessary for cholesterol transfer function of StAR (Rajapaksha et al., 2013; Prasad et al., 2015, **Figure 1**).

In summary, ACTH action on StAR biology requires an immediate response to hormone stimulation, which is seen in a rapid increase in StAR transcription. StAR translation, phosphorylation, and mitochondrial import are likely

coordinated processes, but the mechanisms regulating these steps are lacking. Nevertheless, the work over the past decade supports a model for localized translation at the mitochondria. One proposed scenario is the newly transcribed StAR mRNA binds AKAP1 through 3'UTR-driven interactions localizing the mRNA to the mitochondria where it is bound by ribosomes and translated (**Figure 1**, step a and b). Alternatively, cytoplasmic StAR mRNA is bound by ribosomes and the ribosome-nascent-chain is directed to mitochondria where the interaction is stabilized by 3'UTR mRNA-AKAP1 interaction, similar to co-translational models described for yeast (**Figure 1**, step a' and b'). StAR mRNA 3'UTR driving mitochondrial location and localized translation could explain the amino-terminal truncated form of StAR (N62StAR), which lacks the signal sequence, being localized to the OMM and functional. Translation of the amino-terminal mitochondrial signal sequence, on the other hand, could lead to recognition by the TOM20/22-TOM40 complex, thereby tethering StAR to the TOM complex. StAR translation continues and phosphorylation of S194/195 of the START domain occurs in a co-translational process by the mitochondrial localized PKA. Post-translational mitochondrial targeting of StAR would follow the classical pathway; StAR is synthesized on cytoplasmic polysomes, bound by chaperones, and targeted to TOM20/22 by the N-terminal signal sequence (**Figure 1**, steps c and d). StAR phosphorylation could occur at any step in this classical pathway. In both the co- and post-translational models, there must be a mechanism allowing for folding of the START domain and phosphorylation of S194/195 that are necessary for interactions at the OMM that promote cholesterol transfer. StAR import is slowed by a pause domain (Bose et al., 1999, 2002), and this could permit time necessary to generate phosphoStAR by mitochondrial localized PKA. The retention of phosphoStAR's START domain at the OMM signals for cholesterol transfer. Thus, studies directed at testing StARS194/195-OMM protein interactions may help uncover the ACTH-dependent phosphoStAR-OMM protein interaction specific for cholesterol transfer.

## AUTHOR CONTRIBUTIONS

BC reviewed the literature, generated the figure, and wrote the mini-review.

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# Thirty-Eight-Year Follow-Up of Two Sibling Lipoid Congenital Adrenal Hyperplasia Patients Due to Homozygous Steroidogenic Acute Regulatory (STARD1) Protein Mutation. Molecular Structure and Modeling of the STARD1 L275P Mutation

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**Objective:** Review the impact of StAR (STARD1) mutations on steroidogenesis and fertility in LCAH patients. Examine the endocrine mechanisms underlying the pathology of the disorder and the appropriate therapy for promoting fertility and pregnancies.

**Design:** Published data in the literature and a detailed 38-year follow-up of two sibling LCAH patients. Molecular structure and modeling of the STARD1 L275P mutation.

**Setting:** University hospital.

**Patients:** Patient A (46,XY female phenotype) and patient B (46,XX female) with LCAH bearing the L275P mutation in STARD1.

**Interventions:** Since early-age diagnosis, both patients underwent corticoid replacement therapy. Patient A received estrogen therapy at pubertal age. Clomiphene therapy was given to Patient B to induce ovulation. Pregnancies were protected with progesterone administration.

**Main Outcome Measures:** Clinical and molecular assessment of adrenal and gonadal functions.

**Results:** Both patients have classic manifestations of corticosteroid deficiency observed in LCAH. Time of onset and severity were different. Patient A developed into a female phenotype due to early and severe damage of Leydig cells. Patient B started a progressive pubertal development, menarche and regular non-ovulatory cycle. She was able to have successful pregnancies.

**Conclusions:** Understanding the molecular structure and function of STARD1 in all steroidogenic tissues is the key for comprehending the heterogeneous clinical manifestations of LCAH, and the development of an appropriate strategy for the induction of ovulation and protecting pregnancies in this disease.

**Keywords:** LCAH, spontaneous puberty, fertility, pregnancy, steroidogenic acute regulatory protein (StAR, STARD1), cholesterol, molecular structure, therapy

## INTRODUCTION

Classical lipoid congenital adrenal hyperplasia (LCAH), which was originally described by Prader and Siebenmann (1957) is a severe form of congenital adrenal hyperplasia inherited as an autosomal recessive disease. Patients bearing this disorder show an impaired production of glucocorticoids, mineralocorticoids, and sex steroids. Their basal levels of adrenocorticotropin (ACTH) and plasma renin activity (PRA) are high with no steroid response to ACTH, or human chorionic gonadotropin (hCG) treatment. Signs of adrenal insufficiency and hyperpigmentation occur in phenotypic female infants regardless of the karyotype (Bose et al., 1996).

In LCAH, the destruction in early fetal life of Leydig cells of 46,XY subjects, by the toxic effect of stored cholesterol and derivatives, eliminates testosterone biosynthesis and normal virilization. In such cases, external genitalia are female with blind vaginal pouch and absence of cervix, uterus, and fallopian tubes due to undamaged Sertoli cells producing the Müllerian inhibitory hormone. Occasionally reported development of Wolffian ducts testifies for the presence of testosterone synthesis early in fetal life (Ogata et al., 1989). When adequately treated, 46,XX subjects may undergo spontaneous puberty, feminization and even cyclic vaginal bleeding (Matsuo et al., 1994; Bose et al., 1997; Fujieda et al., 1997). Despite the presence of sufficient amounts of estrogens to produce secondary sexual development and endometrial growth, progesterone levels remain undetectable, indicating that the periodic vaginal bleeding is of a non-ovulatory nature (Fujieda et al., 1997). Thus, affected individuals are all phenotypically female with a severe salt-wasting crisis and hyperpigmentation and may die shortly after birth unless treated with steroid-replacement therapy (Hauffa et al., 1985). Non-classical LCAH has also been reported: Affected individuals present with a phenotype of late onset adrenal insufficiency with only mild or minimally disordered sexual development (Baker et al., 2006; Sahakitrungruang et al., 2010).

**Abbreviations:** StAR, steroidogenic acute regulatory protein (STARD1); LCAH, lipoid congenital adrenal hyperplasia; P450ccc, cytochrome P450 side chain cleavage; Testo, testosterone; Andro, androstenedione; DHEA, dehydroepiandrosterone; Prog, progesterone; E<sub>2</sub>, 17 $\beta$ -estradiol; LH, luteinizing hormone; FSH, follicular stimulating hormone; 17 OH-Prog, 17 hydroxy-progesterone; 17 OH-Preg, 17 hydroxy-pregnenolone; ACTH, adrenocorticotropin; hCG, human chorionic gonadotropin; PRA, plasma renin activity; PCR, polymerase chain reaction; PMSG, post-menopausal stimulating gonadotropin; IVF, *in vitro* fertilization; PPROM, premature preterm rupture of membranes.

Because mitochondria from affected adrenal and gonads fail to transform cholesterol to pregnenolone, it has been postulated for many years that LCAH disease was due to a defect in the cholesterol side chain cleavage enzyme complex cytochrome P450 (P450ccc) (Degenhart et al., 1972; Koizumi et al., 1977; Matteson et al., 1986) which is the first step in steroidogenesis: The conversion of cholesterol to pregnenolone. In searching for such defect, Lin et al. (1991), Sakai et al. (1994) and Fukami et al. (1995) found no mutations in the *CYP11A1* gene of affected individuals. However, mutations in *CYP11A* gene were subsequently reported resulting in indistinguishable clinical and hormonal phenotypes when compared to LCAH cases (Tajima et al., 2001; Katsumata et al., 2002; Hiort et al., 2005). In addition, these patients do not have the massive adrenal hyperplasia that characterizes LCAH (Miller, 2016).

With the discovery of STARD1 (STeroidogenic Acute Regulatory) protein (Clark et al., 1994), the leading cause of LCAH was then attributed to mutations in that protein (Lin et al., 1995; Bose et al., 1996). STARD1 is produced in the cytoplasm and mediates the biosynthesis of steroid hormones by controlling the transfer of cholesterol from the outer mitochondrial membrane (OMM) to the inner mitochondrial membrane (IMM) where P450ccc is located (Farkash et al., 1986; Geuze et al., 1987). Moreover, numerous mutations disrupting STARD1 activity were found in the carboxyl-terminal of the protein (Lin et al., 1995; Bose et al., 1996; Bhangoo et al., 2005, 2006) suggesting that this is a biologically important domain. Another crucial domain for STARD1 is the cholesterol binding pocket which contains a putative salt bridge between Glu<sup>169</sup> and Arg<sup>188</sup> (Mathieu et al., 2002a). Other clinical STARD1 mutations are found at the binding site, in particular Glu<sup>169</sup> and Met<sup>225</sup>, and they lead to LCAH (Miller, 1997).

In the absence of STARD1, steroidogenesis proceeds, for a temporary period, at about 14% of the STARD1 induced level (Lin et al., 1995; Tee et al., 1995; Bose et al., 1996; Miller, 1997; Khoury et al., 2006a). The pathogenesis of the different manifestations of LCAH involves STARD1-dependent and STARD1-independent steps of steroidogenesis (Bose et al., 1996; Miller, 1997). The STARD1-dependent phase would be the loss of the protein activity leading to a decrease of more than 80% of adrenal and gonad steroidogenesis. The STARD1-independent stage is characterized by a destruction of the steroidogenic capacity due to the accumulation of cholesterol, cholesterol esters and oxidation products. This engorges the cells and damages its cytoarchitecture through both physical displacement and biochemical actions.

We have previously communicated the cases of LCAH in two children (46,XX and 46,XY) of a French Canadian family due to a homozygous L275P STARD1 mutation (Khoury et al., 2004). Furthermore, ovulation and pregnancies in the 46,XX patient were possible with Clomiphene stimulation and progesterone administration during the first trimester (Khoury et al., 2006b, 2009). Here, we review data collected over a three-decade follow-up of these patients focusing on their clinical evolution, gonadal function, puberty and fertility. Finally, the STARD1 mechanism of action and the impact of the clinical mutants on the protein's structure/activity will be addressed. An informed consent form was signed by all participants for blood sampling and analysis, and studies were approved by the institutional "Comité d'éthique de la recherche en santé chez l'humain du CHUS".

## PATIENT A (46,XY KARYOTYPE)

Patient A was born in summer 1977 after 41 weeks of normal gestation, weighing 3960 g and measuring 53 cm (Khoury et al., 2004). External genitalia were described as normal female phenotype with bilateral inguinal hernia. This was surgically repaired at the age of 5 weeks with no complications. Both gonads observed during surgery were pushed into the abdomen. At the age of 11 months, the patient suffered from gastroenteritis, hyperthermia, and dehydration. Blood pressure was 100/50, heart rate 120/min, and respiratory rate 24–28/min. Blood glucose was 3.8 mmol/L, sodium 121 mmol/L, chloride 93 mmol/L, potassium 5.4 mmol/L, and serum bicarbonate was 7 mmol/L. Intravenous fluid and glucose were administered to correct this situation. Four days after the cessation of the intravenous therapy, electrolytes disturbance and metabolic acidosis relapsed (sodium 126 mmol/L, chloride 103 mmol/L, potassium 6.3 mmol/L, sodium bicarbonate 14 mmol/L, and blood glucose 4.6 mmol/L). Intravenous rehydration was restarted. Physical examination was normal except for generalized and moderate hyperpigmentation, hypertrichosis on arms and on the lower part of her back. The external genitalia corresponded to those of a normal female with normal clitoris and vaginal orifice. There were no palpable gonads in the inguinal region and the scar of the previous surgical procedure appeared normal.

### Adrenal Function

At the age of 11 months, serum cortisol was 640 nmol/L at 8 h 00 and 361 nmol/L at 16 h 00 ( $N = 165\text{--}635 \text{ nmol/L}$ ); serum aldosterone was respectively 641 and 319 pmol/L ( $N = 168\text{--}2570 \text{ pmol/L}$ ) and plasma renin activity was 11.9 ng/ml/s at 16 h ( $N < 4.17 \text{ ng/ml/s}$ ), and urine pregnanetriol 0.0  $\mu\text{mol/d}$  ( $N = 0.06\text{--}0.6$ ). Further investigations showed that serum cortisol, aldosterone as well as urinary pregnanetriol did not respond to intramuscular ACTH stimulation for 3 days (Table 1). Substitution therapy with mineralocorticoid (fludrocortisone acetate) and glucocorticoid (cortisone acetate) was then started. Four months later, we decided to stop cortisone acetate to obtain more data on her glucocorticoid secretion.

For the following 20 months, the child was growing well (50 percentile for height and weight) and has no specific

symptoms. From 35 to 41 months of age, the patient was admitted to the hospital for 3 episodes of hypoglycemia and acidosis during infectious illnesses. Blood glucose was 1.4–4.0 mmol/L, electrolytes were normal with high renin activity and elevated urinary sodium. Basal steroid hormones (while receiving fludrocortisone acetate 0.025 mg every 12 h) revealed low cortisolemia\* 69 and 8.28 nmol/L at 8 and 16 h respectively, normal aldosterone\*\* 737 and 208 pmol/L, 17 OH-progesterone 0.15 nmol/L ( $N = 0.1\text{--}2.7$ ), DHEA 0.69 nmol/L ( $N = 0.38\text{--}2.52$ ), DHEA-S 0.54  $\mu\text{mol/L}$  ( $N = 0.13\text{--}0.54$ ) testosterone 0.1 nmol/L ( $N = 0.07\text{--}0.9$ ), and ACTH 33 pmol/L ( $N = 2\text{--}11$ ). ACTH stimulation test was repeated at that time (intramuscular injections of ACTH-Gel 25 IU/m<sup>2</sup> every 12 h  $\times$  5 days) under replacement therapy with fludrocortisone acetate 0.025 mg every 12 h and Dexamethasone 0.25 mg every 8 h. The higher values of blood cortisol and aldosterone obtained during this stimulation was 19.3 and 263 pmol/L respectively, urinary free cortisol\*\*\* was  $< 5.5 \text{ nmol/day}$  and pregnanetriol\*\*\*\* 0.11  $\mu\text{mol/day}$  at that moment. The result of previously ordered blood karyotype revealed the suspected 46,XY. Then clinical, biochemical and genetic studies confirmed the suspected diagnosis of what was believed at that time as 20,22 desmolase deficiency. Substitution therapy with fludrocortisone acetate and hydrocortisone continues, doses were regularly adjusted according to clinical symptoms, blood glucose and electrolytes as well as serum level of ACTH and PRA in addition to observations of growth and bone maturation.

Normal basal values from 2–5 years:

\* Cortisol 166–525 nmol/L (mean  $\Delta$  after ACTH stimulation 400 nmol/L)

\*\* Aldosterone 83–971 pmol/L (mean  $\Delta$  after ACTH stimulation 500 pmol/L)

\*\*\* Urinary free cortisol 8.28–24.8 nmol/d

\*\*\*\* Urinary pregnanetriol  $< 1.5 \mu\text{mol/d}$

### Radiological Investigations

At the moment of her first admission, at 11 months of age, the IV pyelography was normal. Several pelvic and abdominal echographies were performed from the age of 3 6/12 to 13 years. These showed no uterus, no visible gonads and no adrenal hypertrophy, the bone age was always significantly delayed (growth curve not shown).

### Gonadal Function

FSH, LH and different androgens were measured at regular intervals from the age of ~3 years until the patient was 13 year old (Table 2). As can be seen, the levels of serum androstenedione, dehydroepiandrosterone, total and free testosterone were low to not detectable. hCG stimulation test (intramuscular injections 4000 IU/day for 5 days) was performed at the age of 13 years (while the patient was receiving fludrocortisone acetate and Dexamethasone). As shown in Table 2, hCG stimulation did not increase circulating levels of 17 OH-pregnenolone, 17 OH-progesterone, DHEA, androstenedione, and total and free testosterone.

**TABLE 1 | Patient A.**

Dates	Serum cortisol nmol/L (165–635)	Serum aldosterone pmol/L (168–2520)	Plasma renin ng/L/s (<4.17)	Urine pregnanetriol $\mu$ mol/day (0.06–0.6)
Day 2	966	670	19.46	0
Day 1	634	621	22.2	<0.03
Day 0	739	474	11.95	<0.03
Day 3	811	244	15.29	<0.03
Day 4	497	357	13.34	<0.09

ACTH stimulation test: Injection of ACTH-Gel 25 IU/m<sup>2</sup> /q 12 h/3 days During this test, the patient received NaCl 70 meq/d, the serum sodium was 120–131 mmol/L, potassium 6.0–6.9 mmol/L and urinary sodium 51–64 mmol/L, at 8 and 16 h respectively. Age of patient A, 11 months.

**TABLE 2 | Patient A.**

Hormones age	FSH IU/L	LH IU/L	17 OH-Preg nmol/L	17 OH-Prog nmol/L	DHEA nmol/L	Andro nmol/L	Testo (total) nmol/L	Testo (free) nmol/L
~3 years	–	–	–	0.15	0.69	–	0.1	–
~4 years	–	–	–	–	< 0.35	< 0.3	< 0.35	–
5 10/12 years	3.9	< 2.7	–	–	1.73	< 0.3	< 0.35	–
6 7/12 years	13.8	< 5	–	–	2.08	< 0.7	< 0.35	–
7 6/12 years	8.6	< 5	–	–	–	–	< 0.35	–
8 years	23	< 5	–	–	–	–	< 0.35	–
8 9/12 years	11.8	5.1	–	–	–	–	< 0.35	< 0.6
10 4/12 years	11.6	< 2	–	–	–	–	< 0.35	< 2
12 6/12 years	4.6	< 0.5	–	–	< 1.7	< 0.3	< 0.7	< 3
hCG (M) 4000 IU/d <sup>5</sup> days	D 0	33.2	1.8	< 0.16	< 0.3	< 1.5	< 0.3	< 0.7
	D 3	–	–	< 0.16	< 0.3	< 1.5	< 0.3	< 0.7
	D 4	–	–	< 0.16	< 0.3	< 1.5	< 0.3	< 0.7
	D 6	–	–	< 0.16	< 0.3	< 1.5	< 0.3	< 2.1

T, total; F, free; D, day; –, not available. Normal values are indicated for different ages:

FSH: Prepubertal 0.26–3 IU/L, puberty Tanner II, III 0.6–10.9 IU/L.

LH: Prepubertal 0.02–0.3 IU/L, puberty Tanner II, III 0.2–5.0 IU/L.

17-OH pregnenolone: Puberty Tanner II, III 0.6–10.9 nmol/L.

17-OH progesterone: Prepubertal 0.09–2.7 nmol/L, puberty Tanner II, III 0.3–3.9 nmol/L.

DHEA: 2–5 years 0.38–2.52 nmol/L, 6–10 years 0.9–5.3 nmol/L, puberty Tanner II, III 0.87–10.5 nmol/L.

Androstenedione: 1–10 years 0.18–1.78 nmol/L, puberty Tanner II, III 0.6–2.87 nmol/L.

Testosterone (total): 3–10 years 0.07–0.66 nmol/L, puberty Tanner II, III 0.63–11.2 nmol/L.

Testosterone (free): Prepubertal 0.52–2.0 pmol/L, adult male 180–971 pmol/L.

Gonadal function of Patient A measured at different interval from age 3 to 13 years old.

## Gonadal Histology

A laparotomy was performed at the age of 13 5/12 years: The left gonad (3 × 1.5 × 1 cm) was removed. The exploration of the right side was negative and showed no Müllerian or Wolffian structures. The histology of the removed gonad is illustrated in Figure 1. Worth noticing is the atrophy and hyalinization of the seminiferous tubules, the presence of some germinal cells without spermatogonia, the presence of some Sertoli cells and the important engorgement of Leydig cells with fat.

## Growth

The patient's growth velocity decreased regularly from the age of 10 years until she was 14 year old. Bone age was always significantly delayed (curve not shown). The thyroid function and growth hormone secretions were normal. Progressive ethinyl estradiol therapy was started at the age of 14 leading to a gradual pubertal development (good breast development, moderate

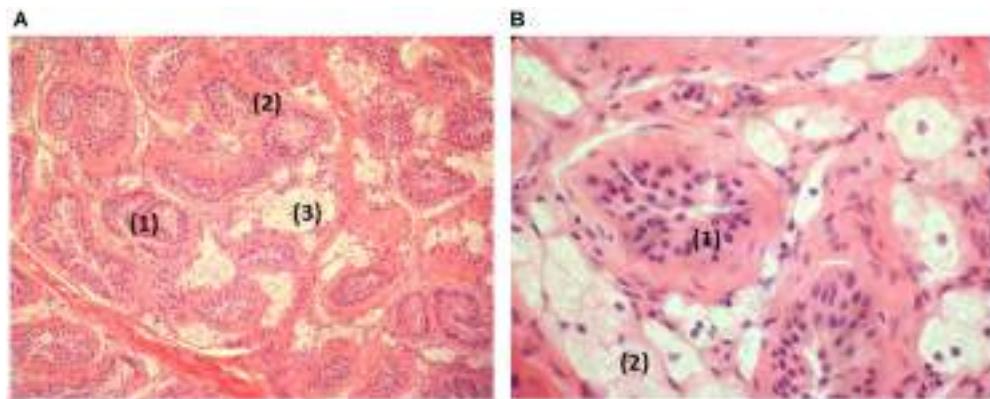
pubic hair) and an increase of the growth rate. Final adult height was in the normal mid-parental range.

## PATIENT B (46,XX KARYOTYPE)

Patient B was born in spring 1979 (Koizumi et al., 1977; Khoury et al., 2004). She had skin hyperpigmentation since the age of 1.5 months. At 4.5 months, she was hospitalized for fever, anorexia, fatigue and weight loss, and was treated with antibiotics (pharyngitis) for 10 days. Furthermore, she suffered from somnolence, vomiting and dehydration with hyponatremia and hyperkalemia (Khoury et al., 2009).

## Adrenal Function

The endocrine investigations revealed a low/normal serum cortisol 174 nmol/L ( $N = 77$ –635), low/normal aldosterone 138.7 pmol/L ( $N = 166$ –1970) and renin activity 3.3 ng/L/s



**FIGURE 1 | Histology of the removed gonad.** (A) Testis in low magnification: atrophic seminiferous tubules (1). Hyaline thickening of the basal membranes (2). Hyperplasia and clarification of the Leydig cells (3). (B) Testis in higher magnification: atrophic seminiferous tubules (1) containing Sertoli cells in the absence of spermatogonia. Lipid overload of the Leydig cells (2).

**TABLE 3 | Patient B.**

Time	Serum cortisol nmol/L (166–525)	Serum aldosterone pmol/L (139–1498)	Plasma renin ng/L/s (<2.8)
Day 1	18.2	136	0.88
Day 0	5.52	161	6.47
Day 2	8.28	136	3.27
Day 4	13.8	180	5.36

ACTH stimulation test: Injection of ACTH-Gel 25 IU/m<sup>2</sup>/12 h/3 days. Age of Patient B, 21 months. Normal values are in parentheses.

( $N < 4.17$ ). The urinary pregnanetriol was 0.59  $\mu$ mol/day ( $N = 0.06\text{--}0.6$ ). The ACTH stimulation (IM injections of ACTH-Gel 25 IU/m<sup>2</sup> q 12 h.  $\times$  3 days) resulted of very small increases in cortisol (220 nmol/L) and aldosterone (277.4 pmol/L). She was growing well with no specific symptoms while treated only with fludrocortisone acetate 0.025 mg bid, up to the age of 15 months. From that age to 21 months she suffered 3 episodes of hypoglycemia during a varicella illness and otitis media. Her serum cortisol level was 8.28 nmol/L ( $N = 166\text{--}690$ ), serum aldosterone and plasma renin activity were 141.3 pmol/L ( $N = 139\text{--}1498$ ) and 0.67 ng/L/s ( $N < 2.8$ ) respectively. Then Dexamethasone 0.25 mg p.o. every 8 h was added to fludrocortisone acetate before to undergo her second ACTH stimulation test. Results illustrated in Table 3 indicate a very low basal cortisol and incapacity of the zona fasciculata-reticularis to respond to ACTH stimulation; the same table shows low level of aldosterone with no response of the zona glomerulosa to ACTH stimulation. Consequently, the doses of glucocorticoids (Dexamethasone changed for hydrocortisone) and fludrocortisone acetate, were adjusted regularly during all the next years, according to her clinical evolution, blood glucose and electrolytes, as well as plasma ACTH and renin activity.

## Gonadal Function

The patient's serum levels\* of FSH and LH were normal up to puberty. Serum levels of 17 OH-Prog, DHEA, androstenedione, and testosterone were very low. Plasma ACTH varied from

43–4.6 pmol/L during that period. She started thelarche and pubarche at the age of 11 7/12 and 12 years, respectively. The hypophyso-gonadal function was previously summarized (Khoury et al., 2009). A spontaneous normal menarche started at the age of 14 2/12 years. The menstruations were regular with no dysmenorrhea. The basal temperature registered between 21 and 24 years indicated a monophasic non-ovulatory curve. Shortly after a Clomiphene stimulation, circulating 17 $\beta$ -estradiol level increased from 703 to 1428 pmol/L and progesterone level increased from 3.4 to 9.4 nmol/L (Khoury et al., 2004).

\*Normal prepubertal values: FSH 1.0–4.2 IU/L, LH 0.02–0.18 IU/L, 17-OH progesterone 0.09–2.72 nmol/L, DHEA 0.4–6.6 nmol/L, androstenedione 0.28–1.75 nmol/L, testosterone 0.07–0.35 nmol/L.

## Radiological Investigations

An abdominal and pelvic echography was performed at the age of 2 5/12 years. It revealed a normal uterus and ovaries. Adrenal glands have no particular aspect. This examination was repeated at 11 1/12 years and 12 3/12 years and showed a prepubertal uterus and normal ovaries with small follicles. A small delay of bone age was noted at different ages.

## Growth

Regular growth was observed. Peak height velocity began around the age of 13 years. Final adult height was in the normal mid-parental range.

## Fertility Studies and Childbirth

Clomiphene stimulation, to increase gonadal activities and induce ovulation, was started at the age of 25 years. First pregnancy occurred at the age of 25 4/12 years, unfortunately interrupted by spontaneous abortion 6 weeks later (Khoury et al., 2009). A second pregnancy (quadruplet) was initiated later on with Clomiphene stimulation, and was protected with progesterone supplementation at the 17th day of the cycle. One fetus was naturally lost at 7 weeks of gestation and one feticide was done 1 ½ weeks later, so progesterone was continued up to the 25th week of gestation. In October 2005, two normal boys

were born prematurely. This first child delivery was previously communicated in an international meeting in 2006 (Khoury et al., 2006b). At 28 years old, a third pregnancy initiated with Clomiphene stimulation, and progesterone was taken from the 17th day of the cycle up to the 17th week of gestation. After 36 weeks of normal gestation, a female child was born in October 2008 (Khoury et al., 2009).

## Molecular Biology Studies

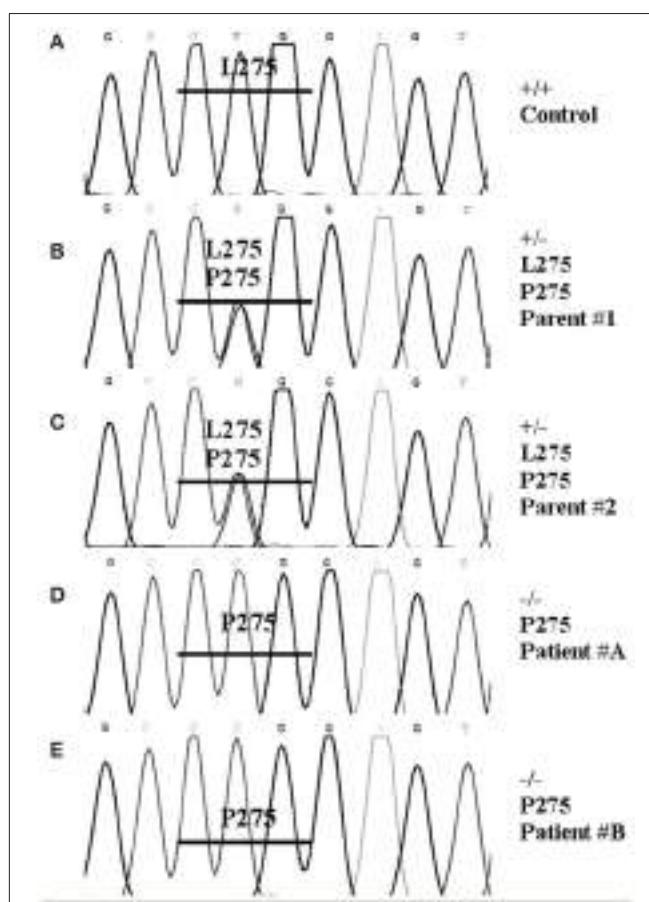
Genomic DNA was extracted from the gonadal tissue of patient A. The seven exons of the STARD1 gene were PCR-amplified using primers and conditions previously described (Bose et al., 1996) and PCR products were sequenced. A mutation was found in exon 7 which was located at the amino acid residue 275, a leucine being substituted by a proline. Genomic DNA was subsequently extracted from the blood leukocytes of the two patients, their two parents and a normal individual. The exon 7 was analyzed for each individual. For the mutation L275P, the control was  $+/+$ , the two parents were  $+/-$ , and the two children were  $-/-$  (Figure 2).

COS-1 cells were used to determine STARD1 activity and quantification. Figure 3A shows results obtained by western blotting analysis, and Figure 3B shows that the L275P mutant activity was 87% impaired compared to wild type (Khoury et al., 2004).

## MECHANISM OF CHOLESTEROL TRANSFER BY STARD1

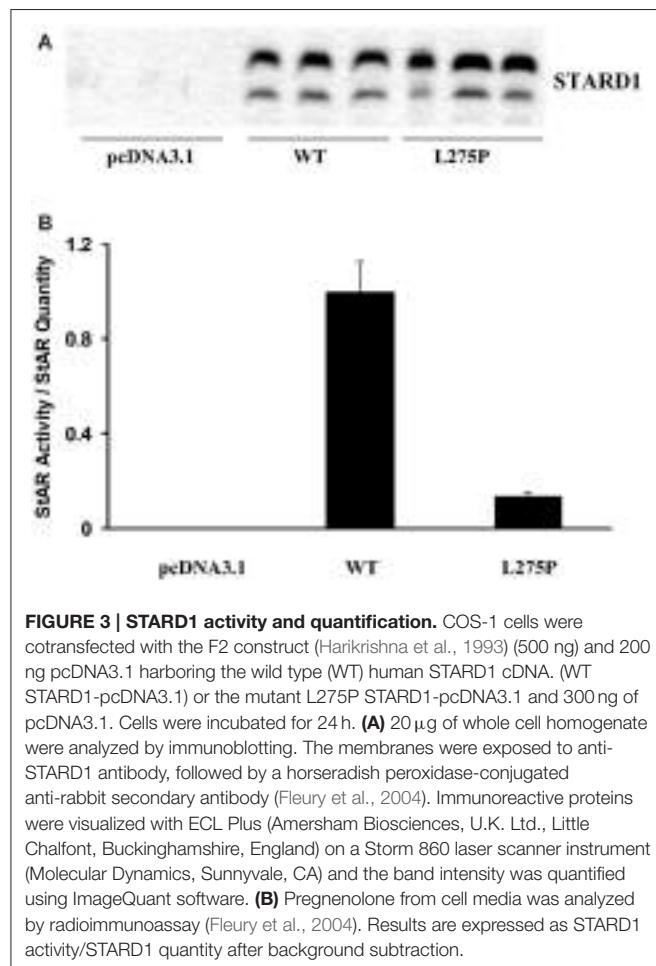
In order to better explain how clinical mutations affect steroidogenesis, we will discuss the mechanism of action of STARD1 at the molecular level. STARD1 delivers cholesterol from the outer to the inner membrane of mitochondria is poorly understood. STARD1 may act as a sterol transfer protein and enhance sterol desorption from the outer to the inner side of mitochondria (Kallen et al., 1998; Petrescu et al., 2001). In this model STARD1 is directed to the mitochondria via its N-terminus recognition sequence and then, utilizing C-terminal sequences, produces as yet unidentified alterations in the OMM that results in the transfer of cholesterol to the inner side. It is established that STARD1 acts on the OMM and that its level of activity is proportionate to the time it remains at that site (Arakane et al., 1998; Bose et al., 2002).

MLN64, a protein involved in cholesterol transfer in malignant breast cancer, has a significant homology to the C-terminal region of STARD1 and can promote cholesterol transfer (Watari et al., 1997). The C-terminus of STARD1 contains the StAR-Related Lipid Transfer (START) domain which is conserved across a large family of proteins including MLN64 and STARD1 (Ponting and Aravind, 1999; Stocco, 2000; Tsujishita and Hurley, 2000). START domain in MLN64 is homologous to the START domain of the STARD1 protein. Both, functionally similar, are able to bind cholesterol in a 1:1 ratio (Tsujishita and Hurley, 2000; Petrescu et al., 2001). It was first proposed that STARD1 acts as a cholesterol shuttle for transferring cholesterol to the IMM via a hydrophobic tunnel



**FIGURE 2 | DNA sequencing.** Genomic DNA was extracted from the blood of two patients, their two parents and a normal individual as control (**A**) using the Invitrogen DNazol BD reagent. The seven exons of the STARD1 gene were PCR and sequenced. A mutation was found in exon 7. For exon 7 oligonucleotides used for PCR were (5' to 3') ATGAGCGTGTTACCACTGACG, (5' to 3') CCTGGCAGCCTGTTGTGATAG; the annealing temperature was 60°C and the reaction processed for 30 cycles. The PCR products were sequenced. The mutation found in exon 7 was located at the amino acid residue 275, a leucine being substituted by a proline. The two parents (**B,C**) were  $+/-$ , and the two children (**D,E**) were  $-/-$ .

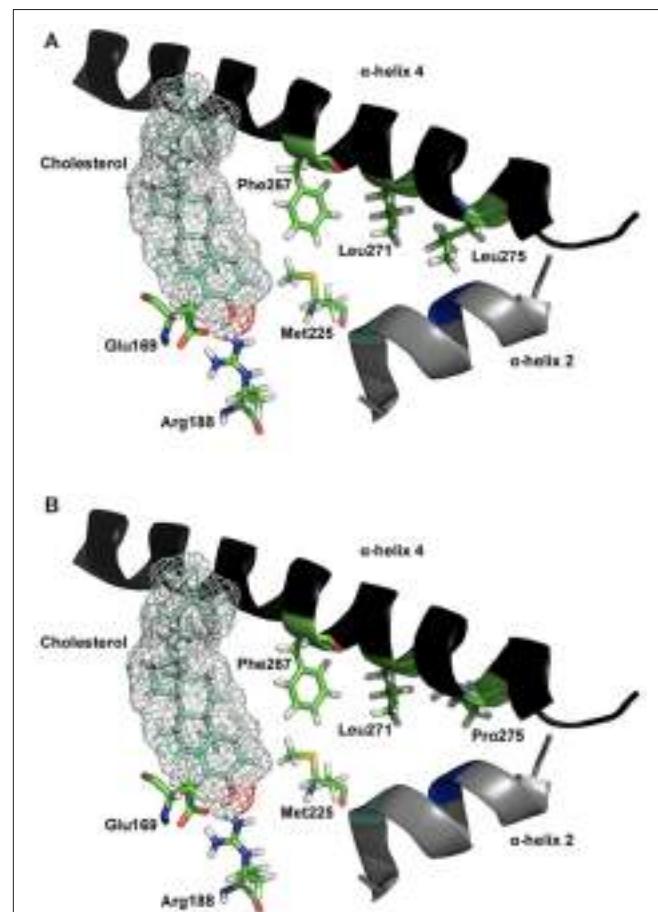
revealed by the crystal structure of MLN64-START (Tsujishita and Hurley, 2000), but the exact mechanism by which STARD1 releases cholesterol to the IMM is unknown (Stocco, 2001). According to computer homology models based on MLN64 structure, STARD1 does not contain a tunnel, but a hydrophobic pocket that can accommodate one molecule of cholesterol (Mathieu et al., 2002a,b; Baker et al., 2005; Yaworsky et al., 2005; Murcia et al., 2006; Lavigne et al., 2010; Thorsell et al., 2011; Létourneau et al., 2014, 2015); the STARD1 crystal structure corroborates this hydrophobic pocket. Several mutations that result in LCAH were mapped onto the MLN64-START structure, in positions residing within the hydrophobic tunnel; these mutations would destabilize the tunnel. In parallel, the residues Glu<sup>169</sup>, Met<sup>225</sup>, and Leu<sup>275</sup> are part of STARD1's sterol binding pocket and the hydrophobic environment provided by  $\alpha$ -helices 2, 3, and 4 (Figure 4). Two regions of STARD1 appear important



for the binding of cholesterol and its release during its transfer to the IMM. A cholesterol gating region containing the C-terminal  $\alpha$ -helix 4 and a cholesterol binding region buried inside the STARD1 molecule (Roostaei et al., 2008). More specifically, the importance of STARD1's C-terminal region in cholesterol transfer is confirmed by many observations; deletion of 28 amino acids from the C-terminal results in a complete loss of steroids production (Arakane et al., 1996; Wang et al., 1998). This C-terminal deletion corroborates with the truncated STARD1 mutant Q258X and lead to LCAH (Miller, 1997).

*In vitro* experiments have highlighted the role of  $\alpha$ -helix 4 as a gate for cholesterol binding (Baker et al., 2005; Roostaei et al., 2008). The helix would be in thermodynamic equilibrium between a partially-unfolded (open) and folded (closed) states (Mathieu et al., 2002a). Upon cholesterol binding, the equilibrium is shifted to the more stable closed state (Baker et al., 2005; Roostaei et al., 2008; Barbar et al., 2009a), until the subsequent release of the ligand by other mechanisms, such as interactions with other mitochondrial proteins, to trigger the transfer of cholesterol from the outer to the inner mitochondrial membrane (Liu et al., 2006; Bose et al., 2008a).

The hypothesis is that the STARD1 C-terminal  $\alpha$ -helix 4 movement is a key factor for STARD1-mediated cholesterol transfer inside mitochondria (Mathieu et al., 2002a,b; LeHoux



et al., 2003). For instance, biochemical and structural studies of the L275P mutation at the  $\alpha$ -helix 4 led to a dramatic reduction in cholesterol binding that correlated with a decrease in the steroidogenic activity of STARD1 (Roostaei et al., 2008).

Several amino acids are in contact with the sterol binding pocket. **Figure 4A** shows Leu<sup>275</sup> and other residues forming a hydrophobic core near the end of the C-terminal  $\alpha$ -helix 4, and **Figure 4B** shows the model for the mutation Leu<sup>275</sup> to proline. Any alterations in these residues, forming a line of hydrophobic residues, would affect the binding and the steroidogenic activity of STARD1. In the case of the L275P mutation, it yielded a 13%

pregnenolone production in isolated mitochondria (Roostaei et al., 2008); such a level of steroidogenesis is in agreement with previous studies (Bose et al., 1996; Khoury et al., 2004).

The far-UV CD spectra of STARD1 WT has a 32.5%  $\alpha$ -helical content, which is close to the 40% maximum helical content calculated for the three-dimensional model. The L271N and L275P mutants showed a reduced  $\alpha$ -helical content of 28.8 and 29.4% respectively compared with STARD1 WT. As discussed above, these mutants were expected to lead to the weakening of the hydrophobic interface and hence to a reduction in the amount of  $\alpha$ -helical content and stable tertiary structure (Roostaei et al., 2008). Also, short molecular dynamics simulations have indicated that the L275P mutation confers a higher flexibility to STARD1's  $\alpha$ -helix 4 (Barbar et al., 2009b), which suggests a more open state of the helix, thereby reducing its gating capacity, which is related to the decrease in cholesterol binding and the steroidogenic activity previously studied (Bose et al., 1998; Roostaei et al., 2008).

Interestingly, another clinical leucine to proline mutation (L260P) at the  $\alpha$ -helix 4 has been found in Swiss LCAH patients and exhibited a similar *in vitro* steroidogenic activity as the L275P mutant (Flück et al., 2005). Hence, one would expect that the L260P mutant might structurally behave in a similar fashion as L275P, by affecting the  $\alpha$ -helix 4 gating mechanism.

## FUNCTIONAL ACTIVITIES

Functional activities determined in transfected COS-1 cells revealed differences between different mutants. On the two LCAH patients reported in the study of Lin et al. (1995), the Q258X STARD1 mutant had a functional activity of 17% and the R193X mutant had 14%. Bose et al. reported that the L275P mutant converted 10% more cholesterol to pregnenolone than the vector control (Bose et al., 1996). In 19 Japanese patients, Nakae et al. reported 8 different mutations and in functional studies only the M225T mutation was found to have a partial activity of 44% (Nakae et al., 1997). All other mutants had activities not greater than that of the vector plasmid. STARD1-independent pregnenolone production in the three above mentioned studies was about 14%. Nakae et al. reported on a patient with Q258X, M225T (heterozygous compound) mutations who had a cliteromegaly at the time of diagnosis and moderate elevation of serum testosterone in response to hCG stimulation (Nakae et al., 1997). This patient had the onset of symptoms of adrenocortical insufficiency at the age of 10 months. These authors suggested that late onset of LCAH results from mutations that do not completely inactivate STARD1. The Canadian patient cited by Bose et al. (1996) had the heterozygous compound mutation A218V, L275P, and 46,XY karyotype; her manifestations of corticosteroid deficiency were noted at the age of 2 months.

## STARD1 INDEPENDENCE

STARD1 is mainly expressed under acute tropic hormone stimulation. In the absence of STARD1, several elements may account for the basal level of steroid hormone production. For

example, MLN64 may be able to enhance the basal low level of steroidogenesis since it is present in human granulosa and theca cells as well as in the fetal adrenal cortex (Watari et al., 1997). Other proteins with a START domain (like STARD4 and D6) might contribute to the basal steroidogenic activity (Soccio et al., 2002; Bose et al., 2008b), but their precise distribution in steroidogenic tissues and role are yet to be clarified. The synthesis or uptake of hydroxysterols might also contribute to the STARD1-independent steroidogenesis; for example, soluble 22R-OH and 25 OH-cholesterol can diffuse to the inner mitochondrial membrane without the participation of STARD1 and be used as substrate for pregnenolone formation (Lin et al., 1995; Roostaei et al., 2008).

## LCAH AND STARD1 MUTATION IN CANADA

In this review, we report the first detailed study of LCAH due to homozygous STARD1 mutation in Canada and, to our knowledge, the first successful pregnancy in a 46,XX female with this disease. Since the discovery of STARD1 in 1994 (Clark et al., 1994), many important works contributed to increase our understanding on the role played by this protein in the steroidogenesis and the physiology of LCAH. We have identified a homozygous recessive mutation on the STARD1 gene of our two patients and the heterozygous mutation in their mother and father. This mutation resulted in the substitution of a leucine by a proline (L275P) in the STARD1 protein (Khoury et al., 2004), which did not conserve more than 13% of its steroidogenesis activity compared to wild type STARD1, as analyzed in COS-1 cells. This is in agreement with Bose et al. (1996) who reported that the mutated L275P STARD1, found in another Canadian, converted 10% more cholesterol to pregnenolone than the control vector. The Canadian patient cited by Bose et al. (1996) had the heterozygous compound mutation A218V, L275P, and 46,XY karyotype; her manifestations of corticosteroid deficiency were noted at the age of 2 months. We did not have the opportunity to obtain a genetic history for this patient; we know however (personal communication from Dr. Rose Girgis, Edmonton, Canada) that the parents are from French Canadian origin who lived in the province of Quebec before moving to the west. It is unknown if any parental connection exists between these two families who share the allele L275P. Although the number of patients bearing the L275P mutation is too small to be conclusive, it might indicate a possible founder effect for this disease in Canada (Bose et al., 1996).

## CLINICAL MANIFESTATION AND EFFICIENCY OF CHOLESTEROL TRANSFER SYSTEM

The two patients, A and B, described in this study had a normal health before their first admission to the hospital for severe manifestations of mineralocorticoid deficiency and hyperpigmentation at the age of 11 and 4 1/2 months respectively. Some residual secretion of aldosterone was present and basal

blood cortisol was in the low normal level, but without any significant response to ACTH stimulation up to the age of 3 years for patient A and only 15 months for her sister. Consequently, patient A seemed to have less severe corticosteroid deficiency than patient B and both patients have less severe mineralocorticoid and glucocorticoid deficiencies than reported in similar cases (Bose et al., 1996).

To date, at least 97 patients with LCAH and more than 35 different mutations have been reported in the STARD1 gene (Bhangoo et al., 2005, 2006; Papadimitriou et al., 2006; Abdulhadi-Atwan et al., 2007). Two distinct genetic clusters were initially reported: More than 70% of Japanese alleles and all Korean alleles carry the Q258X mutation; a second genetic cluster is found among Palestinian Arabs, most of whom carry the R182L mutation. Many publications underline that some correlations exist between the severity of STARD1 mutation and the age of onset of a clinical salt-wasting crisis, hypoglycemia and gonadal function (Bose et al., 1996; Miller, 1997; Bhangoo et al., 2005, 2006). Patients with the mutation Q258X or R182L are generally symptomatic by the age of 1 day to some weeks. Three unrelated patients of Swiss ancestry, L260P mutant, had their onset of symptoms at the age 2.5–5.5 months (Flück et al., 2005). Furthermore, as reported by Chen et al. (2005) patients from eastern Saudi Arabia who carry the R182H mutation, very close to the Palestinian mutation R182L, had a milder disease starting at the age of 1–14 months, and 4 out of 7 of them had the onset of their symptoms from 7–14 months of age; functional study in this group showed a complete loss of STARD1 activity. One patient from Qatar, with R182H mutation, had the onset of clinical symptoms and laboratory evidence of salt loss at the age of 3 weeks (Achermann et al., 2001); the *in vitro* STARD1 activity of this patient was not determined. The patients reported by Chen et al. (2005) as well as our two patients raise the question about the different clinical manifestations and time of onset with the same genetic mutations, in the same ethnic group or family members and the same geographic region. There is, clearly, a limited sensitivity of the different functional assays for determination of STARD1 activity which may explain the poor correlation frequently observed between the clinical findings and the results of *in vitro* studies. There is also a wide spectrum of heterogeneity in the clinical manifestations and biochemistry, as well as, the time of onset of different symptoms in LCAH. However, in our case, since the assays for Patients A and B are relatively standardized, the sensitivity is the same.

Interestingly, both patients carry the same mutation, yet the clinical manifestation of LCAH was delayed between them. The physiological conditions are different from one pregnancy to another and the demand for different steroids is variable during fetal development. Indeed, during pregnancy, conditions such as nutrition, hydration/dehydration, disease and other stress, may have an influence on steroidogenesis. In addition, the circumstances of delivery and the neonatal environment may have different patterns of tropic hormone stimulation which may modulate the extent of acute steroidogenic demands.

The STARD1 -dependent and STARD1 -independent steroidogenesis seem to be good descriptive models to explain the chronology of events in non-placental steroidogenic tissues.

It is not clear however what determines the length of time an infant with LCAH can survive before experiencing a salt-wasting crisis (Hauffa et al., 1985; Fujieda et al., 2003; Bhangoo et al., 2006). We still need more information concerning the function or loss of function of the mutated STARD1 and what determines the longevity and efficiency of the STARD1 -independent system. It is clear, however, that early appeal and continuous stimulation of steroidogenesis in the gonads (by the hormones hCG, FSH, LH) and the adrenals (by ACTH and the renin-angiotensin system) are the starting point initiating the failure of both systems. In this study, our two patients, Patient A, 46,XY and Patient B, 46,XX, members of the same family with the same homozygous (L275P) mutation, have different severity of the disease and different time of onset of corticosteroid deficiency. Clinical manifestations and hormonal data prove the severe deficiency of testosterone secretion in Patient A, the milder ovary dysfunction in Patient B and the presence of some residual corticosteroid secretion and progressive post natal adrenal failure for both.

## GONADAL FUNCTION IN LCAH AND 46,XY GENOTYPE

Patient A in this study is a 46,XY genotype with female external genitalia and blind vaginal pouch. **Figure 1** shows lipid accumulation in Leydig cells typical of LCAH. The absence of androgen secretion, basal and after hCG stimulation, as well as the absence of Wolffian structures testify on the early and severe deficiency of testosterone secretion necessary for differential sexual development and virilization. In fact, during the early weeks of embryonic development, the fetus is exposed to acute hCG stimulation which may lead to lipid accumulation in the cell due to the lack of STARD1 activity, thereby destroying Leydig cells. This is clearly contrasting with the relatively moderate severity of mineralocorticoid and glucocorticoid deficiency of this patient. Generally, all 46,XY reported patients had a profound impairment of testosterone synthesis (Bose et al., 1996), however Wolffian duct derivatives are sometimes well developed as reported by Nakae et al. (1997) on patient 7 (Q258X, M225T) who had a cliteromegaly and moderate secretion of testosterone in response to hCG stimulation.

This seems to be concordant with the hypothesis of the earlier mentioned STARD1-dependent steroidogenesis deficiency and STARD1-independent impairment of steroidogenesis due to Leydig cells destruction following lipid accumulation caused by an early and continuous stimulation (placental hCG, fetal pituitary LH). Such patients should be raised as females (Bose et al., 1996) and should undergo orchidectomy due to the risk of *in situ* testicular carcinoma development (Korsch et al., 1999).

## GONADAL FUNCTION IN LCAH AND 46,XX GENOTYPE

### Spontaneous Puberty

Patient B in this study is a unique case. While she had an earlier and more severe corticosteroid deficiency than her sibling, her

ovarian function was less disturbed than what was observed in other similar cases (Bose et al., 1996). By the end of 1990, at the age of 11 7/12 years, she started a progressive pubertal development; her ovarian response to hCG stimulation was normal and she had menarche at the age of 14 2/12 years (Khoury et al., 2004). This was unexpected for LCAH in 1993. Our observations are in agreement with Matsuo's report in 1994 (Matsuo et al., 1994) that 5/5 46,XX patients with LCAH aged more than 13 years expressed a spontaneous development of secondary sex characters and vaginal bleeding at the time of puberty, their estrogen level ranging from 22–85 pg/ml. Bose et al. (1997) described in 1997 the first genetic analysis in a 46,XX female homozygous for STARD1 shift mutation 261 $\Delta$ T and spontaneous feminization. Three other cases were reported by Fujieda et al. (1997, 2003) who found that the ovaries of such patients were enlarged in the post pubertal stage with many cysts occupying the entire volume of the ovary and with hypertrophied ovarian stroma. This phenomenon was not observed on repeated echographies in our Patient B. The heterogeneity in the pubertal development of 46,XX female patients with STARD1 mutation was also described by Nakae et al. (1997) who reported on 6/10 46,XX patients who experienced spontaneous pubertal changes, irregular menstruations, anovulatory cycles and in some a polycystic aspect of ovaries. All these reports show that the difference in the extent of impairment of the testis and ovaries is dramatic in LCAH. However, although initially unexpected, even the most severely affected 46,XX female patients undergo spontaneous feminization, breast development and cyclical vaginal bleeding at the usual age of puberty (Matsuo et al., 1994; Bose et al., 1997; Nakae et al., 1997).

The fetal ovary cells are quiescent and in affected 46,XX patients these cells should remain undamaged since they should not accumulate cholesterol esters until they first undergo gonadotropin stimulation. This would explain why these cells retain steroidogenic capacity and hence at the time of puberty can make estrogens, albeit, in subnormal amounts, resulting in high gonadotropin secretion. However, only the cells in the individually recruited follicles undergo stimulation, and hence only these cells accumulate cholesterol esters and lose steroidogenic capacity. Regular monthly cycles are possible, because a large number of follicles remain relatively undamaged before recruitment. Such monthly cycles, which may persist for years, are probably anovulatory and can produce a large quantity of ovarian cysts.

## STARD1 EXPRESSION IN THE OVARIES

The study of Sandhoff and McLean (1996) shows that in rat ovaries STARD1 mRNA expression is controlled by tropic hormones PMSG (post-menopausal stimulating gonadotropin) and hCG. hCG increases the steady-state level of STARD1 mRNA in the ovary during follicular and luteal development. The rise in STARD1 expression paralleled the rise in serum progesterone levels, consistent with STARD1's presumed role in the regulation of steroidogenesis. Furthermore, STARD1 gene expression in primary cultures of porcine granulosa cells

is stimulated above control levels by FSH, 8-bromo-cAMP and IGF-1 respectively. FSH and IGF-1 interact synergistically to induce amplification of STARD1 mRNA and protein expression in serum-free monolayer culture of immature swine granulosa cells (Balasubramanian et al., 1997). During follicular development in the rat ovary, Ronen-Fuhrmann et al. (1998) have examined the time-dependent expression of STARD1 mRNA and protein in PMSG/hCG-treated immature rats. They found that in the early phase, before the administration of tropic hormones, granulosa cells did not express STARD1. The first peak of STARD1 expression was generated by PMSG administration. The expression was restricted to the ovarian secondary interstitial tissue as well as to a few scattered theca interna cells producing androgens which can synergistically potentiate FSH-induced actions in granulosa cells, and P450 aromatase for estrogen production in granulosa cells of Graafian follicles. This also seems to initiate the transition of follicles from the small antral stage to the preovulatory stage. After hCG administration, both the granulosa (until now devoid of STARD1) and the theca-interstitial cell types joined in synchronized production of STARD1. At this phase of follicular development, usually perceived as the onset of the luteinization process, high STARD1 mRNA levels resumed in the all-ovarian interstitium and high levels of STARD1 were also expressed in the granulosa cells; however, in the later cell type, STARD1 expression was strictly confined to periovulatory follicles.

## INDUCTION OF OVULATION

The measurement of blood FSH, LH, E2, and other hormones, at different ages in patient B, argues for a normal function of the hypothalamus-pituitary axis with a progressive increase of 17 $\beta$ -estradiol secretion to induce progressive pubertal feminization and regular menstruations. This was probably due to an efficient mechanism of STARD1-independent steroidogenesis still undamaged by the toxic accumulation of cholesterol and its derivatives, during the short life of stimulated follicles. However, data showed that she failed to demonstrate the spontaneous estrogen surge of the end of follicular phase, necessary for the secretion of ovulatory peak of LH, ovulation and consequently the normal increase of progesterone and basal temperature during the next theoretical luteal phase. The menstrual cycles of this patient were anovulatory for many years before consulting for infertility problems. Interestingly, during her early pubertal development, a hCG stimulation test resulted in a small increase in androstenedione and substantial enhancement of 17 $\beta$ -estradiol secretion (Khoury et al., 2009).

The mechanism of this response is subject to speculation, but more importantly it indicates that the steroidogenic systems retained some capacity to respond to tropic hormones. Thus, during the follow-up in the fertility clinic, we proposed to observe the function of the hypothalamus-hypophyso-ovarian axis during a Clomiphene stimulation test. This was done at the age of 25 years. Endocrine data shows that pituitary gonadotrophins, induced by Clomiphene stimulation, enhanced the steroidogenesis process in the granulosa cells, in the same

manner as was observed with the hCG stimulation test, 13 years earlier. Furthermore, the high levels of progesterone and  $17\beta$ -estradiol observed during the second half of her menstrual cycle is a clear confirmation of a process of ovulation and luteinization (Khoury et al., 2006b, 2009). According to Ronen-Fuhrmann et al. (1998) this is due, in the normal rat, to the high STARD1 expression induced by tropic hormones in all ovarian interstitium and the granulosa cells of periovulatory follicles. In our patient, this may indicate that hCG stimulation or increased secretion of pituitary FSH and LH induced by Clomiphene, as well as the ovulatory peak of LH, may have a role of further activation of the STARD1-independent system (Watari et al., 1997). It is also plausible to think that the amplification of the mutated STARD1 expression by those tropic hormones may contribute, via its partial steroidogenic activity, to the production of estrogen surge necessary for the ovulatory peak of LH and ovulation.

## STARD1, CORPUS LUTEUM, AND PREGNANCY

Pregnancy is the ultimate proof of the induction of ovulation. In our patient this was produced by Clomiphene stimulation. We believe that any other protocols of ovulation induction by gonadotrophins will also have a good chance of success. Our patient had a first pregnancy at the age of 25 4/12 years. However, a spontaneous abortion occurred 6 weeks later. The human corpus luteum derived from the ovulated follicle is an active producer of progesterone, an essential hormone for establishing and sustaining early pregnancy (Carr, 1992). Placental progesterone becomes sufficient to maintain pregnancy only after about 6 weeks of gestation, the so-called “luteoplacental shift.” Consequently, human maternal ovariectomy in the first 6 weeks will cause spontaneous abortion, but maternal ovariectomy thereafter will not (Csapo et al., 1973; Csapo and Pulkkinen, 1978). The critical step in luteal progesterone secretion is the movement of cholesterol from the OMM to the IMM (Devoto et al., 2002). Examination of corpora lutea of different luteal phases revealed that the basal expression of STARD1 transcript and protein was greatest in early and mid-luteal phase to decline in the late-luteal phase. Furthermore, under hCG stimulation, the expression of the major 1.6 kb STARD1 mRNA transcript is rapidly enhanced. The rise in STARD1 expression paralleled an increase in progesterone levels (Chung et al., 1998; Devoto et al., 2001). In human corpus luteum, the theca-lutein cells and granulosa-lutein cells exhibited marked heterogeneity in STARD1 protein concentration, with theca-lutein cells expressing greater levels of STARD1 than granulosa-lutein cells, irrespective of the stage of the luteal phase (Devoto et al., 2001; Christenson and Devoto, 2003). Human chorionic gonadotropin treatment during the late-luteal phase causes a pronounced increase in both theca-and granulosa-lutein cell STARD1 gene expression. In some species, including human, PGF $2\alpha$  is believed to be the physiological agent responsible for causing corpus luteum regression at the end of a non-fertile cycle. This luteolytic compound has been shown to cause a pronounced decline in STARD1 gene expression,

altering cholesterol transport to the mitochondria, inhibiting progesterone production and leading to substantial stores of lipids (Devoto et al., 2001; Christenson and Devoto, 2003). The distribution of STARD1 inside theca and granulosa-lutein cells of human corpus luteum was assessed by electron microscopy (Sierralta et al., 2005). Greater levels of STARD1 immunolabeling was found in the cells from early- and mid- than in the late-luteal phase of corpus luteum, and lower levels in cells from women treated with GnRH antagonist in the mid-luteal phase. There is also a substantial amount of mature STARD1 protein (30 kDa) in the cytoplasm of luteal cells (Sierralta et al., 2005). The presence of STARD1 in the cytoplasm was also reported in rat adrenal homogenates (LeHoux et al., 1999). The administration of GnRH antagonist during mid-luteal phase causes a dramatic reduction in STARD1 immunolabeling in both the cytosol and mitochondria of theca and granulosa-lutein cells of mid-luteal corpus luteum suggesting that, the levels of STARD1 protein in both cell compartments are LH dependent. Also, the presence of MLN64 in the supernatant of human corpus luteum extract throughout the luteal phase (Watari et al., 1997; Kishida et al., 2004) supports the idea that in cells with high steroidogenic activities, proteins with a START domain, other than STARD1, might participate in the intracellular trafficking of cholesterol or other lipids.

We conclude that the early spontaneous abortion at the first pregnancy was due to a dysfunction of this mutated STARD1 inside the theca and granulosa cells of the corpus luteum. The STARD1-dependent steroidogenesis was highly solicited during the early and middle phase of the corpus luteum development by an acute and important stimulation of hCG. The incapacity of the mutated STARD1 to transport the high quantity of cholesterol to the IMM will result in an accumulation of cholesterol, cholesterol esters, and oxidative products in the steroidogenic cells of the corpus luteum. This leads to the destruction of both the STARD1-dependent and STARD1-independent systems, in agreement with the two-hit theory concerning STARD1 mutations in steroidogenic tissues. Moreover, this explains the failure of the corpus luteum to produce the progesterone necessary for preparing and stabilizing the endometrium, and protecting the embryo during the early first trimester (Christenson and Devoto, 2003; Sierralta et al., 2005). Substitution therapy with progesterone was then indicated. It was recommended to start progesterone at the 17th day of the cycle and to continue this treatment, during the first trimester, if pregnancy was confirmed later. This technique was successful since a second pregnancy occurred. Progesterone treatment was initially recommended throughout the pregnancy hoping to optimize uterine quiescence and prevent premature delivery in her multiple gestations (Meis, 2005); but for some reason, it was stopped at 25 weeks. Delivery of two normal boys occurred at 30 weeks of gestation. The same treatment was used for a third pregnancy, however progesterone administered up to the 17th week was deemed sufficient to support a single gestation. This led to the delivery of a normal girl at 36 weeks of gestation (October 2008).

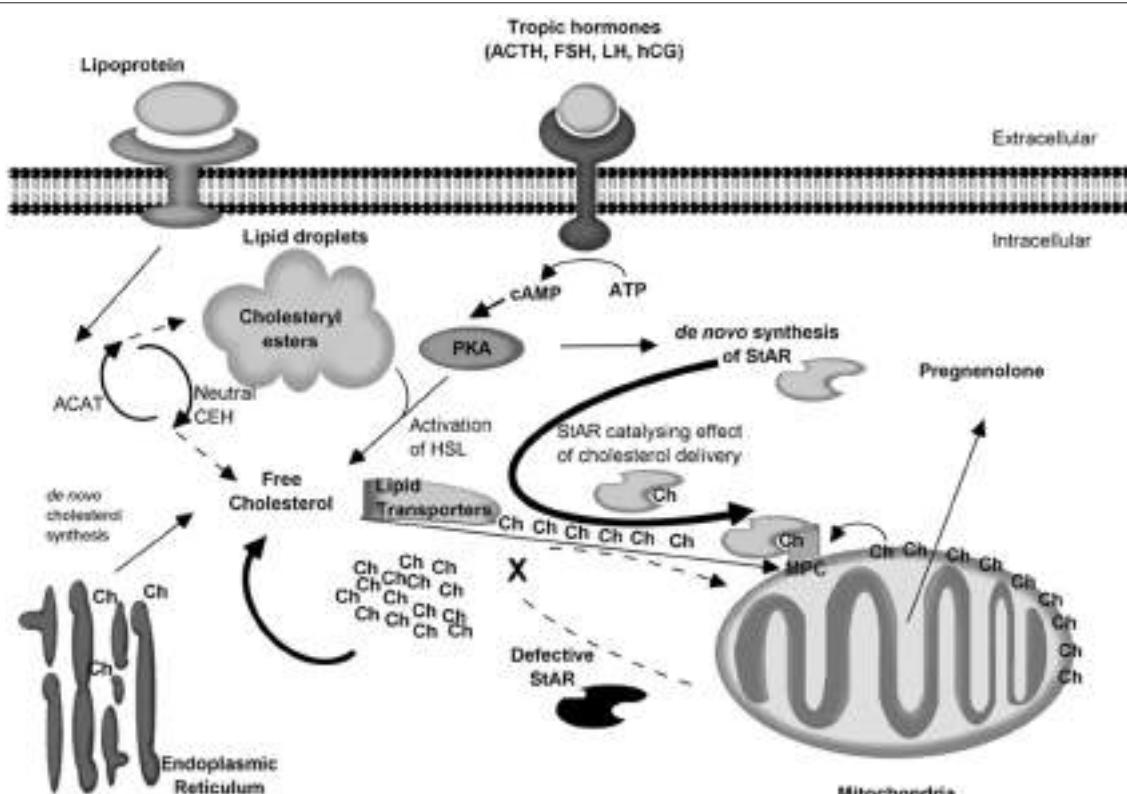
In 2008, Sertedaki et al. reported a female LCAH patient bearing a 11-bp deletion in exon 6 of her STARD1 gene (Sertedaki

et al., 2009). She did have “cysts in the ovaries” and the LH/FSH ratio was very high. After many unsuccessful attempts to conceive, she entered a program of IVF. Follicle development and growth was easily obtained by the administration of recombinant FSH and recombinant LH and follicle maturation was initiated by hCG administration before oocyte retrieval. After IVF and implantation, vaginal administration of progesterone was conducted to support pregnancy up to the 11th week. A female baby was born by cesarean section in 2007 with no perinatal complications. The used protocol was a classic strategy for IVF. Furthermore, it is fully concordant with our strategy for induction of ovulation and protecting pregnancy in LCAH during the first trimester of gestation. In our case, we used Clomiphene for ovulation induction and only 400–600 mg/d of progesterone (Prometrium) (oral and/or vaginal) was given (Smitz et al., 1992; Progesterone supplementation, 2008).

## PREGNANCIES OBSERVATIONS

Ovulation induction with Clomiphene citrate is associated, in 6–8 % of cases, with multiple pregnancies, mainly twins (Messinis, 2005). During her last two pregnancies, the patient B developed high blood pressure. Because it first started during pregnancy and it returned to normal after delivery, we classified it as gestational hypertension. She never met preeclampsia criteria. The onset of high blood pressure was earlier in the second pregnancy (16 + 6 weeks GA) than in the third one (34 weeks GA), perhaps because of the twin pregnancy status which represents a risk factor (Creasy et al., 2004).

We do not know exactly the cause of the two preterm deliveries preceded by premature preterm rupture of membranes (PPROM). Although high dosages and regular daily oral steroid administration during pregnancy has been associated with preterm delivery and PPROM (Laskin et al., 1997), this was



**FIGURE 5 | Cellular model of steroidogenesis and LCAH.** Low and high density lipoproteins (LDL and HDL) are captured by receptors on the cell membrane. Initial metabolism of LDL lipids occurs in the lysosome where cholestryly esters are hydrolyzed by acidic cholestryly ester hydrolases (acidic CEH). The resulting free cholesterol (Ch) is re-esterified by the cytosolic acyl-CoA:cholesterol acyltransferase (ACAT) and stored in lipid droplets. Cholesterol can also be synthesized *de novo* in the endoplasmic reticulum. In the resting cell, cholesterol is constantly hydrolyzed/re-esterified by neutral CEH and ACAT. With the help of lipid transporters, free cholesterol can be conveyed at the OMM. Then cholesterol present at the OMM can be transferred to the IMM for conversion to pregnenolone without STARD1. This low capacity system may explain the basal level of hormone production (10–13%) for homeostasis. Following an acute event (stress, dehydration, puberty, etc.), trophic hormones stimulate steroidogenic cells and activate PKA, which in turn leads to three actions: The activation of the hormone-sensitive lipase (HSL) which releases cholesterol from lipid droplets, the *de novo* synthesis of STARD1, and the formation of a multiprotein complex (MPC) somehow involved in the transfer of cholesterol to the IMM (Liu et al., 2006; Bose et al., 2008a). Then STARD1 may act as a high capacity system by catalyzing the delivery of cholesterol to the OMM and the MPC complex for its transfer to P450ccc in response to the acute demand for steroid hormones. In the case of LCAH, the STARD1 high capacity system is impaired cannot support the substantial throughput of cholesterol, and the latter accumulates in the cytosol; lipid droplets become engorged with cholesterol. As lipid droplets accumulate, the cell becomes less functional and LCAH is onset.

probably not the case for our patient who received physiological doses of hydrocortisone and fludrocortisone acetate during her pregnancies. Furthermore, in this case, we not believe that there is an association between preterm delivery from PPROM and cessation of progesterone, since the patient did not complain of premature contraction or significant uterine activity before the delivery. Also, stimulation with oxytocine has been necessary for delivery. Finally, even if infection is a main causative agent in PPROM, she didn't have any criteria associated with that.

## CONCLUSION

We have described clinical data collected over 38 years of follow-up on two French-Canadian patients with 46,XY and 46,XX karyotype and presenting the classical clinical manifestations of LCAH due to a homozygous STARD1 mutation (L275P).

The two patients presented the classic picture of LCAH. Mineralocorticoids and glucocorticoids deficiencies are less severe in Patient A than Patient B. Both patients have had less severe manifestations of corticosteroid deficiency and a later onset of clinical symptoms than the vast majority of patients suffering from STARD1 mutations described in the literature. Patient A (46,XY) portrayed clinical and biochemical signs of early and severe deficiency of testosterone secretion. Testosterone is necessary for differential sexual development and virilization in the early weeks of fetal life during the period of high stimulation by hCG.

Patient B, 46,XX karyotype, is probably one of the first patients with LCAH in which spontaneous pubertal development was observed. She had menarche and spontaneous regular non-ovulatory menstruations for many years before consulting the fertility clinic. hCG and Clomiphene stimulation tests proved the possibility of increasing estrogen secretion by an acute stimulation of the ovarian theca interstitia and the granulosa cells. Clomiphene stimulation restored the surge of 17 $\beta$ -estradiol and LH secretion of the mid-cycle to induce ovulation and open the door for pregnancy. Our understanding of the physiological role of STARD1 during the different phases of the corpus luteum was essential to explain the miscarriage at 6 weeks of the first pregnancy, and then to develop a preventive therapy by progesterone administration during the first trimester of the following pregnancies. We were able to describe and present the first case of pregnancy in the patient with LCAH. It was a very long way from a fatal disease to a normal healthy life and restoration of the reproductive function in a female with the L275P mutation of STARD1.

The clinical and biochemical data of our two patients are concordant with the STARD1-dependent and STARD1

-independent mechanisms of steroidogenesis. However, the non-STARD1 steroidogenesis system is, in our opinion, the basal physiologic system of steroid synthesis producing normal adrenal and gonadic steroids during the normal physiological circumstances. Furthermore, the STARD1 - dependent steroidogenesis system seems to be a key rate-limiting mediator in the acute regulation of steroidogenesis by tropic hormones. It is acting during the acute need for more production of steroids during physiologic periods of normal development (as sexual differentiation and virilization in the early fetal life of the male), surgical or infection stress, normal surge of estrogen production in the mid-cycle necessary for ovulation, or the high production of progesterone during the first trimester of pregnancy. The incapacity of the mutated STARD1 to assume this high speed function, during stress situations and tropic hormone stimulation, leads to the accumulation of high levels of cholesterol, cholesterol esters and oxidative products in the cell. Early appeal and continuous stimulation of steroidogenesis in the gonads (by the hormones hCG, FSH, LH) and the adrenal (by ACTH and renin-angiotensin system) are the starting points initiating the failure of both systems of steroid production.

**Figure 5** shows an up to date model of steroidogenesis and LCAH. Despite all the progress made during the last 20 years in the physiology of the transfer of cholesterol to the IMM (Liu et al., 2006; Bose et al., 2008a; Miller, 2016), we still need to understand the detailed interplay between STARD1 and its delivery of cholesterol to a multiprotein complex (MPC) (Liu et al., 2006; Bose et al., 2008a) somehow involved in the transfer of cholesterol to the IMM.

Finally, we wish to point the reader to our recent progress on the dynamics and mechanism of ligand binding and release of the START domain of STARD6 (Létourneau et al., 2016).

## AUTHOR CONTRIBUTIONS

KK was in charge of patients. EB and PL contributed to figures. JGL, EB, and KK contributed to the writing of this article. YA and AO did the clinical follow-ups of the pregnancies.

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# Impact of ACTH Signaling on Transcriptional Regulation of Steroidogenic Genes

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The trophic peptide hormone adrenocorticotrophic (ACTH) stimulates steroid hormone biosynthesis evoking both a rapid, acute response and a long-term, chronic response, via the activation of cAMP/protein kinase A (PKA) signaling. The acute response is initiated by the mobilization of cholesterol from lipid stores and its delivery to the inner mitochondrial membrane, a process that is mediated by the steroidogenic acute regulatory protein. The chronic response results in the increased coordinated transcription of genes encoding steroidogenic enzymes. ACTH binding to its cognate receptor, melanocortin 2 receptor (MC2R), stimulates adenylyl cyclase, thus inducing cAMP production, PKA activation, and phosphorylation of specific nuclear factors, which bind to target promoters and facilitate coactivator protein recruitment to direct steroidogenic gene transcription. This review provides a general view of the transcriptional control exerted by the ACTH/cAMP system on the expression of genes encoding for steroidogenic enzymes in the adrenal cortex. Special emphasis will be given to the transcription factors required to mediate ACTH-dependent transcription of steroidogenic genes.

**Keywords:** adrenal cortex, steroidogenesis, cAMP, transcription factors, gene regulation

## INTRODUCTION

The adrenal gland is a key component of the hypothalamus–pituitary–adrenal (HPA) axis, thus playing a crucial role in the adaptation of organism to stressors. The adrenocorticotrophic hormone (ACTH) belongs to this regulatory circuitry, being one of the most potent physiological modulators of adrenal cortex steroidogenesis and trophicity (1, 2). It exerts its role through the binding to the G protein-coupled receptor (GPCR) melanocortin 2 receptor (MC2R), which activates adenylyl cyclase cascade leading to cAMP production and subsequent activation of cAMP-dependent protein kinase A (PKA). PKA is the main kinase responsible for the phosphorylation of specific transcription factors, which in turn regulate free cholesterol availability and activate steroidogenic enzyme expression (3, 4). Among those transcription factors, steroidogenic factor 1 (SF-1), cAMP response element-binding protein (CREB), CRE modulator (CREM), CCAAT/enhancer-binding proteins (C/EBPs), and activator protein 1 (AP-1) have been extensively described for their implication in regulating the expression of the genes encoding for steroidogenic acute regulatory (StAR) protein and steroidogenic enzymes (5–8). Any perturbation of this ACTH/cAMP/PKA-dependent cascade may cause alteration in adrenocortical cell proliferation and dysregulation of steroidogenesis as occur in various human diseases. The aim of the present review is to provide an overview of the ACTH/cAMP-dependent transcriptional regulation of the steroidogenic process in the adrenal cortex.

## ADRENAL STEROIDOGENESIS

Steroid hormones are implicated in the regulation of a plethora of developmental and physiological processes from fetal life to adulthood. Cholesterol is the precursor of all those hormones that hence share a closely related structure based on the cyclopentanophenanthrene 4-ring hydrocarbon nucleus. Cholesterol can be synthesized *de novo* from acetate in the adrenal (9). However, the main adrenal source of cholesterol is plasma low-density lipoproteins (LDLs) provided by dietary cholesterol (10). The sterol response element-binding proteins (SREBPs) are a family of transcription factors implicated in the regulation of genes participating in the biosynthesis of cholesterol and fatty acids (11). They are the main regulators of intracellular cholesterol metabolism. ACTH promotes the activation of 3-hydroxy-3-methylglutaryl coenzyme A reductase, the rate-limiting enzyme in cholesterol synthesis. It also stimulates the uptake of LDL cholesterol esters, which, once taken up by receptor-mediated endocytosis can be converted to free cholesterol for steroid hormone biosynthesis (12) or directly stored. Moreover, ACTH stimulates hormone-sensitive lipase (HSL) and inhibits acyl-coenzyme A (CoA):cholesterol acyltransferase (ACAT), thereby increasing the free cholesterol pool available for steroid hormone biosynthesis.

The initial step of steroidogenesis occurs in the mitochondria. Whereas the movement of cholesterol from the outer mitochondrial membrane (OMM) to the inner mitochondrial membrane (IMM) is known to be promoted by STAR (see below), the molecular mechanism underlying cholesterol transport and loading into the OMM is still under investigation (13). The enzymes that participate in steroid biosynthesis are either cytochrome P450s (CYPs) or hydroxysteroid dehydrogenases (HSDs).

Cytochrome P450s are a group of oxidative enzymes. In the human genome, genes for 57 CYPs enzymes have been described. Seven of them are called "type 1" enzymes. They are targeted to mitochondria and receive electrons from NADPH via a flavoprotein (ferredoxin reductase) and a small iron-sulfur protein (ferredoxin). "Type 2" enzymes are localized in the endoplasmic reticulum and receive electrons from NADPH via a single 2-flavin protein called P450 oxidoreductase (14). Six P450 enzymes participate in steroidogenesis. The mitochondrial P450 side chain cleavage (SCC, encoded by the *CYP11A1* gene) catalyzes breakage of the bond between positions 20 and 22 in the cholesterol side chain (20, 22 desmolase). P450c11 $\beta$  (11 $\beta$ -hydroxylase, encoded by the *CYP11B1* gene) and P450c11AS (aldosterone synthase, encoded by the *CYP11B2* gene) catalyze 11 $\beta$ -hydroxylase, 18-hydroxylase, and 18-methyl oxidase activities. At the level of the endoplasmic reticulum, we distinguish: P450c17 (encoded by the *CYP17A1* gene) that catalyzes both 17 $\alpha$ -hydroxylase and 17,20-lyase activities; P450c21 (encoded by the *CYP21A1* gene) that catalyzes 21-hydroxylation in the synthesis of both glucocorticoids and mineralocorticoids; and P450-Aro (encoded by the *CYP19A1* gene) that catalyzes the aromatization of androgens to estrogens.

The HSDs use nicotinamide adenine dinucleotides or their phosphate forms (NADH/NAD $^+$  or NADPH/NADP $^+$ ) as cofactors to either reduce or oxidize a steroid through a hydride transfer mechanism (15). Differently from steroidogenic reactions

catalyzed by P450 enzymes, which are mediated by a single form of P450, each reaction catalyzed by HSDs involves at least two, often different isozymes. On the basis of their structures, those enzymes are divided into: short-chain dehydrogenase/reductase (SDR) family, which include 11 $\beta$ -HSDs 1, 2, and 17 $\beta$ -HSDs 1, 2, and 3 and aldo-keto reductase (AKR) family, which include 17 $\beta$ -HSD5 (15, 16). Under a physiological point of view, it is preferable to classify the HSDs as dehydrogenases or reductases. The former use NAD $^+$  as cofactors to oxidize hydroxysteroids to ketosteroids, whereas the latter mainly use NADPH to reduce ketosteroids to hydroxysteroids. Those enzymes act *in vitro* typically bidirectionally on the basis of the pH and cofactor concentration, while in intact cells they work mainly in one direction, with the direction established by the cofactors available (15, 16).

The pattern of steroid hormones secreted by each adrenal zone is determined by the enzymes expressed in each zone (17). Specifically, adrenal *zona glomerulosa* expresses angiotensin II receptors and P450c11AS, whereas it does not express P450c17. Indeed, *zona glomerulosa* produces aldosterone under the regulation of the renin-angiotensin system. In contrast, at the levels of *zona fasciculata*, angiotensin II receptors, and P450c11AS are not detected, whereas the ACTH receptor MC2R and P450c11 $\beta$  are expressed (18). The *zona fasciculata* also expresses P450c17, which catalyzes 17 $\alpha$ -hydroxylation, exhibiting only little 17,20-lyase activity. Indeed, *zona fasciculata* secretes the two glucocorticoids, cortisol and corticosterone, under the influence of ACTH, but very little dehydroepiandrosterone (DHEA). Interestingly, patients displaying mutations in P450c17 are not able to produce cortisol, but show increased corticosterone production (19), like in rodents, which normally do not express P450c17 in their adrenals. Regarding the *zona reticularis*, it also expresses MC2R and large amounts of P450c17 and cytochrome b<sub>5</sub>, thus displaying 17,20-lyase activity with subsequent DHEA production, the most part of which is sulfated to DHEAS by SULT2A1. In general, small amounts of DHEA are converted to androstenedione, and very little amounts of androstenedione are converted to testosterone, likely through AKR1C3/17 $\beta$ HSD5. In contrast, *zona reticularis* expresses very little P450c21 and P450c11 $\beta$  (thus producing only a minimal amount of cortisol) and relatively little 3 $\beta$ HSD2.

## ACUTE RESPONSE TO ACTH: THE STEROIDOGENIC ACUTE REGULATORY PROTEIN

Steroidogenic cells are able to store very little amounts of steroids, which imply a rapid synthesis of new steroids in response to a sudden demand. ACTH exerts its role in promoting steroidogenic cell growth and stimulating steroidogenesis at three distinct levels. We first distinguish a long-term exposure to ACTH. It takes weeks or months to stimulate adrenal growth and it is mediated by ACTH-dependent production of cAMP, which in turn triggers IGF-II (20), fibroblast growth factor (FGF) (21), and epidermal growth factor (EGF) (22) synthesis. The concerted action of those growth factors is to promote adrenal cellular hypertrophy and hyperplasia. Second, ACTH can act over days through cAMP to stimulate the transcription of genes, which encode for different

steroidogenic enzymes (see below). Third, ACTH can mediate an acute response, which occurs within minutes and is inhibited by protein synthesis inhibitors (like puromycin or cycloheximide). This ACTH-mediated acute response is accompanied by a rapid stimulation of the StAR gene transcription in steroidogenic cells of the adrenal cortex, testis, and ovary (5, 23) and by the phosphorylation of Ser195 in the existent pool of StAR (24). Those events promote cholesterol flow from the OMM to the IMM, where cholesterol is converted to pregnenolone in the first and rate limiting step of steroid hormone biosynthesis. The first to show that ACTH acute steroidogenic response involved the rapid synthesis of a 37-kDa phosphoprotein were Orme-Johnson and coworkers (25, 26). Just a few years later, Stocco and colleagues cloned this protein and they gave it the name of “StAR” (27). StAR is an acutely regulated, cycloheximide-sensitive protein exhibiting a mitochondrial leader sequence by which it is directed to the mitochondria. Once inside the mitochondria, StAR is cleaved to a 30-kDa protein. It has been shown that the overexpression of a mouse StAR in the mouse Leydig MA-10 cells increased their basal steroidogenesis (27). Moreover, when expression vectors for both StAR and P450scc enzyme are cotransfected in non-steroidogenic COS-1 cells, the synthesis of pregnenolone is augmented respect to that obtained with P450scc alone (28). The pivotal role of StAR in the regulation of steroidogenesis was strengthened after the identification of mutations causing premature stop codons in the StAR gene of patients affected by the most common form of congenital lipid adrenal hyperplasia (CAH) (28, 29), a rare disorder of steroid biosynthesis. In this disorder, glucocorticoid, mineralocorticoid, and sex steroids biosynthesis is impaired, which may lead to adrenal failure, severe salt wasting crisis and hyperpigmentation in phenotypical female infants irrespective of genetic sex (29). Moreover, it has been shown that in mice the targeted disruption of the *Star* gene causes defects in steroidogenesis, with consequent male pseudohermaphroditism and lethality within 1 week after birth (30, 31).

Several studies have been carried out to understand the mechanism of action of StAR (32), which still remains to be fully elucidated. It was hypothesized that the “mature” 30-kDa intramitochondrial form of StAR was the biologically active portion of the protein, due to its longer half-life respect to the short one of the 37 kDa precursor. However, when the two StAR forms are expressed in the cytoplasm or added to mitochondria *in vitro*, they are equally active (33). Moreover, while constitutively active when it is localized on the OMM, StAR results to be inactive at the level of the mitochondrial intramembranous space or the matrix (34). That evidence suggested that StAR exerts its action on the OMM, its steroidogenesis-promoting function tightly depending on the residency time on the OMM itself (33, 34). This implies that StAR activity is linked to its localization rather than to its cleavage to the “mature” form. When StAR interacts with the OMM, it undergoes to conformational changes (35, 36) that allow StAR to accept and discharge cholesterol molecules. Interestingly, steroidogenesis-promoting and cholesterol-transfer activities of StAR are distinct. Indeed, StAR-mediated transfer of cholesterol between synthetic membranes *in vitro* (37) is maintained also by the inactive R182L mutant, which impairs steroidogenesis, causing lipid CAH (38). Finally, StAR activity on the OMM

requires the translocator protein TSPO, also called peripheral benzodiazepine receptor (PBR) (39, 40), which has been identified as a key player in the flow of cholesterol into mitochondria to permit the initiation of steroid hormone synthesis. Moreover, it has been demonstrated that phosphorylated StAR interacts with voltage-dependent anion channel 1 (VDAC1) on the OMM, which in turn promotes processing of the 37-kDa phospho-StAR to the 32-kDa intermediate (41). In the absence of VDAC1, phospho-StAR undergoes degradation by cysteine proteases prior to mitochondrial import and subsequent cleavage to the 30-kDa protein. StAR phosphorylation by PKA requires phosphate carrier protein on the OMM, which seems to interact with StAR before it interacts with VDAC1 (41). Importantly, although StAR is necessary for the ACTH-mediated acute steroidogenic response, steroidogenesis still occurs in the absence of StAR (around 14% of StAR-induced rate) (29, 42). This may account for the steroidogenic capacity of tissues lacking StAR, like placenta and brain.

## CHRONIC RESPONSE TO ACTH

The transcription of steroidogenic genes depends on the slower, chronic response to ACTH in the adrenal cortex. Indeed, ACTH interaction with specific membrane receptors leads to the activation of coupled G proteins, with subsequent stimulation of membrane-associated adenylyl cyclase catalyzing cAMP formation. cAMP-activated PKA hence phosphorylates multiple transcription factors, whose concerted action and interaction with different *cis*-regulatory elements direct StAR and steroidogenic gene expression. Furthermore, after transcription factor binding to gene promoters, posttranslational modifications, like phosphorylation/dephosphorylation and coactivator proteins binding, are required to activate gene expression. In the following section, the main transcription factors that direct the transcription of steroidogenic genes in response to ACTH will be described (Table 1; Figure 1).

### Steroidogenic Factor 1

Steroidogenic factor-1 (SF-1; NR5A1) is an orphan member of the nuclear receptor superfamily, which acts as a key regulator of adrenogonadal development and tissue-specific gene expression in steroidogenic cells. Parker and Morohashi groups identified SF-1 by its capacity to bind to and activate transcription from multiple P450 steroidogenic enzyme promoters, which display one or more SF-1-binding sequences in close proximity to the TATA box (72, 73). Furthermore, it has been shown that SF-1 interacts with multiple coactivator and corepressor proteins that function as bridges between transcription factors and the basal transcription system (74–76).

In the human adrenocortical cancer cell line H295R *StAR* gene transcription is induced by both angiotensin II and cAMP via increased SF-1-binding to a cAMP-responsive region within the first 350 bp upstream of the transcription initiation site (45, 77–79). On the other hand, the mutation of the SF-1 response elements in the mouse promoter of the *StAR* gene does not impair cAMP-dependent StAR induction in MA-10 Leydig cells or Y1 adrenocortical cells (78).

**TABLE 1 | Promoter elements implicated in basal and ACTH/cAMP-regulated expression of steroidogenic genes.**

	<b>Basal regulation</b>	<b>ACTH/cAMP-dependent regulation</b>
<b>StAR</b>	Three SF-1-binding sites [−135; −95; −45; <b>mouse</b> promoter, Ref. (7)] Three SF-1-binding sites [−926/−918; −105/−96; −43/−36; <b>human</b> promoter, Ref. (43, 44)] Two Sp1-binding sites [−1159/−1153; −157/−151; <b>human</b> promoter, Ref. (44, 45)]	CRE2/AP-1 [−81/−75, <b>mouse</b> promoter; Ref. (6)] Two C/EBPs putative-binding sites [−119/−100; −50/−41; <b>human</b> promoter, Ref. (43)] Two SF-1-binding sites [−105/−65; −43/−36; <b>human</b> promoter, Ref. (43)] Highly conserved overlapping motif [−81/−72, <b>mouse</b> promoter, which recognizes the CRE/AP1 and C/EBPs family of proteins, Ref. (7, 46–48)]
<b>CYP11A1</b>	Proximal SF-1-binding site [P site, −46/−38; <b>human</b> promoter, reviewed in Ref. (49)] Imperfect Sp1-binding site [−111/−101; <b>human</b> promoter, reviewed in Ref. (49)] TRE-P-132 [−155/−131; <b>human</b> promoter, reviewed in Ref. (49)] Adrenal enhancer (AdE, −1850) composed of two binding regions (a) AdE1 (−1845) containing an imperfect Sp1 and an NF-1-binding site [ <b>human</b> promoter, reviewed in Ref. (49)] (b) AdE2 (−1898) containing an imperfect Sp1-binding site [ <b>human</b> promoter, reviewed in Ref. (49)] AP-1 motif [−319/−313; <b>mouse</b> promoter, Ref. (50)] TGAGTCA motif [termed SF-3-binding site, −120/−114; <b>mouse</b> promoter, Ref. (50, 51)] AGGTCA motif [termed SF-2-binding site, −73/−68; <b>mouse</b> promoter, Ref. (50, 51)] AGCCTTG motif [termed SF-1-binding site, −45/−39; <b>mouse</b> promoter, Ref. (50, 51)]	Proximal SF-1-binding site [P site, −46/−38; <b>human</b> promoter, reviewed in Ref. (49)] Upstream cAMP responsive sequence (U-CRS, −1600 bp), which includes (a) SF-1-binding site (−1617/−1609) (b) CREB/ATF-binding site (CRE; −1685/−1606) (c) Two AP-1-binding sites (−1559/−1553; −1633/−1626) (a)/(b)/(c) <b>human</b> promoter, reviewed in Ref. (49) AP-1 motif [−319/−313; <b>mouse</b> promoter, Ref. (50), although available data indicate that this motif is not a major contributor to the induction of CYP11A1 expression by ACTH/cAMP] TGAGTCA motif [termed SF-3-binding site, −120/−114; <b>mouse</b> promoter, Ref. (50, 51)] AGGTCA motif [termed SF-2-binding site, −73/−68; <b>mouse</b> promoter, Ref. (50, 51)] AGCCTTG motif [termed SF-1-binding site, −45/−39; <b>mouse</b> promoter, Ref. (50, 51)] (mutation of those elements reduced the expression levels of <i>Cyp11A1</i> gene following treatment with 8-Br-cAMP, although all mutated plasmids retained appreciable responsiveness to cAMP)
<b>CYP11B1</b>	CRE-binding site [termed Ad1/CRE and resembling a consensus CRE, −71/−64; <b>human</b> promoter, Ref. (52, 53) reviewed in Ref. (54)] Ad5 [−119/−111; <b>human</b> promoter, ERRAalpha has been shown to be the nuclear protein interacting with this element under basal conditions, reviewed in Ref. (54)] SF-1-binding site [termed Ad4, −242/−234; <b>human</b> promoter, it seems to be less important for both CYP11B1 and CYP11B2 basal expression, reviewed in Ref. (54)] CRE-binding site [−74/−67; <b>mouse</b> promoter, reviewed in Ref. (54)] Two Ad5-binding sites [one at −122/−114 and the other one at −208/−200; <b>mouse</b> promoter, reviewed in Ref. (54)] SF-1-binding site [−247/−240; <b>mouse</b> promoter, reviewed in Ref. (54)]	CRE-binding site [termed Ad1/CRE and resembling a consensus CRE, −71/−64; <b>human</b> promoter, Ref. (52, 53), reviewed in Ref. (54)] Functional CRE consensus sequence [−56/−49, <b>mouse</b> promoter, Ref. (55)]
<b>CYP11B2</b>	CRE-binding site [termed Ad1/CRE and resembling a consensus CRE, −71/−64; <b>human</b> promoter; Ref. (53), reviewed in Ref. (54)] Ad5 [−119/−111; <b>human</b> promoter, ERRAalpha has been shown to be the nuclear protein interacting with this element under the basal conditions, reviewed in Ref. (54)] Two SF-1-binding sites [one at −129/−114, <b>human</b> promoter, Ref. (56)]; The other one termed Ad4, −344/−337, <b>human</b> promoter. It seems to be less important for both CYP11B1 and CYP11B2 basal expression; reviewed in Ref. (54) Chicken ovalbumin upstream promoter transcription factor [COUP-TF, −129/−114; <b>human</b> promoter, Ref. (56)] CRE-binding site [−67/−60; <b>mouse</b> promoter, reviewed in Ref. (54)] Ad5 [−108/−100; <b>mouse</b> promoter, reviewed in Ref. (54)] SF-1 [−330/−323; <b>mouse</b> promoter, reviewed in Ref. (54)]	CRE-binding site [termed Ad1/CRE and resembling a consensus CRE, −71/−64; <b>human</b> promoter; Ref. (53, 56) reviewed in Ref. (54)] SF-1-binding site [−129/−114; <b>human</b> promoter, Ref. (56)] Chicken ovalbumin upstream promoter transcription factor [COUP-TF, −129/−114; <b>human</b> promoter, Ref. (56)] CRE-binding site [−67/−60; <b>mouse</b> promoter, Ref. (56), reviewed in Ref. (54)]
<b>CYP17A1</b>	ASP/Sp1-binding site [−8/−19; <b>human</b> promoter, Ref. (57)] SF-1-binding site [−58/−50; <b>human</b> promoter, Ref. (57)] Two nuclear factor 1 (NF-1)-binding sites [−107/−85; −178/−152, <b>human</b> promoter, Ref. (59)] −184/−206 region [The site within this sequence that confers basal activity is not known, although it does contain a sequence resembling an SF-1 site at −195/−200; <b>human</b> promoter, Ref. (57)] Sp1/Sp3-binding site [−227/−184, <b>human</b> promoter; Ref. (59)] SF-1 (−62/−40), Sp1 (−186/−177), and Pbx/Meis (−243/−225) binding sites [ <b>bovine</b> promoter; Ref. (59–63)]	cAMP-regulatory sequence [CRS, −57/−38: SF-1, p54rb/NonO, and poly-pyrimidine tract-binding protein-associated splicing factor (PSF) are the transcription factors shown to be associated to this region; <b>human</b> promoter, Ref. (58)] SF-1 (−62/−40)- and Pbx/Meis (−243/−225)-binding sites [ <b>bovine</b> promoter; Ref. (60–63)]

(Continued)

**TABLE 1 | Continued**

	<b>Basal regulation</b>	<b>ACTH/cAMP-dependent regulation</b>
<b>CYP21</b>	Two Sp1-binding sites [−118/−112 and −106/−100 within the recognition site −129/−96; <b>human</b> promoter, Ref. (64)] Two SF-1-binding sites [a putative one within the −300 bp proximal promoter and a second one within a distal region, lying approximately 4.8 kb upstream of the transcription start site; <b>human</b> promoter, Ref. (65)] Enhancer element [−330/−150; <b>mouse</b> promoter, Ref. (51)] Essential regulatory element [−210/−170; <b>mouse</b> promoter, highly conserved in the genes from human and bovine; Ref. (51, 66, 67)] A and B elements located 5.3 and 6 kb upstream of the transcriptional start site [ <b>mouse</b> promoter, Ref. (69)]	Adrenal-specific protein (ASP)-binding site [−129/−113, within the recognition site −129/−96; <b>human</b> promoter, Ref. (64)] Enhancer element [−330/−150; <b>mouse</b> promoter, Ref. (51)] Essential regulatory element [−210/−170; <b>mouse</b> promoter, highly conserved in the genes from human and bovine; Ref. (51, 66, 67)] cAMP consensus sequence [−68/−62; <b>mouse</b> promoter, it matches part of the consensus sequence proposed for cAMP-regulated expression of other genes, Ref. (68)] Nuclear-binding response element (NBRE)/Nurr77 binding site [−65, <b>mouse</b> promoter, Ref. (66)] Regulatory elements containing variation of an AGGTCA motif at −170, −210, −140, −65 [ <b>mouse</b> promoter; they show similarity to the CRE consensus, although they do not function as classical CREs, Ref. (68); variations of these same AGGTCA-bearing elements are also involved in the expression of <i>Cyp11a</i> and <i>Cyp11b</i> in Y1 adrenocortical cells, see above and Ref. (70)]
<b>HSD3B2</b>	Two SF-1/LRH-1-binding sites [−64/−56; −315/−307; <b>human</b> promoter, reviewed in Ref. (71)] Nuclear-binding response element (NBRE)/Nurr77 binding site [−131; <b>human</b> promoter, reviewed in Ref. (71)] GATA-binding site [−196/−190; <b>human</b> promoter, reviewed in Ref. (71)]	Nuclear-binding response element (NBRE)/Nurr77 binding site [−131, <b>human</b> promoter, reviewed in Ref. (71)]

Blue, red and green colours distinguish the different species (human, mouse and bovine, respectively).

Adrenocorticotropic hormone/cAMP-dependent transcription of the human *CYP11A1* gene, encoding for the mitochondrial enzyme P450scc responsible for cholesterol conversion to pregnenolone, requires the binding of SF-1 at two sites (−40 and −1600 bp) on the gene promoter (80). Remarkably, Omura and Chung laboratories have shown that the upstream promoter elements confer a large portion of *CYP11A1* responsiveness to cAMP in Y1 or I-10 Leydig tumor cells and primary adrenal cells of transgenic mice (80, 81). SF-1-dependent activation of the *CYP11A1* promoter can be potentiated by cotransfection with c-Jun in steroidogenic JEG3 cells, but not in COS-1 cells (82). Thus, c-Jun and SF-1 act synergistically to activate the transcription of *CYP11A1*. It has also been shown that in the human adrenal cortex *CYP11A1* can be strongly activated by GATA-6 in a SF-1-dependent and DAX1-sensitive fashion (83).

Moreover, it has been shown that SF-1 interacts with the homeodomain-containing transcription factor pituitary homeobox 1 (Ptx/Pitx1) to synergistically promote *CYP11B1* gene transcription (84). Interestingly, in the bovine adrenal only one *CYP11B1* gene is expressed. Its promoter is characterized by the presence of both a SF-1- and a CRE (cAMP-response element)-like binding site, which are essential for cAMP-driven transcription (85, 86).

Adrenocorticotropic hormone/cAMP signaling also regulates the expression of the human *CYP11B2* gene in the *zona glomerulosa*, where it is responsible for mineralocorticoid production via an SF-1-binding site and a CRE (56).

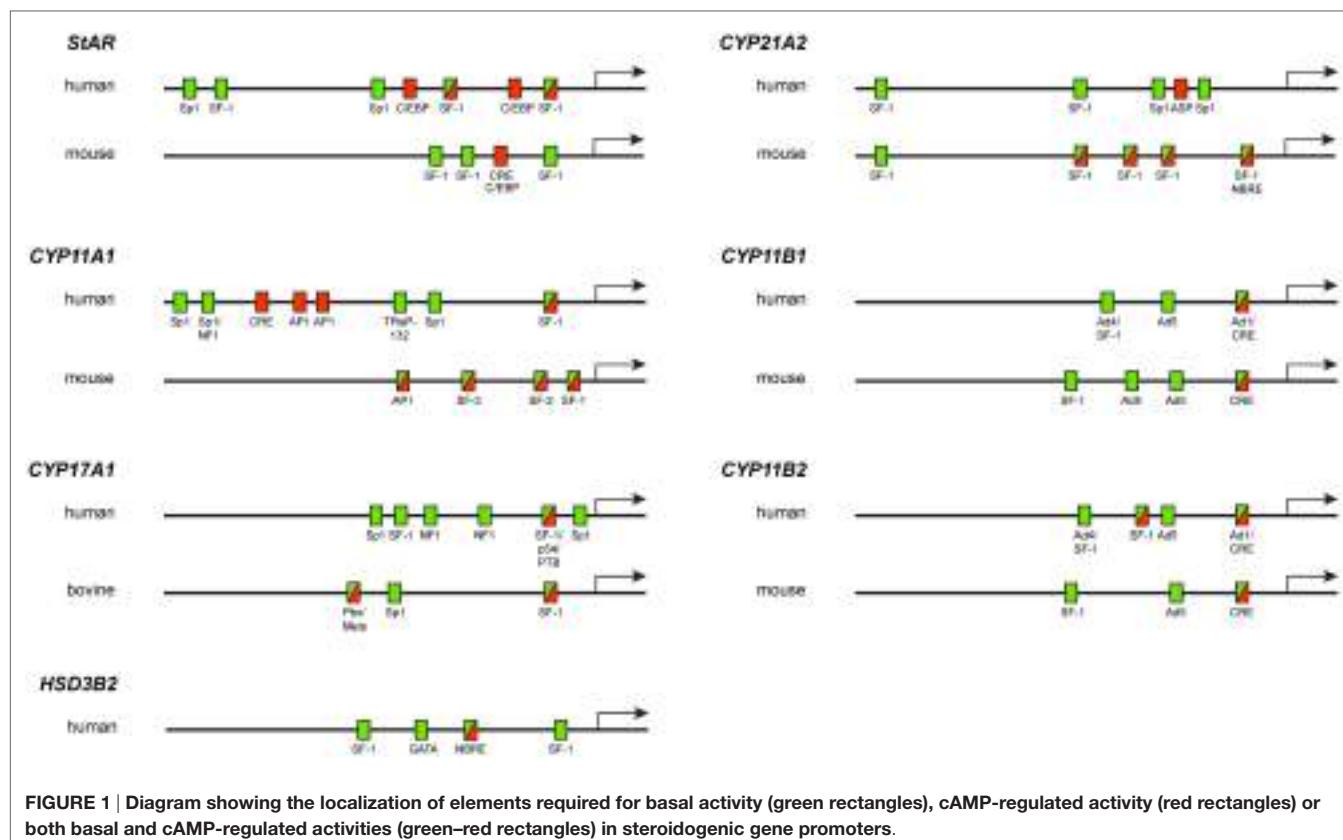
Furthermore, SF-1 is implicated in the transcriptional regulation of *CYP17A1*, the gene encoding the P450c17 enzyme, which catalyzes both the 17 $\alpha$ -hydroxylation of pregnenolone and progesterone (required for cortisol biosynthesis) and the 17,20-lyase reaction of 17 $\alpha$ -hydroxylated steroids (leading to androgen production). Studies carried out on H295R cells revealed that CREs are located within the first 63 bp upstream of the transcriptional

initiation site and that a second basal transcription element lies between −184 and −206 bp (87). SF-1 forms a complex with p54<sup>nrb</sup>/NonO and polypyrimidine tract-binding protein-associated splicing factor (PSF), which binds within those first 60-bp upstream of the transcriptional start site, stimulating *CYP17* expression in response to ACTH/cAMP signaling (58). As for *CYP11A1*, GATA-6 promotes the SF-1-dependent transcription of *CYP17* in H295R cells (83).

Many studies have revealed that the ACTH-regulated expression of the *CYP21A1* gene, coding for the P450c21 enzyme, which has a key role in the production of cortisol and aldosterone, requires the binding of the nuclear receptors SF-1 and Nur77 to its promoter (88–90). Interestingly, SF-1 binds to a distal region that lies approximately 4.8 kb upstream of the *CYP21A1* transcription start site driving adrenal-specific expression of the human gene (65).

## cAMP Response Element/Binding Protein/CRE Modulator/Activating Transcription Factor

A family of cAMP-responsive nuclear factors mediates transcriptional regulation by ACTH/cAMP signaling pathway. This family is composed by a large number of proteins, which are encoded by the CREB, CREM, and ATF genes. Those proteins recognize and bind the 8-bp 5'-TGACGTCA-3' palindromic sequence or a minor variation, called the CRE, which lie within 100 nucleotides of the TATA box in the promoters of eukaryotic cAMP responsive genes (91–93). The members of the CREB family are characterized by their DNA-binding leucine zipper (bZIP) domains and generally they interact with each other to mediate cAMP-dependent transcriptional response (91). Interestingly, the sequences of the mouse (27), human (94), and rat *StAR* promoters, which exhibit an extensive homology, lack a consensus CRE, similarly



**FIGURE 1 |** Diagram showing the localization of elements required for basal activity (green rectangles), cAMP-regulated activity (red rectangles) or both basal and cAMP-regulated activities (green-red rectangles) in steroidogenic gene promoters.

to the promoters of different steroid hydroxylase genes whose transcription is regulated by ACTH/cAMP signaling (57). It has been shown that the cAMP-responsive region of the StAR gene promoter exhibits a highly conserved motif (50-TGACTGATGA-30 corresponding to 281/272 bp in the mouse promoter) to which different bZIP families of transcription factors, like not only CREB, CREM, and ATF1, but also AP-1 and C/EBPs (see below) bind to drive StAR transcription (7, 46, 47, 95, 96).

cAMP response element-binding protein has been demonstrated to be the principal player in mediating stimulus-transcription coupling in the ACTH/cAMP pathway. However, knockout mouse CREB studies showed that this action can be compensated by other CRE-binding proteins like CREM and ATF-1 (97). This mechanism also seems to work in the regulation of steroidogenesis, as CREB family members directly induce StAR gene transcription (6, 7, 47). Interestingly, whereas CREB gene products usually function as positive transactivators, CREM can either activate or repress CRE-mediated transcription (98, 99). Alternative splicing of the CREM gene originates multiple isoforms that can act as either activators ( $\tau$ ,  $\tau_1$ , and  $\tau_2$ ) or repressors ( $\alpha$ ,  $\beta$ , and  $\gamma$ ) of transcription (100). Identical functional regions have been identified in CREB and CREM $\tau$  proteins (99). When overexpressed, either CREB or CREM $\tau$  display qualitatively comparable effects toward cAMP-dependent StAR gene transcription in murine adrenal and gonadal cells (6, 7, 101), whereas CREM $\alpha$  and CREM $\beta$  have been shown to repress StAR transcription (7). Further, CREM proteins can

bind to CREs as homodimers or as heterodimers with CREB/ATF displaying similar functional outcomes to those of CREB (91). Remarkably, it has been shown that CREB and CREM associate with the proximal rather than the distal StAR promoter upon cAMP analog treatment (47, 48). Sugawara and coworkers compared the implication of CREB and CREM in cAMP-mediated StAR gene expression and identified CREM as the main mediator in H295R cells (101). In contrast, another group showed that CREB and ATF-1, but not CREM, mainly bound to the StAR promoter upon ACTH/cAMP stimulation (47). Besides CREB and CREM, also the CRE-binding protein, ATF-1 is a key regulator of StAR gene expression. ATF-1 differs from CREB and CREM as it lacks the glutamine-rich Q1 domain, although this does not affect its ability to work as a transcriptional activator (91, 102). Interestingly, two paralogs of ATF-1, called CRE-binding protein 1(CRE-BP1 or ATF-2) and ATF-a, display alternative exon splicing and bind to CREs, but they are not able to mediate cAMP-responsive transactivations (91).

cAMP response element-binding protein/CREM/ATF are activated by PKA, PKC, and other kinases that phosphorylate them at specific residues within their N-terminus. Indeed, phosphorylation of CREB at Ser133/119 or CREM at Ser117 leads to CREB-CREM interaction with coactivators like CREB-binding protein/p300 (CBP/p300) (see below) with subsequent stimulation of their transcriptional activity (47, 103–105). It has been reported that cAMP analogs increase CREB phosphorylation in a time-dependent manner in steroidogenic cells. This

phosphorylation event correlates with the association of both phosphorylated CREB and CBP to the proximal promoter of the StAR gene (47, 48, 106). The phosphorylation of Ser133 is required for CREB activation; however, it is not sufficient for full activation of the protein. Indeed, it has been shown that a short region C-terminal to the PKA phosphorylation site within CREB protein is required for CREB transcriptional activation (91, 107, 108). The crucial role of CREB phosphorylation by PKA has been also observed *in vivo* where transgenic mice, which express a non-phosphorylatable mutant of CREB (called CREB-M1, Ser133Ala), exhibit somatotroph hypoplasia and dwarfism (109). Moreover, expression of CREB-M1 in adrenal and gonadal cells strongly decreases cAMP-induced StAR gene expression (6, 7, 101).

cAMP response element-binding protein participates together with GATA-6 and AP-1 to the SF-1- and AP-2-dependent *CYP11A1* gene transcription in rodent placenta and ovary (110). Interestingly, the ACTH-stimulated transcription of the human *CYP11B1* gene depends on the CREB family member ATF-2 (52). Moreover, as reported above, ACTH/cAMP signaling regulates *CYP11B2* gene transcription via a CRE and a SF-1-binding site (56). Similarly, CREB binds to a CRE at the level of the mouse *Cyp11b1* gene promoter to drive ACTH-dependent transcription (55).

## CCAT/Enhancer-Binding Proteins

CCAT/enhancer-binding proteins are a family of transcription factors containing a highly conserved bZIP at the C-terminus that is involved in dimerization and DNA binding. C/EBPs bind with different affinities to a consensus site consisting of a dyad symmetrical repeat (A/GTTGCGC/TAAC/T) (111). At least six members of the family have been cloned and characterized, named from C/EBP $\alpha$  to C/EBP $\zeta$ . C/EBP $\alpha$  and C/EBP $\beta$  are expressed in steroidogenic cells, the expression levels of C/EBP $\beta$  being increased in the nucleus by the action of LH and analogs of cAMP (112, 113). The cAMP-inducible domains of C/EBPs, with the exception for C/EBP $\beta$ , lack a PKA phosphorylation site, which implies that C/EBPs are able to mediate cAMP-dependent responses by indirect mechanisms. In contrast, C/EBP $\beta$  phosphorylation by PKA within its bZIP domain affects its DNA-binding activity (114).

C/EBP $\beta$  targets a binding region in the promoter of the mouse *Star* gene (281/272 bp), which is also bound by CREB/ATF (see above) and Fos/Jun (see below). Further, two putative C/EBP-binding sites have been identified within the human StAR promoter (43). Consequently, an implication for both C/EBP $\alpha$  and C/EBP $\beta$  in StAR gene transcription has been reported (43, 115, 116). C/EBP $\beta$  phosphorylation on Thr325 increases its association to the proximal StAR promoter, thus inducing StAR transcription (117, 118). Interestingly, it has been shown that GATA-4 and C/EBP $\beta$  directly interact *in vitro* and *in vivo* and synergistically activate the StAR promoter only in the presence of PKA (95, 117). This suggests that GATA-C/EBP transcriptional cooperation might promote ACTH/cAMP-dependent StAR transcription in all steroidogenic tissues, as this kind of PKA-dependent synergy has been shown for other members of GATA and C/EBP families. Finally, the disruption of either C/EBP $\alpha$  or C/EBP $\beta$  impairs normal reproductive development in

female mice, with consequent reduced or altered ovulation and corpus luteum formation inability (112, 119).

## AP-1 Family of Transcription Factors

The AP-1 family of transcription factors participates in the regulation of cellular responses to multiple stimuli regulating proliferation, transformation, and cell death (120). It recognizes and binds to a DNA sequence known as the AP-1/phorbol 12-O-tetradecanoate 13-acetate responsive element [AP-1/TRE; TGA(C/G)TCA]. AP-1 is composed by a mixture of homo- and hetero-dimers formed between Jun (c-Jun, JunB, and JunD) and Fos (c-Fos, FosB, Fra1, and Fra2) family members (121–123). Fos members heterodimerize with Jun proteins and with specific members of the CREB/ATF family, but they are not able to form homodimers, whereas Jun members function as homodimers or heterodimers among themselves or with members of the Fos and CREB/ATF families (124). Both Jun and Fos family proteins belong to the bZIP group of DNA-binding transcription factors and their dimerization is necessary for specific and high affinity binding to the palindromic DNA sequence TGAC/GTCA (see above) (125). Studies carried out on the mouse *Star* promoter identified a highly conserved element (TGACTGA, -81/-75 bp), which shows homology with the AP-1/TRE sequence and overlaps also with the CRE2 sequence (6). Interestingly, it has been shown that Fos and Jun bind to this element, called CRE2/AP-1, thus regulating *Star* gene transcription (46, 96, 126). Moreover, two additional putative AP-1-binding sites have been identified within the rat *Star* promoter. c-Fos reduces basal, cAMP-, and c-Jun-mediated rat *Star* gene transcription in Y-1 adrenocortical cells (127). Indeed, a functional comparison between Fos and Jun revealed that c-Jun is the most powerful AP-1 family member for *Star* gene transactivation. Accordingly to this, it has been shown that only c-Jun, but not other AP-1 members, plays a pivotal role in the regulation of PKC-mediated *Star* transcription and steroidogenesis in Leydig and adrenal cells (96, 128).

Not only Protein kinase A but also PKC phosphorylate several Ser and Thr residues on c-Jun and c-Fos. In particular, the treatment with a cAMP analog or a growth factor increases the phosphorylation of c-Jun Ser63 and c-Fos Thr325. Those phosphorylation events are associated to StAR gene transcription and steroidogenesis in mouse Leydig cells (47, 96, 126, 129). Interestingly, ACTH/cAMP-dependent c-Jun and c-Fos phosphorylation increases the association between p-c-Jun/p-c-Fos and the CBP/p300 cofactors (see below), with consequent recruitment of CBP/p300 to the StAR promoter (47, 96, 126). The phosphorylation of c-Fos and c-Jun can alter their capacity to interact with other transcription factors, affecting their dimerization and DNA-binding specificity (124). This explains why the crosstalk between CREB and c-Fos/c-Jun can be associated with both gain and loss of function on the same *cis*-element in the context of a fine regulation of the transregulatory elements which participate in StAR gene transcription (126).

## Sp Family of Transcription Factors

The Sp family of transcription factors is characterized by the presence of three conserved Cys2His2-type zinc fingers at their

C-terminus that form the sequence-specific DNA-binding domain (130). They can bind and exert their action through GC/GT-rich promoter elements to regulate the expression of multiple target genes (130, 131). Sp1 is the most well characterized member of the family and it exhibits similar structure and a high homology with Sp3 at the level of its DNA-binding domains, both being able to enhance or repress promoter activity. However, although Sp1 and Sp3 recognize the same consensus-binding sites, it has been reported that both their DNA-binding properties and regulatory functions differ and depend on the promoter context and cellular background (130, 132, 133).

Regulatory elements for Sp1 and Sp3 have been identified within the human TSPO proximal promoter (134). They have been demonstrated to be strong positive elements for the promoter activity, although differences have been detected in the mechanism by which TSPO is regulated in non-steroidogenic versus steroidogenic cells (134). Sp1 is also involved in the cAMP-dependent transcription of the *CYP11A1* gene in human (135), bovine (136, 137), and porcine (138) adrenals. Furthermore, a cooperation between SF-1, Sp1, and CBP has been reported to drive cAMP-dependent *CYP11A1* transcription in bovine adrenal (136). Sp1 and Sp3, together with the nuclear factor-1C (NF-1C), bind to the second basal element of the *CYP17* gene promoter. This event is crucial for optimal basal transcription (59). Sp1 can also form a complex with GATA-4 or GATA-6 to regulate the expression of *CYP17* in the adrenal gland (139). Finally, Sp1 and adrenal-specific protein (ASP) bind to the *CYP21* gene promoter to regulate its cAMP-dependent transcription (140). This synergistic action seems to be required for maximal *CYP21* induction (140).

## DAX-1

*DAX-1/NR0B1* encodes an unusual member of the nuclear hormone receptor family of transcription factors. Its mutations cause adrenal hypoplasia congenita (AHC) associated with hypogonadotropic hypogonadism (HHG) (141, 142). *DAX-1* expression pattern, mostly restricted to steroidogenic tissues, suggested that it may have a role in the regulation of steroidogenesis. Indeed, in adrenocortical cells, DAX-1 works as a global negative regulator of basal and cAMP-regulated transcription of steroidogenic genes, both through direct binding to gene promoters and interaction with SF-1 and other transcription factors [(143–145); reviewed in Ref. (146)]. In addition, consistently with DAX-1 negative action on steroidogenesis, activation of the PKA pathway by ACTH in adrenocortical cells (147) and FSH in Sertoli cells (148) downregulates Dax-1 expression.

## ROLE OF COREGULATORS

Transcriptional coregulators are crucially implicated in nuclear receptor-mediated transcriptional activation (149–151) and transactivation by other factors, exerting their roles in multiple processes, like histone modification (152–154), chromatin remodeling (155), post-translational modification of transactivator complex members (156, 157), and ordered recruitment of basal transcriptional machinery (158, 159).

The role of coactivators and corepressors in the transcriptional regulation of steroid hydroxylase genes and *StAR* has been shown by several studies (74, 76). CBP and its functional homolog p300 are transcriptional coactivators that contain multiple functional domains and display intrinsic histone acetyltransferase activity (151, 160), by which they increase transcription factor accessibility to nucleosomal DNA. Classically, ACTH/cAMP signaling triggers the phosphorylation of specific transcription factors, which in turn can bind and recruit CBP/p300 (105, 160–162). As already reported (see above), when phosphorylated at Ser133 CREB is able to interact with CBP (104, 105). Interestingly, CBP/p300 contain PKA consensus sites, the phosphorylation of which is involved in regulating their functions. CBP/p300 play a key role in the transcriptional regulation of the *StAR* gene. Different studies have shown that cAMP-dependent phosphorylation of CREB at Ser133, c-Jun at Ser63, and c-Fos at Thr325 promotes the association and recruitment of CBP/p300 to the proximal *StAR* promoter (47, 48, 95, 126). cAMP-stimulated phosphorylation of C/EBP $\beta$  at Thr325 also increases C/EBP $\beta$  association to the proximal *StAR* promoter (117). Other factors like SF-1 and GATA-4 (see above), that are bound to the proximal *StAR* promoter, once phosphorylated in response to ACTH might enhance CBP/p300 recruitment to the promoter. This correlates with the CBP/p300-dependent increased effects of C/EBP $\beta$  and GATA-4 on *StAR* expression (95). Further, when overexpressed, CBP/p300 potentiated CREB, Fos/Jun, C/EBP $\beta$ , and GATA-4 transcriptional activity on the *StAR* gene (95, 126). This event is attenuated by the adenovirus E1A oncoprotein, which acts impairing CBP/p300 histone acetyltransferase activity and/or their interaction with other transcription factors or with the basal transcription machinery.

As already reported, coactivators and corepressors also play a key role in the transcriptional regulation of steroid hydroxylase genes. We report here some examples. Coexpression of CBP/p300 with the zinc finger protein TReP-132 has an additive effect on human *CYP11A1* gene promoter activity (163). Similarly, it has been shown that the coactivators CBP/p300, steroid receptor coactivator (SRC)-1, and transcriptional intermediary factor-2 (TIF-2) enhance SF-1-mediated bovine *CYP17* transcription, whereas the corepressors nuclear receptor corepressor (N-CoR) and the silencing mediator of retinoic acid and thyroid hormone receptor (SMRT) increase the repressive activity of chicken ovalbumin upstream-transcription factor 1(COUP-TF-1) (164).

Finally, Sewer and coworkers have shown that a corepressor protein called mSin3A inhibits human *CYP17* gene transcription by the recruitment of a histone deacetylase to the SF-1/NonO/PSF complex that binds to the *CYP17* promoter (58). They have also described that coregulator exchange and sphingosine-sensitive cooperativity of SF-1, general control non-derepressed 5 (GCN5), p54, and p160 coactivators regulate cAMP-dependent *CYP17* transcription rate in H295R cells (165). The same group has shown that ACTH/cAMP signaling pathway promotes acid ceramidase (*ASAHI*) gene transcription via the binding of CREB to multiple region of the *ASAHI* promoter. This event triggers the recruitment of CBP/p300 with a related increase in the trimethylation of Lys4 on histone H3 (H3K4) on the *ASAHI* promoter in H295R cells (166).

## ROLE OF PHOSPHATASE ACTIVITY ON ACTH/cAMP-DEPENDENT STEROIDOGENIC GENE TRANSCRIPTION

As already discussed, the ACTH-dependent increase in intracellular cAMP levels leads to the activation of PKA, which, in turn, phosphorylates specific nuclear factors to drive steroidogenic gene transcription. Remarkably, in the adrenal cortex ACTH regulation of steroidogenesis depends not only on PKA-mediated Ser/Thr phosphorylation, but also on the activity of protein tyrosine phosphatases (PTPs), which have been implicated in StAR expression and steroidogenesis (167–172). Indeed, the phosphodephosphorylation of intermediate proteins is considered as a key event in the regulation of steroid biosynthesis. In 1999, Paz and coworkers showed that *in vivo* treatment with ACTH leads to an increase in total PTPs activity in adrenal *zona fasciculata*. The stimulation was characterized by a rapid onset (5 min), reached a peak after 15 min of ACTH administration (around twofold) and returned to basal levels after 30 min (168). They showed that the increase in PTPs correlated with a decrease in phosphotyrosine proteins (168). Moreover, the PTPs inhibitors perva-nadate (PV) and phenylarsine oxide (PAO) inhibited ACTH- and 8Br-cAMP (a permeant analog of adenosine 3',5'-phosphate)-dependent steroidogenesis in a dose-dependent fashion, whereas they did not affect steroid production supported by a cell-permeant analog of cholesterol (168). Those studies clearly indicated that PTPs activity has a key role in ACTH/cAMP signaling pathway, acting downstream of PKA activation and upstream of cholesterol transport across the mitochondrial membrane. The same group evaluated steroid production and StAR protein levels in Y1 cells upon PTP inhibition. They reported that PAO reduced ACTH-dependent stimulated steroidogenesis in those cells in a concentration-dependent manner and abrogated StAR protein induction (169). Those effects have been reproduced by a second PTPs inhibitor, benzyl phosphonic acid, which has a different mechanism of action (169). Altogether, those results show that PKA-mediated PTP activation in the steroidogenic system exerts the functional role of mediating StAR protein induction (169). The ACTH/cAMP/PKA signaling pathway stimulates also the release of arachidonic acid (AA) in adrenal and Leydig cells by the concerted action of two enzymes: an acylCoA-thioesterase (Acot2) and an acyl-CoA-synthetase (ACS4) (170, 173). Several reports have shown that AA and its metabolites play a key role in the hormonal control of steroidogenesis by regulating both the expression and function of StAR protein (174, 175). The ACTH/PKA system has been shown to control this pathway upregulating the ACS4 protein levels in adrenal and Leydig cells (175). Two

PTP inhibitors both abrogate the ACTH/PKA-dependent ACS4 induction and reduced the effects of cAMP on steroidogenesis and StAR protein levels (175). Interestingly, exogenous AA is able to overcome this PTP-dependent inhibitory effect on StAR protein expression and steroidogenesis (176, 177). Furthermore, Sewer and Waterman have shown that PTP activity is essential for cAMP-dependent transcription of the human *CYP17* gene in H295R cells (178). They also investigated whether the inhibition of PTP activity can impair cAMP-dependent mRNA expression of other steroidogenic genes in the adrenal cortex. They have reported that *CYP11A1*, *CYP11B1/2*, and *CYP21A1* also require PTPs for cAMP-dependent mRNA expression, as the inhibition of both serine/threonine and tyrosine phosphatase activities negatively affected this event (178). Those evidences led those authors to propose a model whereby PKA phosphorylates and activates a dual-specificity phosphatase (DSP), which is able to mediate ACTH/cAMP/PKA-dependent transcription of steroidogenic genes (178, 179). The specific DSP has been identified as mitogen-activated protein phosphatase 1 (MKP-1), whose levels are increased by ACTH and cAMP in H295R cells (180). Moreover, the evidence showing that MKP1 overexpression promotes the transcriptional activity of a human *CYP17* promoter-reporter construct and its silencing decreases cAMP-stimulated *CYP17* gene expression, suggest a role for MKP-1 in cAMP-dependent *CYP17* transcriptional activation (180).

## SUMMARY AND CONCLUSION

The studies outlined here have given an important contribution to the understanding of the impact of ACTH on the regulation of steroidogenic gene expression in the adrenal cortex. Given the pivotal role played by ACTH/cAMP signaling in the acute and chronic regulation of steroid secretion and the implication of those hormones in diverse arrays of fundamental physiological processes, an in-depth investigation is needed to completely elucidate the ACTH-dependent transcriptional regulatory mechanisms that control steroid secretion. Indeed, some aspects addressed in this review still represent a challenge for future studies, which might provide the scientific community with a broader picture of the complex signaling pathways and the intricate transcriptional circuitries that coordinately ensure optimal hormonal output.

## AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct, and intellectual contribution to the work, and approved it for publication.

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# ACTH Action on Messenger RNA Stability Mechanisms

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The regulation of mRNA stability has emerged as a critical control step in dynamic gene expression. This process occurs in response to modifications of the cellular environment, including hormonal variations, and regulates the expression of subsets of proteins whose levels need to be rapidly adjusted. Modulation of messenger RNA stability is usually mediated by stabilizing or destabilizing RNA-binding proteins (RNA-BP) that bind to the 3'-untranslated region regulatory motifs, such as AU-rich elements (AREs). Destabilizing ARE-binding proteins enhance the decay of their target transcripts by recruiting the mRNA decay machineries. Failure of such mechanisms, in particular mis-expression of RNA-BP, has been linked to several human diseases. In the adrenal cortex, the expression and activity of mRNA stability regulatory proteins are still understudied. However, ACTH- or cAMP-elicited changes in the expression/phosphorylation status of the major mRNA-destabilizing protein TIS11b/BRF1 or in the subcellular localization of the stabilizing protein Human antigen R have been reported. They suggest that this level of regulation of gene expression is also important in endocrinology.

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## INTRODUCTION

Transcriptional regulation of the cellular responses to hormones has been the primary focus of many endocrinological research studies during the past decades. Although the transcriptional mechanisms that regulate the production of specific mRNAs are undoubtedly important, it has become increasingly evident that processes regulating the stability of mRNAs also represent critical steps in the control of dynamic gene expression. In particular, acute changes in gene expression are now recognized to be controlled by RNA-binding proteins (RNA-BP) and microRNAs through their binding to target transcripts and their positive or negative regulation of mRNA turnover. Because the ability to respond to rapid changes in ACTH levels is essential for maintaining steroid hormone homeostasis, posttranscriptional mechanisms are expected to be involved in ACTH action. The pleiotropic effects exerted by ACTH on adrenocortical cell functions are regulated through a multiplicity of mechanisms. Through binding to its adenylate-cyclase-coupled receptor MC2R, ACTH stimulates the release of cAMP and the activation of the cAMP-dependent protein kinase A (PKA), which in turn phosphorylates and regulates a number of specific substrates including the steroidogenic acute regulatory protein (Star) (1) and the cAMP response element-binding protein (CREB) (2). ACTH also strongly regulates the transcription of a number of genes involved in the

**Abbreviations:** TIS11b, TPA-inducible sequence 11b; BRF1, butyrate response factor 1; ZFP36, zinc finger protein; TTP, tristetraprolin; CCR4, carbon catabolite repressor protein 4; CNOT1, CCR4-NOT transcription complex subunit 1; Dcp1a, decapping enzyme 1a.

steroidogenic response including those encoding several steroidogenic enzymes (3), components of the extracellular matrix (4) and many others. Another less characterized level of regulation through which ACTH exerts its actions is the control of mRNA stability through the activity of specific proteins that bind the 3'-untranslated region (3'-UTR) of target mRNAs. We were first to observe that the increase in vascular endothelial growth factor-A (VEGF-A) mRNA induced by ACTH in primary adrenocortical fasciculata cells did not result from increased transcription (5) but from stabilization of its mRNA (6). Here, we will present the proteins that mediate the regulation of short-lived mRNA stability/degradation and focus on those which are regulated by ACTH in adrenocortical cells.

## mRNA STABILITY MECHANISMS: AN INTERPLAY BETWEEN C/S-ACTING ELEMENTS AND TRANS-ACTING FACTORS

The steady-state level of any mRNA in an eukaryotic cell results from the balance between its synthesis through gene transcription and its degradation through the mRNA decay machinery. Regulation of mRNA stability implies both *cis* elements mainly located in the 3'-UTR of mRNAs and *trans*-acting factors. These latter factors comprise a number of RNA-BP that specifically bind distinct *cis* elements, form multimolecular scaffolds that favor or prevent the subsequent recruitment of the mRNA deadenylation and mRNA degradation machineries (Figure 1A) (7). Eukaryotic mRNAs are protected at both extremities by a 7-methyl guanosine cap at their 5'-end that confers resistance to 5' to 3' exonucleases and by a poly(A)-tail recruiting the poly(A)-binding protein (PABP) at their 3'-end that confers resistance to 3' to 5' exonuclease attack by the exosome complex (8). The most frequently distributed and best-studied *cis* elements are the AU-rich elements (AREs) that are often arranged in mRNA 3'-UTRs as repeated AUUUA pentameric sequences that eventually overlap (9). More than 6,000 human ARE-containing mRNAs have been listed in the last upgrade of the ARED 3.0 database (<http://brp.kfshrc.edu.sa/ARED/>) (10). Many of them have a short half-life, rendering this regulatory process highly effective to rapidly turn down a cellular function.

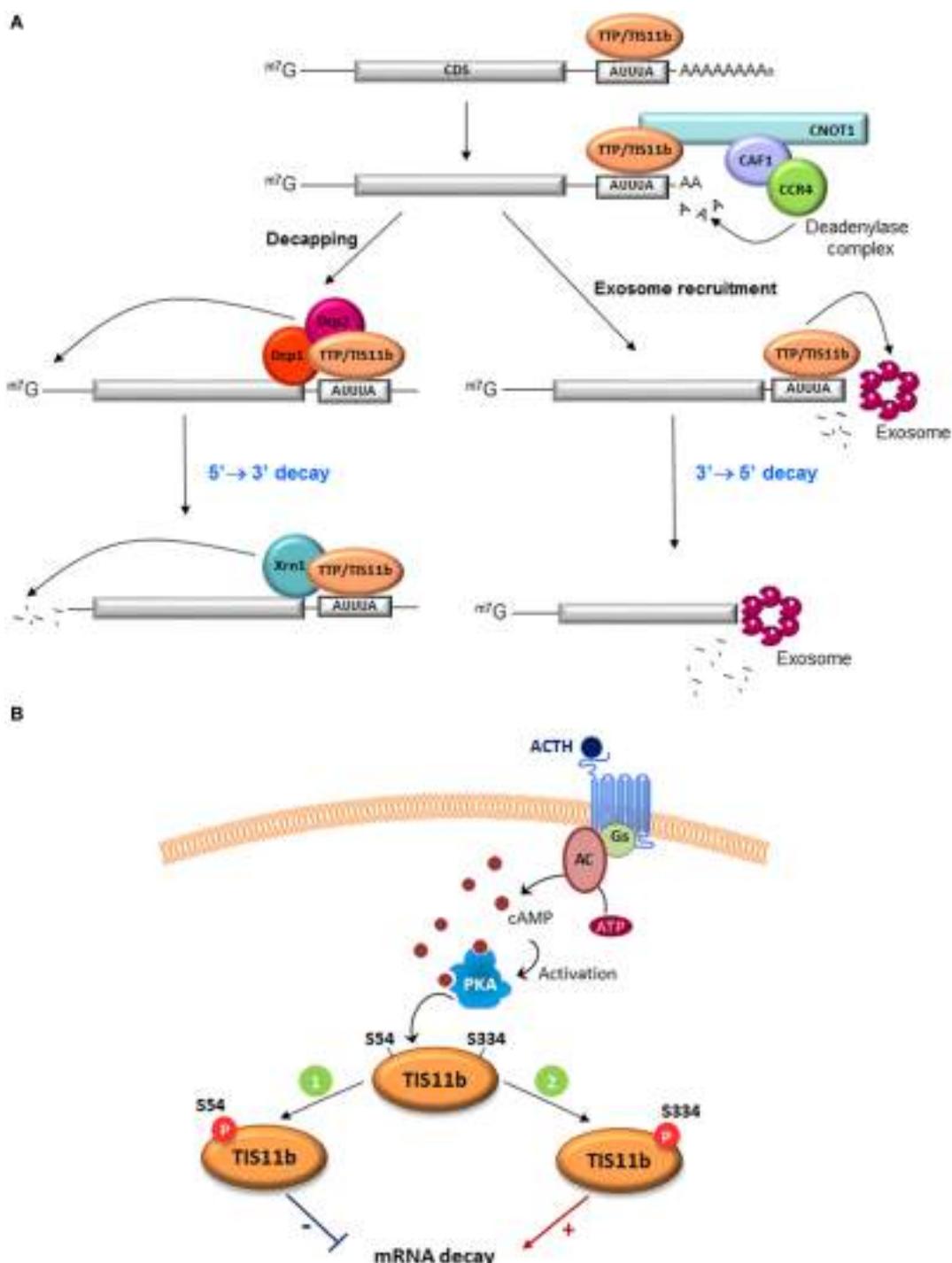
## AREs BINDING PROTEINS

Among the more than 20 ARE-binding proteins (ARE-BP) identified so far, some are destabilizing the target mRNA by recruiting deadenylases and exonucleases and some are stabilizing them by protecting them from these degradation enzymes. The competition between both types of proteins for a given ARE will ultimately determine the fate of the target mRNA. The best characterized stabilizing ARE-BP is human antigen R (HuR), which is a member of the ELAV (homologs of the *Drosophila* proteins *embryonic lethal abnormal vision*) family (11). HuR is ubiquitously expressed and is predominantly localized in the nucleus of non-stimulated cells where it forms messenger ribonucleoprotein complexes that are assembled during splicing of primary transcripts, prior

to transport of mature mRNAs to the cytoplasm (12). Upon cell activation by various stimuli, HuR undergoes CRM1-dependent nuclear-cytoplasmic shuttling, directed by localization signals (13). Binding of HuR to ARE may play important roles in controlling the processing, splicing, and polyadenylation of the nuclear transcript, together with the nuclear export and stabilization/translation in the cytoplasm. The exact mechanism by which HuR stabilizes target mRNAs is still unclear, but HuR has been reported in many cell types to prevent the degradation of target mRNAs by competing with destabilizing proteins and thereby preventing their recruitment of the exosome machinery (6, 14). A larger number of destabilizing proteins has been described. Among them, the tristetraprolin (TTP) family, which comprises three distinct members in mammals (TTP/TIS11/ZFP36, TIS11b/BRF1/ZFP36-L1, and TIS11d/BRF2/ZFP36-L2) is one of the best characterized. Although they all bind to similar synthetic sequences *in vitro*, members of the TTP family present specific sites of action and preferential targets *in vivo*, as demonstrated by the distinct phenotypes of the mice that have been genetically invalidated for each of these genes. In TTP-KO mice, the TNF $\alpha$  mRNA is significantly stabilized in macrophages, resulting in a fatal postnatal inflammatory syndrome (15). Deletion of TIS11b/BRF1 gene in mice results in embryonic lethality due to abnormal placentation and major vascular defects (16, 17). This is mainly caused by the failure of TIS11b to repress the expression of VEGF at the end of the developmental angiogenic process. The complete invalidation of the TIS11d gene causes postnatal lethality due to defective definitive hematopoiesis (18). Structural studies have shown that the tandem zing finger repeats of TTP family proteins bind to the 5'-UUAUUU(U/A)(U/A)-3' recognition motif while the C-terminal and N-terminal domains of the proteins participate in the recruitment of the mRNA deadenylation and mRNA degradation enzymes (19, 20). Other destabilizing ARE-BP include KH-splicing regulatory protein (KSRP) and the AU-rich RNA-binding factor 1 (AUF1/HnRNP D).

## ACTH ACTION ON mRNA DECAY MECHANISMS: FROM TRANSCRIPTION TO PHOSPHORYLATION OF ARE-BP

TIS11b, also named BRF1 or ZFP36-L1, was identified in the adrenal cortex through a differential display RT-PCR analysis of ACTH-stimulated versus non-stimulated adrenocortical cells (21). HuR and alternatively spliced isoforms of AUF1/hnRNPD are also expressed in adrenal cells (6, 22). Recently, it was established that ACTH induced *zfp36-L1* gene transcription in bovine adrenocortical cells (BAC) through phosphorylation of CREB transcription factor and CREB-mediated activation of TIS11b promoter (23). A highly conserved cAMP response element (CRE) was found at -402 to -394 relative to the transcription start site (TSS) of human *zfp36-L1* gene. TIS11b mRNA is also rapidly stimulated by 8-bromo-cAMP in murine adrenocortical Y-1 cells or Leydig MA-10 cells (22), suggesting that the protein is a key regulator of endocrine tissue biology. Interestingly, no classical CRE was identified within the -2,000 bp upstream of the TSS of the two other family members TTP and TIS11d. In agreement



**FIGURE 1 | Function of tristetraprolin (TTP) and TIS11b in ARE-mediated mRNA decay. (A)** TTP and TIS11b bind to AU-rich elements in the 3'-UTR of target mRNAs and recruit the deadenylase complex directly (CCR4-CAF-NOT1 complex) to trigger mRNA deadenylation. Deadenylated transcripts are degraded through TTP- or TIS11b-mediated recruitment of the exosome, a multiprotein complex that promotes the 3' to 5' mRNA decay. Alternatively, deadenylation can be followed by mRNA decapping by the decapping enzymes Dcp1/Dcp2 and the 5' to 3' mRNA degradation by the Xrn1 exonuclease. CDS, coding sequence. **(B)** Two putative protein kinase A (PKA) phosphorylation sites, S554 and S334, were identified in TIS11b protein sequence with important roles in protein activity and stability. ACTH stimulation increases intracellular cAMP levels through the action of the G protein Gs and the adenylyl cyclase. This leads to activation of PKA (1). Phosphorylation of TIS11b at S554 by PKA inhibits protein activity. TIS11b-phospho-S554 is sequestered in the cytoplasm due to enhanced interaction with 14-3-3 proteins. This mechanism would promote vascular endothelial growth factor (VEGF) mRNA induction (2). To turn down VEGF production, phosphorylation of TIS11b at S334 by PKA increases protein stability and activity. Dephosphorylation of both serines presumably by the phosphatase PP2A leads to degradation of TIS11b via the proteasome.

with this observation, TTP or TIS11d mRNA levels were not changed upon ACTH challenge, pointing at a specific induction of TIS11b by the hormone in the adrenal cortex (23).

Two major target transcripts of TIS11b have been identified in the adrenal cortex. The first one is the message encoding the angiogenic cytokine VEGF (24). Knowing that VEGF mRNA was rapidly but transiently induced by ACTH in fasciculata cells through a transcription-independent mechanism, the contribution of TIS11b and HuR to this transient induction was investigated. Using HuR- and TIS11b-specific siRNAs, it was established that (i) ACTH induced nucleo-cytoplasmic translocation of HuR to trigger VEGF mRNA stabilization in the cytoplasm and (ii) TIS11b, which is induced later by ACTH, participates in the downregulation of VEGF mRNA levels. A short 75-bp long sequence in VEGF mRNA 3'-UTR was shown to bind TIS11b through two adjacent UUAUUUAU and AUUUA motifs. The second identified target of TIS11b in endocrine cells is the StAR mRNA whose PKA-stimulated transcription paralleled TIS11b induction (22). StAR mediates intramitochondrial cholesterol transport in most steroidogenic tissues in response to hormonal changes (1). Cyclic AMP stimulates two major StAR transcripts of 3.5 and 1.6 kb, which arise from differential use of polyadenylation signals and therefore differ only in their 3'-UTR (lengths 0.7 and 2.8 kb, respectively). In mouse MA-10 and Y-1 cells, 8-bromo-cAMP stimulates StAR 3.5-kb mRNA as the predominant form. In BAC, similar long and short forms appear equally (21), whereas in human adrenocortical carcinoma cells H295R, di-butryryl-cAMP selectively stimulates the short form of StAR mRNA (25). Following translation, the 3.5-kb StAR message is preferentially degraded after removal of the stimulus through the action of TIS11b binding to UAUUUAUU repeats in the extended 3'-UTR. This attenuation process provides a rapid mechanism to inactivate StAR when hormonal stimulation ceases.

ARE-binding proteins are also distal targets of several signaling pathways. TTP and TIS11b are phosphorylated by a variety of protein kinases. However, the impact of these phosphorylations on their mRNA-destabilizing activity is still a matter of debate, with some studies indicating that phosphorylations of TTP decrease its affinity for ARE-rich mRNAs (26) and other studies reporting that phosphorylations by ERK, p38 MAP-Kinase/MK2, or JNK kinase do not impact this affinity (27). Similarly, phosphorylation of TIS11b by PKB/Akt or MK2 at conserved serine residues did not affect its ability to bind AREs but nevertheless inhibited its ability to promote ARE-mRNA degradation (28, 29). The current accepted model suggests that protein-kinase activation leads to phosphorylation of TTP or TIS11b, favors their sequestration by 14.3.3 proteins, and thereby reduces their mRNA-destabilizing action. Upon signal extinction, dephosphorylation of TTP and TIS11b occurs probably *via* protein phosphatase PP2A and allows the recruitment of the mRNA decay ribonucleases. A new ACTH-regulated phosphorylation site recently identified in TIS11b seems, however, to mediate a different biological response. ACTH-mediated activation of PKA was reported to induce the phosphorylation of TIS11b on two serine residues, S54 and S334 (23) (**Figure 1B**). Analysis of phospho-dead (S54A, S334A) and phospho-mimick (S54D, S334D) TIS11b mutants revealed that S54 regulates the binding of TIS11b to 14.3.3 protein

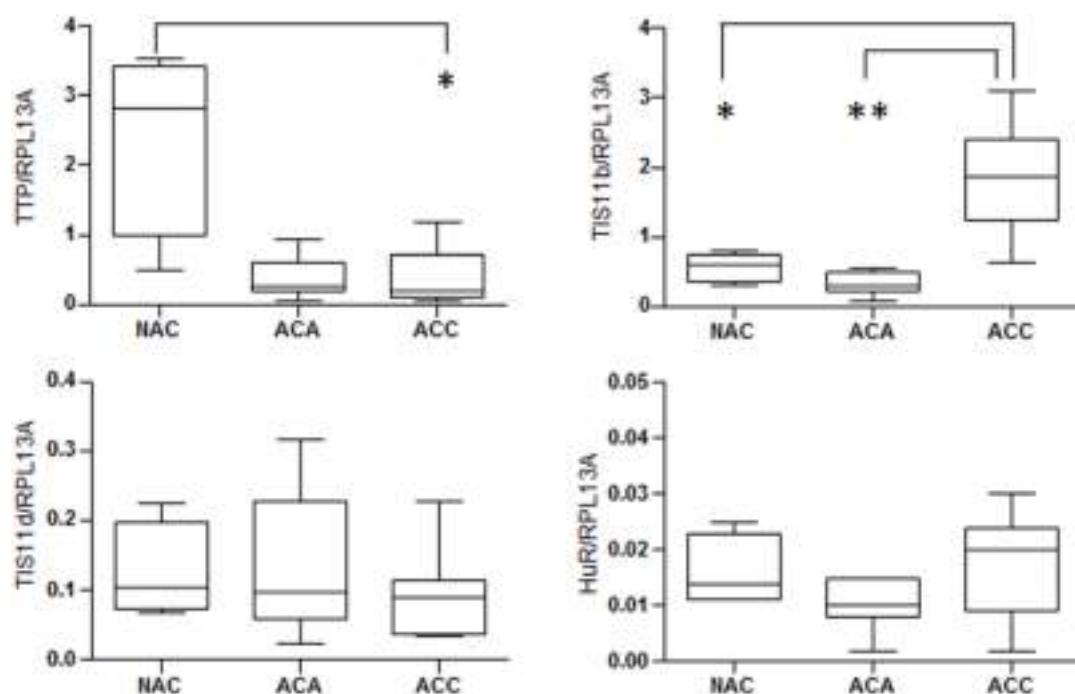
but that S334 does not play a significant role in this interaction. In contrast, the C-terminal S334 phospho-site appears to be involved in the interaction with the CCR4-NOT1 deadenylation complex. TIS11b S334D phospho-mimick mutants presented a reduced association with CNOT1, a core subunit of the CCR4-NOT deadenylase complex, but however displayed an enhanced interaction with the decapping enzyme Dcp1a. Unexpectedly, this interaction was associated with an increased mRNA-destabilizing activity of TIS11b S334D as compared to the dephospho-form of TIS11b (23). These observations suggest that combinatorial phosphorylations of TIS11b on specific residues do not systematically abrogate their mRNA-destabilizing capability but rather fine-tune their interactions with the mRNA decay machineries.

## EXPRESSION OF TTP FAMILY MEMBERS IN ADRENOCORTICAL TUMORS

Overexpression of ARE-containing transcripts encoding factors promoting growth, inflammation, angiogenesis, and invasion has been observed in carcinogenesis (30). These aberrant expressions results from dysfunctional ARE-mediated posttranscriptional control, which seems to be mainly due to deregulations in ARE-binding proteins rather than to ARE mutations. Downregulation of TTP expression has been found in a variety of human malignancies including breast, colon, prostate, and lung cancers (31–33). The loss of TTP expression seems to be an early event during tumorigenesis. Nevertheless, apart from single-nucleotide polymorphisms associated with decreased translation efficiency, the mechanisms leading to TTP suppression in cancer remain obscure. Analysis of the mRNA expression levels of TTP family members (TTP, TIS11b, and TIS11d) and HuR in human adrenocortical tumors revealed that TTP mRNA was dramatically decreased in adrenocortical adenoma (ACA) and adrenocortical carcinomas (ACC) as compared to normal adrenal cortex (NAC) (**Figure 2**). By contrast, TIS11b mRNA is highly expressed in ACC as compared to ACA and NAC while the expression of the third member of the family TIS11d was similar in all the tissues examined. No significant difference in HuR expression was found between normal cortex and adrenocortical tumors. Remarkably, the expression patterns of TTP and TIS11b are symmetrically opposite in normal adrenal cortex and malignant tumors. The relevance of these variations to human physiology and the pathology of adrenocortical cancer remains to be determined. In addition, these results will require validation by measurement of TTP and TIS11b protein levels.

## CLOSING REMARKS AND PERSPECTIVES

Transcriptional regulation has been considered the primary control point of protein production in eukaryotic cells. However, there is growing evidence of pivotal posttranscriptional regulation for many genes, including those involved in differentiated functions of the adrenal cortex such as the StAR gene. This has prompted extensive investigations to elucidate the mechanisms controlling RNA processing, mRNA nuclear export and localization, mRNA stability, and turnover, in addition to translational



**FIGURE 2 | Expression of mRNA stability regulators in human adrenocortical tumors.** Relative expression of mRNA stability factors in adrenocortical tumor samples from the French COMETE (CORTico et MEDullosurrénales, Tumeurs Endocrines) Network. TTP family members (TTP, TIS11b, and TIS11d) and HuR were quantified by reverse transcription-quantitative PCR in 4 normal adrenal cortex, 11 cortisol-producing adrenocortical adenomas, and 15 adrenocortical carcinomas. RPL13A was used as housekeeping gene for normalization. The graphs show median with interquartile range. All data were analyzed using the GraphPad Prism Software and were considered as statistically different when  $p < 0.05$  (\*) and  $p < 0.01$  (\*\*).

rates and posttranslational events. The regulation of mRNA stability has emerged as a critical control step in determining the cellular mRNA level, which is regulated through specific RNA sequence elements–protein interactions. In this context, study of the hormonal control of mRNA stability regulatory proteins and their activity in adrenal cortex function is just beginning. Considering that acute ACTH treatment affects a large number of transcripts, it seems very likely that mRNA stability regulations might play an important role in these transient gene expressions. These mechanisms are expected to also operate in response to other cAMP-mobilizing hormones in their respective target organs. For example, parathyroid hormone has been shown to decrease sodium/hydrogen exchanger 3 mRNA stability through the action of the destabilizing protein KSRP in kidney epithelial cells (34). Importantly, few mRNA stability regulatory factors have been identified so far that appear to control a large pool of target mRNAs. This suggests that a slight alteration in the control mechanism may generate large-scale effects that could contribute

to the development of complex disorders, including adrenal diseases. Efforts in studying mRNA stability regulators in adrenal cortex and their hormonal regulations should be made in order to better understand their potential contribution to adrenocortical pathologies and possibly discover potential biomarkers and therapeutic targets.

## AUTHOR CONTRIBUTIONS

All authors listed have contributed to the work.

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# Role of Protein Phosphorylation and Tyrosine Phosphatases in the Adrenal Regulation of Steroid Synthesis and Mitochondrial Function

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In adrenocortical cells, adrenocorticotropin (ACTH) promotes the activation of several protein kinases. The action of these kinases is linked to steroid production, mainly through steroidogenic acute regulatory protein (StAR), whose expression and activity are dependent on protein phosphorylation events at genomic and non-genomic levels. Hormone-dependent mitochondrial dynamics and cell proliferation are functions also associated with protein kinases. On the other hand, protein tyrosine dephosphorylation is an additional component of the ACTH signaling pathway, which involves the “classical” protein tyrosine phosphatases (PTPs), such as Src homology domain (SH) 2-containing PTP (SHP2c), and members of the MAP kinase phosphatase (MKP) family, such as MKP-1. PTPs are rapidly activated by posttranslational mechanisms and participate in hormone-stimulated steroid production. In this process, the SHP2 tyrosine phosphatase plays a crucial role in a mechanism that includes an acyl-CoA synthetase-4 (AcsL4), arachidonic acid (AA) release and StAR induction. In contrast, MKPs in steroidogenic cells have a role in the turn-off of the hormonal signal in ERK-dependent processes such as steroid synthesis and, perhaps, cell proliferation. This review analyzes the participation of these tyrosine phosphates in the ACTH signaling pathway and the action of kinases and phosphatases in the regulation of mitochondrial dynamics and steroid production. In addition, the participation of kinases and phosphatases in the signal cascade triggered by different stimuli in other steroidogenic tissues is also compared to adrenocortical cell/ACTH and discussed.

**Keywords:** PKA, PTPs, ERK1/2, SHP2, mitochondrial dynamics, MKP-1, AcsL4

**Abbreviations:** AA, arachidonic acid; ACBD3, acyl CoA-binding domain 3; Acot 2, acyl-CoA thioesterase; AcsL4, acyl-CoA synthetase 4; ACTH, adrenocorticotropin; Ang II, angiotensin II; ERK1/2, extracellular signal-regulated kinases 1 and 2; LH, luteinizing hormone; MAM, mitochondria-associated ER membrane; MAPKs, mitogen-activated protein kinases; MEK1/2, mitogen-activated protein kinase kinase 1 and 2; Mfn, mitofusin; MAKp, mitogen-activated protein kinase phosphatase; PAO, phenylarsine oxide; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; PTPs, protein tyrosine phosphatases; PV, pervanadate; SHP2, Src homology domain 2-containing PTP; StAR, steroidogenic acute regulatory protein; ZF, zona fasciculata.

## INTRODUCTION

Steroid hormones are synthesized in steroidogenic cells of the adrenal gland, ovary, testis, placenta, and brain and are required for normal reproductive function and body homeostasis. Unlike cells producing polypeptide hormones, which store large amounts of hormone in secretory vesicles ready for rapid release, steroidogenic cells store low amounts of steroids. Thus, a rapid steroidogenic response requires a rapid synthesis of new steroid molecules.

The transport of cholesterol from the outer to the inner mitochondrial membrane (IMM) is the rate-limiting step of steroidogenesis (1, 2), and it is controlled by a complex mechanism that includes phosphorylation–dephosphorylation processes and the interaction of several proteins. Among these, the steroidogenic acute regulatory protein (StAR along this review, also known as STAR or more precisely STARD1) is the most widely studied (3, 4). Indeed, specialized reviews have focused and deeply covered the role of StAR protein in steroidogenesis (3, 4).

Steroid biosynthesis is finely regulated by the phosphorylation–dephosphorylation of intermediate proteins (5–9). In this regard, it is well accepted that steroidogenic hormones act through the activation of serine/threonine (Ser/Thr) protein kinases. In all steroidogenic tissues, phosphorylation-dependent events are required for the acute stimulation of steroid biosynthesis through the activation of protein kinases, including cAMP-dependent protein kinase (PKA), protein kinase C (PKC), calcium/calmodulin-dependent protein kinase, and mitogen-activated protein kinases (MAPKs). Adrenocorticotrophic hormone (ACTH) and luteinizing hormone (LH) [or its surrogate chorionic gonadotropin (CG)] signal transduction pathways include PKA-dependent phosphorylation events in adrenal and Leydig cells, respectively (10–12). In the adrenal zona glomerulosa, aldosterone secretion is stimulated by angiotensin II (Ang II) and K<sup>+</sup>, in addition to ACTH. These stimuli promote phosphorylation events, which are not dependent on cAMP/PKA. Indeed, K<sup>+</sup> activates voltage-operated Ca<sup>2+</sup> channels, while Ang II, bound to Ang II type 1 receptors, acts through the inositol 1,4,5-trisphosphate IP<sub>3</sub>-Ca<sup>2+</sup>/calmodulin system (13). In other words, steroid biosynthesis is modulated by hormones, ions, or growth factors through the posttranslational phosphorylation of proteins, while the question that arises is how these phosphorylation events can lead a specific signal to its mitochondrial site of action.

Signal transduction pathways in eukaryotic cells include protein phosphorylation as an integral component regulated by the delicate balance between protein kinases and phosphatases activity. Thereby, many cellular responses require a coordinated cross talk between Ser/Thr and Tyr kinases and phosphatases activity.

In this context, this article will discuss the role of protein phosphorylation–dephosphorylation in cellular biology and endocrine function of steroidogenic cells.

## PROTEIN PHOSPHORYLATION

### Kinases Involved in StAR Phosphorylation

Since the middle 80s, Orme-Johnson and her group were pioneers describing the relevance of the rapid induction of a 30-kDa protein

in adrenal cortex after ACTH stimulation (14) and Leydig cells stimulated by cAMP (15). After these first discoveries, this group demonstrated that this protein is accumulated in mitochondria after hormone stimulation and processed to render two isoforms of 32 and 30 kDa (16, 17). Later, Stocco and Clark provided important data on the crucial role of this protein on the acute regulation of steroidogenesis and also on its molecular aspects. Since this protein is essential for cholesterol transport to the IMM and consequently for steroid synthesis, it was named StAR (3). Even when StAR has been widely identified as a phosphoprotein, the exact role of phosphorylation in StAR protein activity and hence cholesterol transport to the IMM still remains to be fully elucidated.

It is well established that non-genomic effects of PKA mainly involve posttranslational modifications of StAR protein. In fact, PKA phosphorylates murine and human StAR at specific residues such as Ser56/57 and Ser194/195 (18, 19). Moreover, genomic effects of PKA are known to include not only STAR gene (also known as STARD1 gene) transcription but also the transcriptional regulation of several steroidogenic-related genes (20). Even if cAMP-dependent signaling is the major pathway in steroid biosynthesis stimulated by ACTH and LH/C<sub>G</sub>, and PKA phosphorylation sites in StAR protein are well described, it is noteworthy that StAR sequence also contains putative phosphorylation sites for PKC, cGMP-dependent protein kinase or protein kinase G (PKG), casein kinase I and II, and cyclin-dependent kinase 5 (Cdk5), as it was described elsewhere for eukaryotic phosphoproteins using database Expasy Prosite<sup>1</sup> (21). Although the presence of these consensus sites might indicate StAR as a possible substrate for the respective kinases, the occurrence of this phosphorylation *in vivo* and its impact on steroid production remain uncertain.

Recent studies by Sasaki et al. – using a transgenic model with a bacterial artificial chromosome expressing either wild-type (WT) StAR or mutant StAR S194A to rescue StAR knockout mice – have demonstrated that Ser194, a conserved site among species, is an essential residue for normal StAR function in mice adrenal cortex and testis (22). These data indicate that phosphorylation of the Ser194/195 residues of StAR may account, at least in part, for the immediate increase in cholesterol side chain cleavage as a result of enhanced StAR protein activity. Consistent with these results, it has been demonstrated that the mutation in Ser195 in human mature StAR protein, which lacks the leader peptide, reduces pregnenolone production, as determined by an *in vitro* assay using mitochondria isolated from MA-10 Leydig cells (23). Strikingly, when cholesterol binding to StAR is measured with fluorescent or radioactive cholesterol, purified mutant S195A and WT StAR display equal binding activity. As determined by StAR structural analyses, Ser195 lies in a short loop opposite to the C- $\alpha$  helix, which is essential for cholesterol binding. Therefore, the addition of phosphate-negative charge in this Ser might influence StAR activity by modifying its interaction with hypothetical mitochondrial partners such as ACBD3 (previously known as peripheral benzodiazepine receptor-associated protein, PAP7)

<sup>1</sup><http://expasy.org>

(24). This could, in turn, anchor PKA to Ser194/195, rather than alter cholesterol binding to the sterol-binding pocket (23).

In the same line, work by Stocco's group has explored the regulation mechanism of StAR expression and steroidogenesis in conjunction with PKA and PKC pathways in MA-10 Leydig cells (25). This study shows that PKC-dependent induction of steroid synthesis is low when compared to that observed with PKA signaling, but it is capable of enhance LH/CG- and/or cAMP-stimulated steroidogenic response. On the one hand, the activation of PKC markedly increases StAR expression, but not phospho-StAR, with only a modest increase in progesterone production. On the other hand, PKA activation triggers a substantial increase in the band of StAR phosphorylated in Ser194 (25).

## Role of ERKs in the Regulation of StAR Protein and Steroid Biosynthesis

In addition to PKA activation importance for trophic hormone-stimulated steroid biosynthesis, it is also known that extracellular signal-regulated kinases 1 and 2 (ERK1/2) and upstream activator mitogen-activated protein kinase kinase 1 and 2 (MEK1/2) participate in the regulation of steroidogenesis. Indeed, several reports describe the role of members of the MAPK family in the regulation of steroid synthesis acting at both genomic and non-genomic levels.

One of the first published works in the field indicates that cAMP-induced steroid synthesis depends on ERKs phosphorylation and activation (26). These authors show that adenylyl cyclase activation with forskolin promotes a time-dependent increase in ERK activity and translocation of this enzyme to the nucleus in mouse adrenocortical Y1 cells. Similarly, Roy et al. have demonstrated that ACTH receptor activation leads to rapid ERK1/2 phosphorylation in primary cultures of human fasciculata cells (27), an effect also observed in a human adrenocortical H295R cell line (28). Moreover, Ang II also promotes MAPK activation in adrenal glomerulosa cells (29, 30). Thus, ERK activation seems to be a common event in the stimulation of different steroidogenic systems.

Although already demonstrated, PKA involvement in ERK activation continues to generate controversy; Le and Shimmer have shown that ACTH increases MEK and ERK phosphorylation in Y1 adrenocortical murine cells. This effect has also been detected in Kin-8 cells, a PKA-deficient mutant Y1-derived cell line (31), which suggests that ERK activation is independent of PKA activity. H295R adrenocortical cells exhibit only a very modest cAMP response to ACTH, yet ERK1/2 response is immediate and consistent. ERK activation is minimally reduced by PKA inhibitor H89, but unaffected by PKC and calcium inhibitors. Thus, ACTH-induced ERK1/2 activation in H295R cells does not appear to depend on the mechanism by which most G protein-coupled receptors activate ERK1/2, but does seem to depend on receptor internalization (28). On the other hand, Roy et al. have demonstrated the participation of PKA in ERK activation in human fasciculata cells (27).

A role for ERK activity has also been demonstrated in adrenal and gonadal steroidogenesis. Gyles et al. have shown that ERK activation results in enhanced phosphorylation of steroidogenic

factor 1 (SF-1) and increased steroid production through increased transcription of the *STAR* gene in Y1 cells (26). The activation of the ERK/MEK pathway correlates with an increase in StAR mRNA levels, StAR protein accumulation, and steroidogenesis. Similarly, ERK inhibition leads to a reduction in the levels of forskolin-stimulated StAR mRNA, StAR protein, and steroid secretion (26).

Luteinizing hormone receptor cascade activation in Leydig cells also promotes ERK1/2 phosphorylation, which is mediated by PKA through Ras activation (32). More recently, and using mice with a Leydig-specific deletion of MEK1/2 as an experimental model, Yamashita et al. have concluded that the MEK/ERK pathway is critical for maintaining a functional population of adult Leydig cells and fertility (33).

In agreement with findings in adrenocortical cells (26), Martinelle et al. have demonstrated the functional role of the ERK cascade in human CG (hCG)-induced steroidogenesis in primary cultures of immature rat Leydig cells (34). In this system, inhibition of MEK1/2 by U0126 suppresses several cellular responses to hCG.

In turn, 3-day treatment with Ang II in cultured rat adrenal glomerulosa cells increases aldosterone secretion through a mechanism involving both ERK1/2 and p38 MAPK pathways (30). In addition, the effect of Ang II on aldosterone synthesis also requires ERK1/2 activity in primary cultures of glomerulosa bovine cells (29).

Even though several reports support a role for ERK1/2 in StAR mRNA induction and steroid biosynthesis, other studies show controversial results. Indeed, it has been demonstrated that MEK1/2 inhibitors, such as U0126 and PD98059, enhance the expression of StAR protein in MTLC-1 and primary Leydig cells (35). Also, in MA-10 Leydig cells stimulated with dibutyryl cAMP, inhibition of ERK1/2 activity increases *STAR* gene expression (25). Similarly, Seger et al. have demonstrated that ERK signaling cascade inhibits CG-stimulated steroidogenesis in granulosa-derived cell lines (36). Taken together, the discrepancies on the role of ERK1/2 in StAR transcription might be due to different experimental conditions and cellular types, which generate different factor availability, such as transcription factors required for StAR expression. Nevertheless, the results of Yamashita et al. strongly support the requirement of ERK for StAR expression and steroidogenesis (33). Indeed, they analyzed the role of ERK1/2 on steroidogenesis and fertility using as experimental models knockout mice carrying a deletion for MEK1/2 in Leydig cells and primary culture of Leydig cells isolated from these knockout mice. This study demonstrates that the deletion of MEK1/2 and concomitant reduction of phospho-ERK1/2 levels decreased testicular expression of several Leydig cells markers, including StAR protein. Then, a similar experimental model based on transgenic mouse should be a powerful tool to univocally demonstrate the role of ERK1/2 on ACTH action on steroidogenesis and cell growth.

Extracellular signal-regulated kinase activity seems to regulate key steroidogenic transcription factors by non-genomic and genomic actions. *STAR* gene transcriptional regulation requires transcription factors already present in the cell, which are activated by posttranslational modifications, such as SF1, and others which

must be *de novo* synthesized, e.g., NUR77, encoded by *Nr4a1* gene (37). Finally, it has been pharmacologically and molecularly demonstrated that ERK1/2 participates in cAMP-induced *Nr4a1* expression in both MA-10 Leydig and Y1 adrenocortical cells (38), in addition to SF-1 activation (26).

In addition to their role in steroid biosynthesis, ERK1/2 is also involved in adrenal cell proliferation and growth (39–41). ACTH stimulates adrenal growth *in vivo*, whereas *in vitro* ACTH has an inhibitory effect on adrenal cell proliferation. In serum-starved Y1 cells, a short pulse of ACTH produces a mitogenic effect, which is preceded by the rapid activation of ERK1/2 (39). This result is in accordance with the requirement of ERK activation for cell proliferation. However, it is well documented that ACTH-mediated ERK activation is a transient process in Y1 cells. Thus, the early ERK activation could trigger StAR induction, steroidogenesis, and also cell proliferation, while the following decrease in pERK levels could contribute to the inhibition of cell proliferation mediated by prolonged exposure to ACTH.

## ERK-Mediated Phosphorylation of StAR Protein

The activation of the MEK1/2–ERK1/2 cascade appears to enhance steroid synthesis; nevertheless, the requirement of MEK1/2 and ERK1/2 cascade for the induction of STAR gene expression is less evident. Although it is well known the regulatory role of PKA on StAR protein activity, also this MEK1/2 and ERK1/2 cascade has been unveiled as a new mechanism of StAR activity modulation.

Our group has reported the role of MEK1/2–ERK1/2 cascade in the hCG/LH stimulation of StAR protein activity and steroidogenesis (42). In line with reports by Manna et al. and Martinelle et al. (25, 34), our work has shown that PKA acts upstream the stimulation of MEK and ERK activities. The inhibition of MEK1/2 on stimulated progesterone synthesis is not mediated by inhibition of PKA, as this enzyme activity is not altered in the presence of both inhibitors, U0126 and PD98095 (42).

Using a different strategy to study the role of active ERK1/2 in steroidogenesis, the overexpression of a WT form of ERK2 in MA-10 Leydig cells was performed. We observed an increase in steroid production stimulated by submaximal concentration of cAMP (42). Furthermore, an inactive form of ERK2, the H230R variant, which fails to interact with MEK1, but retains the ability to interact with MEK2 in a weakened fashion, does not produce the effect of WT ERK2 (42).

In short, both kinases, PKC and PKA, are capable of phosphorylating ERK1/2 through MEK1/2 activation (25, 32). ERK1/2 activity is involved in STAR gene expression induced by PKC or PKA activation, while a relevant role in StAR protein phosphorylation is attributed to PKA signaling pathway (25).

In summary, it is recognized that the ERK1/2 signaling cascade involved in regulating StAR expression and steroid synthesis is mediated by multiple factors and pathways, and is stimulus-specific.

## MEK1/2 and ERK1/2 at the Mitochondria

The site of action of MEK inhibitors appears to be downstream of PKA activation and before of cholesterol transport, which implies

that one of the targets may be located at the mitochondria. Gyles et al. have observed that activation of adenylyl cyclase causes a time-dependent increase in ERK activity and its localization from cytoplasm to nucleus (26), and our group has further proven a temporal ERK1/2 activation localized in the mitochondria, which is obligatory for PKA-mediated steroid synthesis in Leydig cells (42). Worth pointing out, the phosphorylation of mitochondrial ERK occurs before the increase in steroid production, and the hormone dose that is required for ERK activation at the mitochondria is the equivalent for eliciting steroid synthesis. Phosphorylated ERK1/2 (pERK1/2) is located in the cytosol, mitochondria, and, in lower proportion, in the nuclear fractions after cAMP stimulation. In the mitochondria and the cytosol, an early peak in ERK1/2 phosphorylation is followed by a slow progressive signal reduction during the first hour of cAMP incubation, a profile similar to that observed in hCG stimulation, leading to pERK1/2 activation. In contrast, pERK1/2 is mainly localized in the cytosol and nucleus, after epidermal growth factor (EGF) stimulation. Two different pools of MEK1/2 and pMEK1/2 have been found to be constitutively present in the cytosol and mitochondria. Remarkably, MEK1/2 differential distribution triggers different responses upon cellular stimulation (42).

Poderoso and coworkers have also shown that cAMP clearly induces sustained MEK1/2 phosphorylation in mitochondria, with a minor effect on the cytosolic kinases. Conversely, EGF induces a prolonged and strong cytosolic MEK1/2 activation, but only a discrete phosphorylation, in mitochondria. Although both EGF and cAMP increase total cytosolic MEK1/2, only EGF promotes its phosphorylation in this subcellular fraction (42).

The inhibition of PKA activity with the compound H89 and by PKA knockdown experiments diminishes the increase in mitochondrial pMEK1/2 and pERK1/2 after cAMP action (42). In agreement, the increase in mitochondrial PKA activity occurs after 5 min of cAMP action in parallel with the appearance of the phosphorylated forms of MEK1/2 and ERK1/2 in this organelle.

In regard to PKA activity and subcellular organization, a family of proteins named A-kinase anchor proteins (AKAPs) enhances cAMP-dependent pathways (43, 44). AKAPs raise cAMP signal by anchoring PKA near its cellular substrate, while mouse-derived AKAP121 binds PKA to the mitochondrial outer surface (45, 46). In addition, purified AKAP121 KH domain binds the 3'-untranslated regions of transcripts encoding the Fo-f subunit of mitochondrial ATP synthase and manganese superoxide dismutase (47). A special member of the AKAP family, AKAP121, can be anchored to mitochondria and may compartmentalize PKA and other proteins on the outer mitochondria membrane (OMM) (48). In Leydig cells, cAMP-induced StAR expression and steroidogenesis were found to correlate with the extent of AKAP 121 expression (49). Expression and role of AKAP121 in H295R cells deserve elucidation. Another relevant AKAP in steroidogenic tissues is the ACBD3 protein, an acyl CoA-binding protein, known previously as PAP7 (24). Human ACBD3 is highly expressed in steroidogenic tissues, where it follows the pattern of PRKAR1A expression, suggesting that it participates in PRKAR1A-mediated tumorigenesis and hypercortisolism (50). Therefore, StAR protein is likely to be phosphorylated at the mitochondria by the activation of a cascade of kinases, including ERK.

## StAR Protein as a Substrate of ERK1/2

Steroidogenic acute regulatory protein structural analyses have revealed a consensus sequence that would allow the docking of StAR protein to ERK1/2 and a consensus site for ERK1/2 phosphorylation. A typical docking site known as the D domain (**KTKLTWLLSI**) lies between amino acids 235 and 244 and is conserved among MEK1/2, MAPK phosphatase, and ERK substrates (51). Regarding the ERK1/2 phosphorylation site in StAR protein, it was possible to detect only two Ser-Pro motifs, at Ser232 and Ser277, targets for ERK1/2 phosphorylation in the mature form of the murine StAR protein. In accordance with the database Expasy Prosite,<sup>2</sup> Ser232 (PLAGS<sup>232</sup>PS) has a 90% probability of phosphorylation and is adjacent to the docking D domain, while the probability of Ser277 is only 5%. Besides, Ser277 is relatively less conserved among species. In agreement of a predicted StAR–ERK binding, the treatment of subcellular fractions with pERK–GST has shown that StAR protein interacts with pERK1 just in the mitochondrial fraction, but not in the cytosol. Together, MEK phosphorylation PKA-dependent, mitochondrial StAR and pERK1/2 activity increase cholesterol transport and mitochondrial synthesis of progesterone in cell-free assays (42).

*In vitro* phosphorylation assays using recombinant 30-kDa form of StAR protein and WT and the inactive mutant K71A forms of ERK1 demonstrated that the StAR protein is indeed phosphorylated by ERK1 and not by the K71A mutant. Remarkably, phosphorylation of StAR by ERK1 is dependent on the presence of cholesterol, while phosphorylation by PKA is not. Besides, StAR phosphorylation by PKA does not require previous ERK phosphorylation. By means of directed mutagenesis of Ser 232 (S232A), we demonstrated that this residue is indeed the target of ERK (42).

Expression of S232A mutated form of StAR partially blocks progesterone production enhanced by cAMP treatment in MA-10 cells. In contrast, the StAR mutant in which Ser 232 is replaced by a glutamic acid (S232E) does not produce such effect, which suggests that the negatively charged amino acid partially mimics the negative charge of the phosphate group present in the phospho-Ser (42).

Taking together, PKA phosphorylates StAR protein and also activates mitochondrial MEK1/2. Then, phosphorylated MEK1/2 activates a non-phosphorylated mitochondrial pool of ERK1/2 when the three kinases interact at the OMM, a crucial site for cholesterol transport forming a mitochondrial multi-complex with StAR.

## PROTEIN DEPHOSPHORYLATION

### Regulation of Protein Tyrosine Phosphatases by Steroidogenic Hormones

The degree of tyrosine phosphorylation of a given protein is the result of the action of protein tyrosine kinases and protein tyrosine phosphatases (PTPs). Protein kinases have been the focus of the research for a long time. Proportionately much less research has focused on protein phosphatases.

Whereas PTPs were initially regarded as household enzymes with constitutive activity and capable of all-substrate dephosphorylation, evidence in favor of tight regulation of PTP activity by various mechanisms is now accumulating. Like protein phosphorylation, dephosphorylation by PTPs is required in a cell compartment-specific manner. Protein–protein interaction domains and compartment-specific targeting domains in PTPs serve to localize PTPs all over the cell compartments (52).

Based on the amino acid sequence of their catalytic domain, PTPs are classified into four groups, Class I, II, III (all Cys-based PTPs), and IV (Asp-based PTPs), each with a specific range of substrates. The largest family is the Class I (Cys-based PTPs), comprising the 38 “classical” PTPs, and the 61 “dual-specificity” PTPs (DSPs), which is the most diverse group in terms of substrate specificity. The group of the classical PTPs, with strict tyrosine specificity, consists of the receptor-like (transmembrane) and non-receptor (intracellular) classes. In the human genome, these PTPs comprise 21 and 17 genes, respectively (52, 53).

The cross talk between pathways that involve Ser/Thr phosphorylation and Tyr dephosphorylation has been described in the regulation of steroid synthesis. Our group has reported that ACTH treatment causes an increase in the activity of PTPs located in the cytosol of adrenal zona fasciculata (ZF). The stimulation is detected very soon after ACTH stimulation (5 min), reaches a maximum (twofold) after 15 min, and returns to basal levels after 30 min (54). Incubation of adrenal ZF with 8Br-cAMP (permeant analog of cAMP) also produces PTPs activation, suggesting that it can be mediated by PKA-dependent phosphorylation. Moreover, detection of PTP activity by *in-gel* assays has shown at least two ACTH-stimulated soluble PTPs with molecular masses of 115 and 80 kDa (54).

Protein tyrosine phosphatases are regulated by Ser/Thr or Tyr-kinases. Indeed, several PTPs are known to be phosphoproteins *in vivo* (55, 56), which reflects the potential of cross-regulation between kinases and phosphatases, either PTPs or Ser/Thr phosphatases, for the fine control of cellular activity. Among those phospho-PTPs, there is a membrane-bound form, which can be activated by treatment of intact cells with isoproterenol, forskolin, or cAMP analogs (55), and a soluble form of PTP, known as PTP-PEST, which is inhibited after *in vitro* phosphorylation by PKA and in HeLa cells after forskolin or methylisobutylxanthine treatment (56). Our current studies demonstrate the expression of PTP-PEST in Y1 cells and in rat adrenal ZF and suggest that the ACTH-activated PTP of 115 kDa could be PTP-PEST. Furthermore, when paxillin is precipitated from the cytosol of ACTH-treated rats, a PTP of 115 kDa is coprecipitated according to the analyses of precipitate by *in-gel* PTP assay (57).

The Src homology domain (SH) 2-containing PTP (SHP2) is classified among the non-receptor, classical PTPs. It is widely expressed and plays an essential role in many organisms from lower eukaryotes to mammals (58). In contrast to other PTPs that inactivate intracellular signaling pathways, SHP2 activates them (59). The ACTH-activated PTP of 80 kDa from adrenal ZF has been recognized by a commercially available antibody against SHP2 in Western blot analyses (unpublished results), which

<sup>2</sup><http://expasy.org/prosite/>

suggests that SHP2 is a PTP activated by ACTH in rat adrenal ZF. In line with our results, Rocchi et al. have demonstrated SHP2 expression in bovine adrenocortical cells and its activation by ACTH through PKA-dependent phosphorylation (60). Finally, our group has also shown that SHP2 is expressed in MA-10 Leydig cells (61).

## PTP Activity and Steroidogenesis

The rapid increase in PTP activity induced by ACTH may prove that this activity is necessary in the stimulation of steroidogenesis. Studies on PTPs role in the acute steroidogenic response to hormones have been performed using incubation of rat adrenal ZF cells with two powerful cell permeant PTP inhibitors, phenylarsine oxide (PAO) and pervanadate (PV), and evaluation of the steroid production upon stimulation by ACTH and 8Br-cAMP. It has been proven that those PTP inhibitors block ACTH- and cAMP-stimulated corticosterone production, but exert no effects on basal steroidogenesis (54). Similar conclusions were obtained using Y1 cells (62, 63).

Phenylarsine oxide and PV also reduce LH/hCG- and cAMP-stimulated steroid production in testicular interstitial cells (64) and in MA-10 cells (65). PTP inhibitors affect StAR induction at the protein and mRNA levels in MA-10 Leydig cells (65), as well as in Y1 cells (63). PTP inhibitors affect neither cell viability nor mitochondrial enzymatic activity evaluated as steroidogenesis triggered by 22-OH-cholesterol treatment. Thus, hormone-dependent steroid synthesis requires PTP activity in a site localized beyond PKA actions and before cholesterol transport across the IMM.

Phenylarsine oxide oxidizes the thiol group of a cysteine present in the active site of all PTPs. Benzyl phosphonic acid (BPA) has a structure very similar to the PTP substrate, exerting its inhibitory action on PTPs by competitive inhibition of the enzyme. However, both inhibitors, PAO and BPA, inhibit Ang II- or K<sup>+</sup>-induced steroid synthesis in a dose-dependent fashion in H295R cells, a cell line derived from human ZG tumor (66), and in Y1 cells (63).

Collectively, our group's work demonstrates that steroidogenic stimulus (ACTH, LH, Ang II, and K<sup>+</sup>), acting by different signal transduction pathways, conveys on PTPs as common intermediaries (63–66).

In regard of substrates downstream PTP activity, *in vivo* ACTH treatment decreases phosphotyrosine contents in several proteins, one of them identified as paxillin, a focal adhesion protein (54). In Y1 cells, ACTH and cAMP elicit a rapid morphological transition from a flat epithelioid morphology to rounded cells (11). cAMP causes a rapid and selective Tyr dephosphorylation of paxillin in these cells (67). Moreover, the inhibition of PTP activity blocks changes in cell shape promoted by ACTH (67). Taken together, these results indicate that PTP activity is involved in cAMP-dependent paxillin dephosphorylation and this might mediate hormone-stimulated cell shape changes in adrenocortical cells.

In summary, results presented here support the view that the morphological and functional responses to ACTH in adrenocortical cells are intimately linked to and mediated by PTP activity.

## Links between PTP Activity and Arachidonic Acid Release

cAMP- and PKA-dependent pathways triggered by trophic hormones in steroidogenic cells stimulate arachidonic acid (AA) release (68, 69). AA and its metabolites take part in the acute stimulation of steroid production. The effect is exerted on both the expression and function of StAR (70, 71). Previously, we proposed that free AA levels in steroidogenic cells are determined by a novel hormone-regulated mechanism (69, 72, 73). This mechanism involves the concerted action of an acyl-CoA synthetase (AcsL4) and an acyl-CoA thioesterase (Acot2). AcsL4 is a long chain fatty acid acyl-CoA synthetase, with high affinity for AA, and it is preferentially expressed in steroidogenic tissues (72, 74). Acot2, a thioesterase that acts on long chain fatty acyl-CoA, associates with the matrix face of mitochondrial cristae (75–77). Acot2 mRNA and protein are present in adrenal cortex, ovary, testis, placenta, and brain, among other tissues. The activity of both enzymes is acutely modified after hormone stimulation of steroidogenic cells. Acot2 is activated by phosphorylation and substrate availability (78), and AcsL4 is rapidly induced after hormone treatment (79).

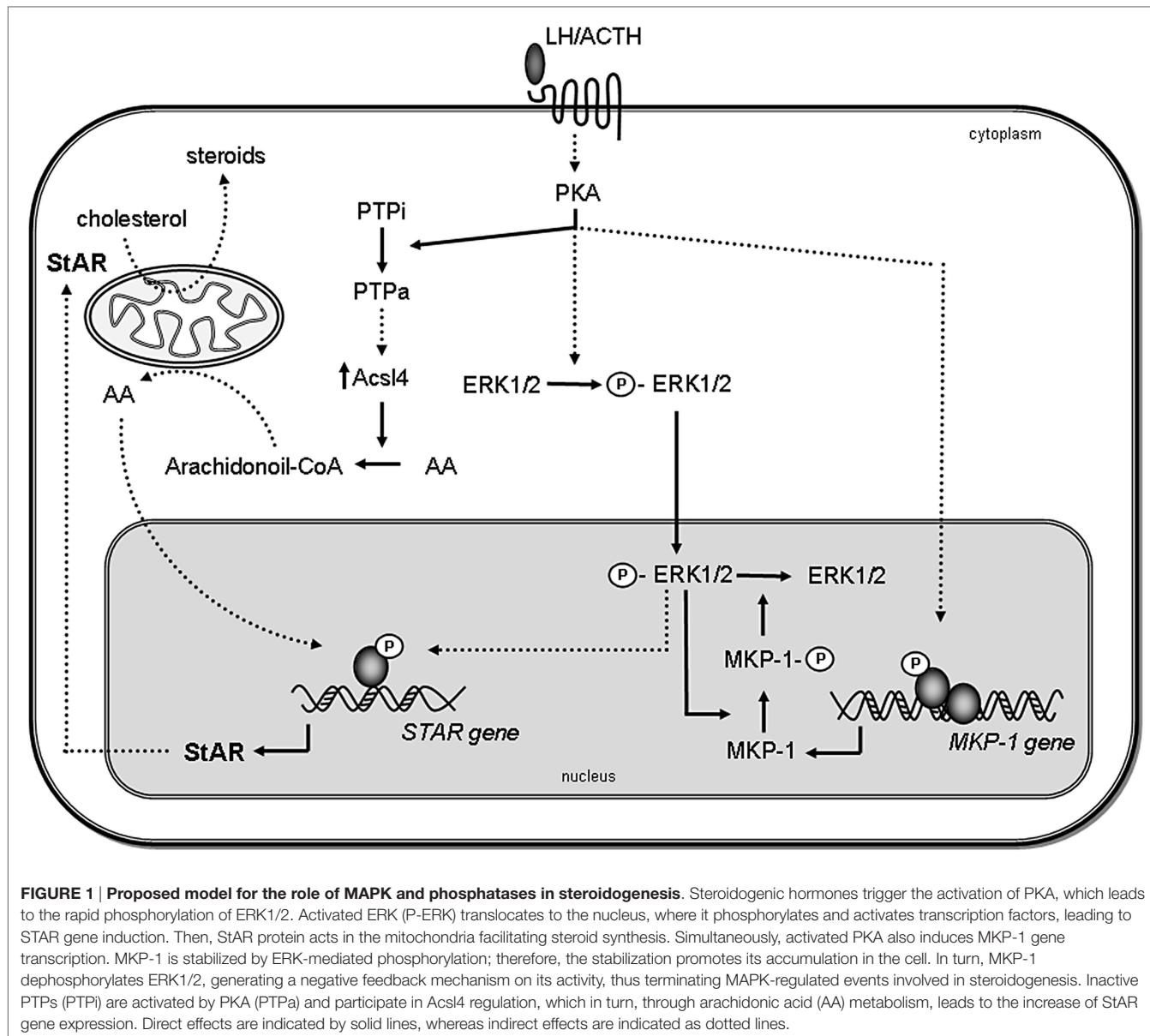
The activity of AcsL4 and Acot2 are needed for AA release, StAR induction, and steroidogenesis. This statement is supported by the fact that the reduction of the expression of both, AcsL4 and Acot2, causes an inhibition of steroid production in two steroidogenic systems (79, 80). Moreover, this effect is overcome by addition of exogenous AA (80). On the basis of these results, we propose that upon hormone treatment, AcsL4 would convert free AA in AA-CoA. The action of mitochondrial Acot2 on AA-CoA would release AA, specifically in the mitochondria, to increase StAR and steroidogenesis (69).

Our group has also linked the sequential action of PTPs, AcsL4, and StAR to the hormone-stimulated steroid production (66, 81). In Y1 cells, inhibition of PTP activity prevents AcsL4 and StAR induction exerted by 8Br-cAMP (81). Moreover, the effect of PTP inhibition is overcome by addition of exogenous AA (81). These results indicate that there is a consecutive action of PTP and AcsL4 to release AA before StAR induction. Moreover, the effect of PTPs on AcsL4 is also described in Leydig (81) and adrenocortical ZG cells (66) (Figure 1), indicating that the action of PTPs on AcsL4 may be a regulatory event that controls the steroidogenesis.

These results brought about a challenge to determine the identity of the PTP involved in the stimulation of steroid synthesis through AA release.

## SHP2 Involvement in Steroid Synthesis

By means of overexpression and suppression approaches, SHP2 has been proven to be at least one of the PTPs playing an obligatory role in steroidogenesis. NSC87877, a specific inhibitor of the tyrosine phosphatase SHP2, has been shown to reduce AcsL4 protein levels in AcsL4-rich breast cancer cells and steroidogenic cells. In addition, overexpression of an active form of SHP2 has increased AcsL4 protein levels in MA-10 Leydig cells. SHP2 has to be activated through a cAMP-dependent pathway to exert its effect on AcsL4, which could be specifically attributed to SHP2, as phosphatase knockdown reduces AcsL4



mRNA and protein levels. Through the action on Acsl4 protein levels, SHP2 affects AA-CoA production and metabolism and, finally, the steroidogenic capacity of MA-10 cells: overexpression (or knockdown) of SHP2 leads to increased (or decreased) steroid production (82).

The downregulation of SHP2 also modifies StAR expression. StAR expression increases in MA-10 Leydig cells treated with cAMP, an effect impaired by a short hairpin RNA (shRNA) against SHP2. Also, cAMP treatment causes a significant increase in StAR levels in mock-transfected cells, whereas SHP2 shRNA treatment prevents this effect. The involvement of AA in this process receives strong support from the fact that AA addition to SHP2 shRNA-treated cells bypasses the inhibitory effect produced by SHP2 knockdown (61). In this

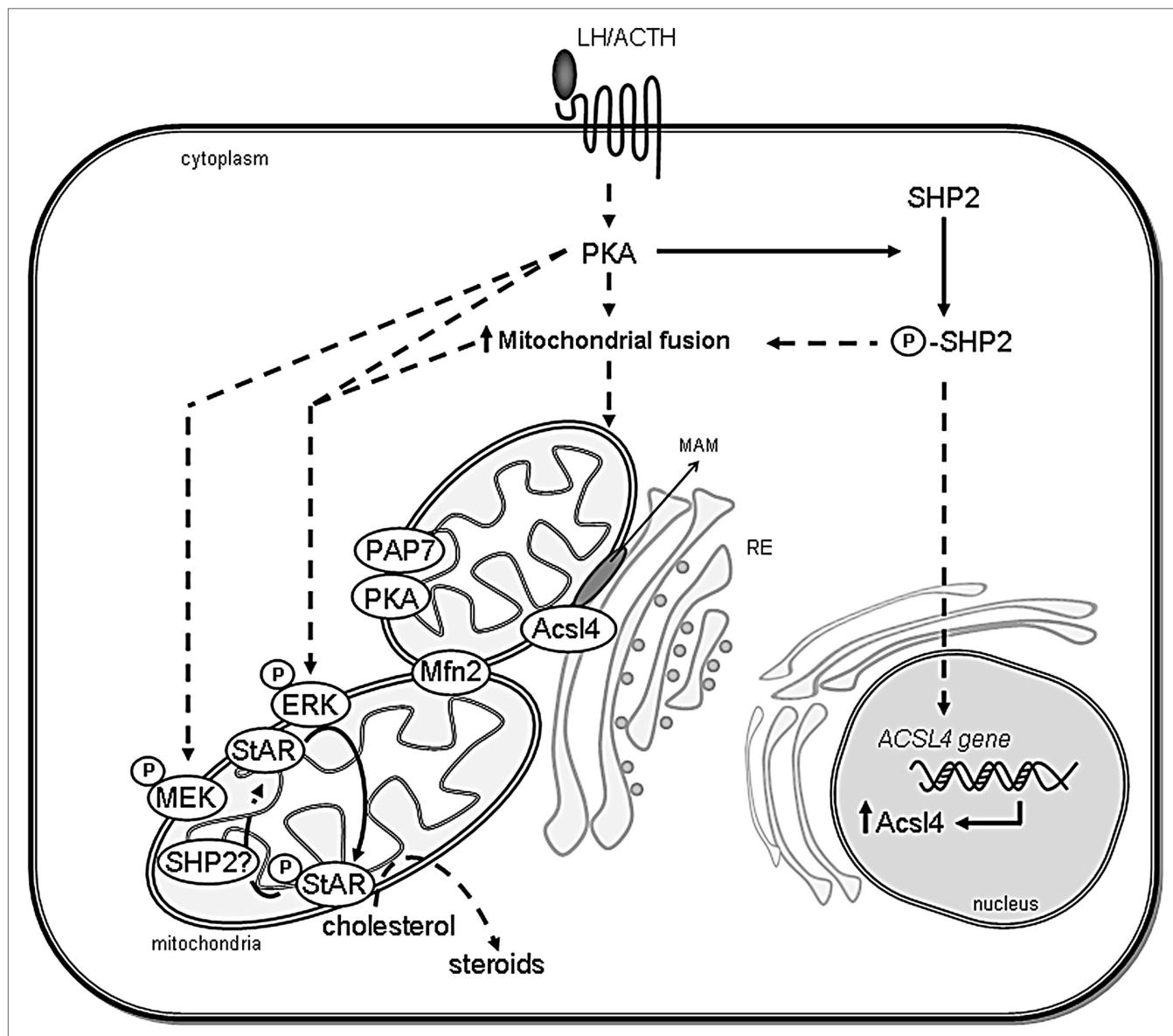
context, the hypothesis that arises is that a putative transcription factor, inhibited by tyrosine phosphorylation, is involved in ACTH-mediated Acsl4 induction. SHP2 could promote the tyrosine dephosphorylation of this factor, thus increasing steroid synthesis.

The Src homology domain (SH) 2-containing PTP is phosphorylated upon ACTH treatment (60). Moreover, *in vitro* phosphorylation of SHP2 by PKA dramatically increases its phosphatase activity (60). Although the phosphorylation of SHP2 by cAMP-independent kinases has not been demonstrated in steroidogenic cells, SHP2 may be phosphorylated by several different kinases to become Ser/Thr- or Tyr-phosphorylated. Indeed, SHP2 itself is Tyr-phosphorylated and activated through the action of different growth factors (83–86).

The Src homology domain (SH) 2-containing PTP has been identified in mitochondria, being the first evidence of the presence of a tyrosine phosphatase in such organelle (87). Several tyrosine kinases are present in the mitochondria (88). Particularly, c-Src is involved in the modulation of the efficiency of the mitochondrial electron transport chain (88). These data become important as stimulation of steroidogenesis needs energized mitochondria, and AA export and StAR induction need the activity of complexes III and V (73). In case, SHP2 is also located in the mitochondria

of steroidogenic cells, this phosphatase would be involved in the regulation of mitochondrial respiration.

The translocation of ERK to mitochondria is abolished by SHP2 knockdown in MA-10 cells. Moreover, the pronounced rearrangement of mitochondria that occurs after hCG stimulation is reduced by the downregulation of SHP2 expression (89). Then, the complete description of the steroid synthesis and secretion after hormone stimulation needs the study of SHP2 activation and mitochondrial reorganization (Figure 2).



**FIGURE 2 | Scheme showing the association between ER and mitochondria in steroidogenic cells.** The proposed interaction between organelles and the involved proteins in the regulation of cholesterol delivery is shown. After steroidogenic hormone action, PKA is activated and Mitofusin 2 (Mfn2) is localized in mitochondria. SHP2 is phosphorylated (P-SHP2) by active PKA, then it participates in both ACSL4 gene induction and the increase of mitochondrial fusion. Next, Acsl4 is localized in MAM subdomains in an Mfn2-dependent manner to exert its enzymatic activity. Mitochondrial ERK is activated by phosphorylation (P-ERK) in PKA- and MEK-dependent mechanism and phosphorylates mitochondrial StAR (P-StAR) to achieve maximal steroid production. A putative mechanism involves StAR dephosphorylation by SHP2 in mitochondria to release a cholesterol molecule to be substrate of P450ccc. Direct effects are indicated by solid lines, whereas indirect effects are indicated as dotted lines.

## MAPK Phosphatases in Steroidogenic Cells

Given that MAPK activation depends on Thr/Tyr protein phosphorylation, the magnitude and duration of their activity are related to protein phosphatases. MAPK phosphatases (MKPs) are a family of dual activity (Thr/Tyr) protein phosphatases, which dephosphorylate specifically members of MAPKs (90, 91). Several distinct mammalian MKP family members have been identified and characterized and can be divided into two broad classes. One group, typified as MKP-1, comprises nuclear enzymes rapidly induced by growth factors or stress signals. This group also includes MKP-2, a nuclear enzyme induced by the same stimuli that induce MKP-1, but with a slower kinetics. The second group, typified as MKP-3, includes predominantly cytosolic enzymes, and their transcripts are induced with delayed kinetics by specific stimuli, but not by environmental stress.

On the basis of evidence showing that ACTH can regulate the activity of MAPKs (31, 92), the regulation of MKPs by this hormone is expected. Analyses on MKP-1 induction in serum-starved Y1 cells demonstrated that ACTH stimulation results in a transient increase in MKP-1 mRNA followed by an increase in protein levels (93). Sewer and Wateman have also described the regulation of MKP-1 expression by cAMP in H295R cells. In this regard, they demonstrated that MKP-1 mRNA and protein levels are induced by cAMP, and overexpression of this phosphatase stimulates hCYP17 reporter gene activity. Besides, this study also demonstrates that PKA phosphorylates MKP-1 (94).

The hormone-dependent expression of MKP-1 has also been analyzed in MA-10 Leydig cells, where hCG/cAMP rapidly increases MKP-1 gene induction in a transient manner (95). Besides, MKP-1 protein levels increased in both nuclear and mitochondrial compartments. Moreover, MKP-1 increase (95) and ERK1/2 dephosphorylation in the mitochondria (42) are temporally coordinated events. In addition, our group has demonstrated that, in cells expressing flag-MKP-1 protein, hCG/cAMP trigger the phosphorylation and the accumulation of the recombinant protein in a time-dependent manner. Altogether, these results indicate that hCG modulates MKP-1 expression by transcriptional and posttranslational actions.

The functional role of MKP-1 in the regulation of steroidogenesis has also been analyzed in MA-10 Leydig cells. Work by our group demonstrates that MKP-1 overexpression downregulates the effects of cAMP on phospho-ERK1/2 levels, StAR expression, and steroidogenesis, while MKP-1 downregulation produces opposite effects. In summary, these data demonstrate that in Leydig cells, MKP-1 expression is regulated at multiple levels as a negative feedback regulatory mechanism to modulate the hormonal action on ERK1/2 activity and steroidogenesis (95).

Casal et al. have demonstrated the expression and regulation of MPK-1 also in primary cultures of bovine adrenal glomerulosa cells (29). These authors show that Ang II markedly increases MKP-1 protein levels in a time- and concentration-dependent manner. Ang II-induced phosphorylation of ERK1/2 leads to MKP-1 phosphorylation and, in turn, MKP-1 promotes ERK1/2 dephosphorylation. MKP-1 overexpression in bovine adrenal glomerulosa cells results in decreased phosphorylation of ERK1/2

and aldosterone production in response to Ang II stimulation. These results strongly suggest that MKP-1 is induced by Ang II and that it is involved in the negative feedback mechanism, ensuring adequate ERK1/2-mediated aldosterone production in response to the hormone.

In MA-10 Leydig cells, LH receptor stimulation also induces MKP-2 (96) and MKP-3 (97) through multiple mechanisms. While MKP-2 completes the ERK1/2 dephosphorylation in the nucleus initiated by MKP-1, MKP-3 dephosphorylates ERK1/2 in the cytoplasm.

In conclusion, stimuli promoting MAPK activity also regulate MKPs expression at multiple stages as a negative feedback regulatory mechanism to modulate hormonal actions on ERK1/2 activity and steroidogenesis (Figure 1).

## KINASES AND PHOSPHATASES IN THE REGULATION OF MITOCHONDRIAL DYNAMICS: ROLE IN StAR ACTIVITY AND STEROIDOGENESIS

### StAR Structural Changes

Several protein kinases, such as PKA, MEK, and ERK – which are essential to complete steroidogenesis – form a mitochondrial-associated complex and are completely required for mitochondrial cholesterol transport along with StAR and other proteins such as Acsl4, voltage-dependent anion channel (VDAC1), and adenine nucleotidetranslocase (ANT) (42, 98).

The precise mechanism of StAR action has been widely explored, but still remains elusive. StAR is synthesized as a 37-kDa protein with a typical mitochondrial leader sequence that directs the protein to the mitochondria for the import and cleavage to an intramitochondrial form of 30-kDa (99–102). After reaching the matrix, the 30-kDa StAR is controlled by the ATP-dependent Lon protease (103) and proteolysed, its half-life being 4–5 h (101, 104). A tight regulation of mitochondrial StAR levels is imperative since excessive accumulation of StAR protein in the matrix provokes mitochondrial damage and a “mitochondria to nucleus” signaling which, in turn, activates transcription of genes that encode mitochondrial proteases crucial for complete clearance of StAR (105). In this regard, we have observed that the presence of mitochondrial ERK is strictly necessary for protecting StAR from unknown proteases to avoid further degradation, which constitutes one of the mechanisms playing a role in mitochondrial StAR levels regulation (106).

This mechanistic model of StAR action suggests that the active form of StAR is partially unfolded, with the N-terminal domain entering the mitochondria and the partially unfolded C-terminus interacting with the OMM. Direct evidence has been presented showing that StAR exists as a molten globule. While certain native structure is retained at the N-terminal domain, the C-terminal domain folding appears to be less tight at the low pH that StAR may undergo on the mitochondrial membrane. Then, the tightly folded N-terminal domain could make StAR halt as it enters the mitochondria, extending the time window for the C-terminus to act.

Steroidogenic acute regulatory protein exhibits constitutive activity on the OMM, but no activity when localized to the inter-membrane space (IMS) or to the matrix (107). Mitochondrial StAR protein import experiments using a modified leader peptide confirmed StAR exclusive activity on the OMM, as reflected by a negative correlation between the time of StAR mitochondrial entry and its activity. Once again, StAR role in promoting steroidogenesis is proportional to the time it spends on the OMM (107, 108).

Even when N-62 StAR form (which lacks mitochondrial peptide leader) does not access to the mitochondria, a few molecules of this protein are associated with the OMM as it is shown by immuno-electron microscopy (108, 109). This truncated StAR form would transport several cholesterol molecules, while complete StAR protein is able to bind just one. This suggests that StAR could transport several cholesterol molecules before entering mitochondria and be processed.

## Mitochondrial Dynamics and Its Regulation by Protein Kinases in Steroidogenic Cells

“Mitochondrial dynamics,” which includes fusion/fission events, is relevant for maintaining mitochondrial integrity. Indeed, mitochondrial plasticity is important for several cellular functions and for protection against aging-related changes. Among these functions, mitochondrial dynamics play a role in mitochondrial replication and repair, propagation of intramitochondrial calcium waves, and in the elimination, *via* mitophagy, of depolarized mitochondria (110). Two GTPases located on the OMM have a crucial role in mitochondrial fusion, mitofusin (Mfn) 1 and 2. These proteins, structurally related to dynamin, are expressed in several tissues, as brain (mainly Mfn2), liver, adrenal glands, and testis. Mfn1 and Mfn2 modulate the interactions mitochondria–mitochondria and endoplasmic reticulum (ER)–mitochondria and also mediate mitochondrial fusion acting in a concerted fashion with another GTPase located in the IMM, optic atrophy 1 (OPA1).

Mitochondria have been shown to be in constant movement within the cells, and this movement can be induced after steroidogenic hormone action. This event would allow the contact between mitochondria and other membranes. It is well known that the contact between mitochondria and ER plays an important role in cell metabolism and signaling transduction pathways. Indeed, it is considered as a unique subdomain termed the mitochondria-associated ER membrane (MAM), with a vast importance in regulation of  $\text{Ca}^{2+}$  signaling, mitochondrial bioenergetics, apoptosis, and lipid metabolism (111–113).

Mfn2 in the ER bridges mitochondria and ER by forming homotypic and heterotypic complexes, with Mfn2 or Mfn1 on the mitochondrial surface. Therefore, Mfn2 is critical for MAM formation by tethering ER to the mitochondria. A mitochondrial ubiquitin ligase, MITOL, has been described as the regulator of the ER–mitochondria interaction by controlling Mfn2 activity (114). Interestingly, Acsl4, the key enzyme involved in the regulation of steroidogenesis through AA release and induced by

steroidogenic hormones (66, 79, 80), is localized and active in the MAM subdomain (115).

Dynamin-related protein 1 (Drp1) is required for mitochondrial fission. It is a cytosolic protein, which is recruited to the OMM by a poorly characterized multiprotein complex. In neurons, Drp1 phosphorylation by PKA in the mitochondria results in its inactivation and concomitant mitochondrial elongation (116). On the other hand, Drp1 phosphorylation by PKC $\delta$  at Ser579 increases mitochondrial fragmentation (117). In summary, several works support a key regulatory role for phosphorylation in mitochondrial morphology maintenance.

Although it is well recognized the relevance of mitochondrial dynamics in several cellular processes, its role in steroid synthesis is poorly described. Nevertheless, a work published 30 years ago described hormone-induced changes in intracellular location of the mitochondria and in the morphology of this organelle (118). Later, it was described that mitochondria move across the cell in a PKA-dependent manner after ACTH stimulation in H295R adrenocortical cells (119). This work demonstrates that ACTH/cAMP-stimulated mitochondrial movements depend on microtubules and have a role in the regulation of cortisol production, facilitating the shuttle of steroidogenic substrates between the ER and mitochondria (119). In this cell line, the reduction of OPA1 facilitates the transfer of cytosolic  $\text{Ca}^{2+}$  signal into the mitochondrial matrix (120), which results in turn in enhanced aldosterone production (121). The authors stated that this is probably due to the altered diffusion conditions under OPA1 knockout. The study of an extramitochondrial form of OPA1 closely related to the lipid droplets ruled out any role of this fraction of OPA1 in cAMP-mediated steroid hormone production, the specific biological function of adrenocortical cells (66, 120). Moreover, the reduction of OPA1 in Leydig cells did not affect steroid production (98), suggesting that OPA1 is not critical for hormone-induced steroidogenesis. Then, the contribution of OPA1 and cristae remodelation to steroid synthesis needs further investigation.

## Mitochondrial Dynamics and Steroidogenesis

Steroid synthesis requires mitochondrial fusion induced by in a hormone-dependent fashion (89). The fact that Mfn2 is rapidly induced after the steroidogenic stimuli, and that blocking mitochondrial fusion by Mfn 2 knockdown expression reduce steroid synthesis, further supports a role of mitochondrial dynamics on steroidogenesis (89). The hormone-induced mitochondrial fusion might also be crucial for the generation of the mitochondrial multiprotein complex that facilitates the access of cholesterol to the P450scc system, since the mitochondrial rearrangement after cell stimulation is necessary for the relocalization of ERK1/2 to mitochondria. Moreover, the abrogation of mitochondrial fusion prevents the association of Acsl4 with the mitochondria, showing clearly that MAM formation depends on mitochondrial fusion (89). As previously mentioned in this review, SHP2 modulates mitochondrial fusion, suggesting that protein tyrosine dephosphorylation could be involved in the mechanism of mitochondrial dynamics (89). According to a

published work (119), mitochondrial fusion might represent a limiting step in the onset of processes that require transport of intermediate products, e.g., liposoluble steroid hormones between organelles, probably mediated by MAM. In agreement with the previous results from our group, recent work demonstrate that hormone-induced MAM formation participates in the optimum transfer of cholesterol from the ER into the IMM increasing steroidogenesis rates (122). Then, steroid hormones might reach the plasma membrane without moving across the hydrophilic cytoplasm. Our group has shown that mitochondrial fusion is an essential process in regulating StAR mRNA levels and driving StAR to the mitochondrial context, probably participating in StAR mRNA stabilization and/or tethering the protein to the OMM (106) (**Figure 2**).

## Role of StAR Phosphorylation and Mitochondrial Fusion in StAR Localization

*In silico* molecular modeling has demonstrated that cholesterol binding to StAR could elicit a conformational change in StAR C-terminal domain, which in turn might favor the exposure of StAR Ser 232 and the docking domain for ERK. Therefore, StAR could be a substrate for ERK binding and phosphorylation, only when this protein is bound to cholesterol (42). This model is sustained by the fact that cholesterol binding to StAR promotes a decrease in its helical structure (123). The OMM is the most probable environment for the interaction between StAR and ERK since this submitochondrial domain anchors both StAR and ERK, as demonstrated in the previous work (42, 53). In turn, the overexpression of the mutated form of StAR, S232A, in steroidogenic cells prevents StAR phosphorylation by active ERK, thus proving that the kinase indeed phosphorylates this residue.

Cholesterol acts as an allosteric modulator of its own binding to StAR (123) and is strong stabilizing of the partially unfolded state in the StAR molecule (124). However, when cholesterol has to reach the P450scC, its release from StAR hydrophobic pocket is obligatory. Since ERK phosphorylation of StAR requires cholesterol, it is conceivable to think that StAR phosphorylation at Ser232 occurs after cholesterol binding. Thus, a conformational change in StAR induced by a negative charge at the Ser232 might reduce StAR affinity for cholesterol, favoring its release. This might in turn facilitate cholesterol transport into mitochondria to achieve high rates of pregnenolone synthesis.

Steroidogenic acute regulatory protein molecular structure has been partially studied (123–126), and the Ser232 residue is predicted to be localized in one of the last  $\beta$  barrels of the StAR-related lipid-transfer (START) domain (126, 127). It is well known that protein stability and interaction with several components are modulated by phosphorylation. Phosphorylation of proteins promotes acidic loops formation in their structure, as it has been described (128). The pH-dependent transition to the molten globule structure in the mitochondrial context (OMM) could provoke a weakened association between StAR C-terminal  $\alpha$ -helix and lipid molecules, thus releasing cholesterol from StAR hydrophobic pocket. Under acidic pH conditions, the cholesterol

affinity for START domain is significantly decreased (127). Thus, the addition of a phosphate group to StAR by ERK could establish a local decrease in pH, directing a conformational change in StAR, to a form with a lower affinity for cholesterol.

Our group has shown that StAR S232A expression significantly diminishes the localization of StAR in the mitochondria induced by hCG or cAMP. ERK phosphorylation affects mitochondrial StAR levels posttranscriptionally, as the expression of transfected StAR S232A is independent of cellular endogenous regulation (106). The mitochondrial module includes MEK, ERK, and cholesterol with a direct physical association between StAR and ERK (42). Their interaction facilitates StAR phosphorylation by ERK. Therefore, it could lead to phospho-StAR retention in the mitochondria, particularly on the OMM where ERK resides (53) (**Figure 2**).

Steroidogenic acute regulatory protein activity is determined by its localization on the OMM, and not its cleavage from the 37- to 30-kDa form (107). Hence, the longest StAR retention time on the OMM might render the maximal StAR activity in cholesterol transport, in agreement with the previous data (102). As described above, ERK is transiently activated after hormone stimulation in MA-10 cells (42). Its dephosphorylation could be mediated by MKP-1, since the temporal profile of mitochondrial MKP-1 and ERK dephosphorylation are compatible (95). Mitochondrial phospho-StAR and ERK interaction could avoid ERK dephosphorylation and inactivation. The temporal frame of ERK activity in this organelle correlates with highest StAR activity and cholesterol transport after hormone stimulation. These results agree with the fact that MKP-1 downregulation leads an increase in progesterone levels (95). The hormone-dependent induction of Mfn2 and mitochondrial fusion play an essential role in localization of ERK and StAR on the OMM and on the steroidogenesis (89, 129).

Taken together, these results offer new insights into StAR regulation by kinases and phosphatases and their impact on StAR site of action. The phosphorylation–dephosphorylation of StAR would contribute to modulate its affinity for cholesterol and to increase pregnenolone synthesis with a few molecules of StAR. In this work, we have reviewed StAR mechanism of action on cholesterol transport to the P450scC to achieve maximal steroid production. We have also described the role of phosphorylation–dephosphorylation events and mitochondrial fusion as novel regulators of the localization of StAR protein in order to carry out its action in steroidogenic cells.

## CONCLUDING REMARKS

Serine/threonine phosphatases have an important role in the regulation of adrenocortical cell functions, mainly steroid synthesis. In this context, the participation of PKA and PKC appears relevant, as phosphorylation events mediated by these kinases are involved in the expression and activation of StAR. Although StAR activation mechanism has not been fully described, it is known to require hormonal action on mitochondrial dynamic. Studies from other and our laboratory show that MAPKs, particularly ERK1/2, play an important role in StAR induction as

well as in its posttranslational regulation in the mitochondria. In addition, ACTH-activated ERK1/2 regulates adrenal cell proliferation.

A field scarcely described is the role of PTPs in steroidogenic cells. We presented data on PTP activation triggered by ACTH through a PKA-dependent mechanism. In this context, PTP SHP2 has a role in the stimulation of steroidogenesis involving Acsl4 protein induction. In turn, Acsl4 promotes AA release, StAR induction, and steroidogenesis. Moreover, SHP2 along with ERK could also have a role in steroidogenesis promoting mitochondrial fusion. MKPs, a group of dual activity phosphatases that inactivate MAPK, are also regulated by steroidogenic hormones at multiple levels. While MAPK activation is linked to steroid production activation and cell proliferation, MKP induction is associated with the turn-off of hormonal signal through MAPK inactivation and, consequently, the downregulation of ERK-dependent events.

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## AUTHOR CONTRIBUTIONS

Ernesto J. Podesta, Cristina Paz, Cecilia Poderoso and Ana F. Castillo wrote the paper. All authors provided critical revisions of the paper.

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# Role of EPAC in cAMP-Mediated Actions in Adrenocortical Cells

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Adrenocorticotrophic hormone regulates adrenal steroidogenesis mainly via the intracellular signaling molecule cAMP. The effects of cAMP are principally relayed by activating protein kinase A (PKA) and the more recently discovered exchange proteins directly activated by cAMP 1 and 2 (EPAC1 and EPAC2). While the intracellular roles of PKA have been extensively studied in steroidogenic tissues, those of EPACs are only emerging. EPAC1 and EPAC2 are encoded by the genes *RAPGEF3* and *RAPGEF4*, respectively. Whereas EPAC1 is ubiquitously expressed, the expression of EPAC2 is more restricted, and typically found in endocrine tissues. Alternative promoter usage of *RAPGEF4* gives rise to three different isoforms of EPAC2 that vary in their N-termini (EPAC2A, EPAC2B, and EPAC2C) and that exhibit distinct expression patterns. EPAC2A is expressed in the brain and pancreas, EPAC2B in steroidogenic cells of the adrenal gland and testis, and EPAC2C has until now only been found in the liver. In this review, we discuss current knowledge on EPAC expression and function with focus on the known roles of EPAC in adrenal gland physiology.

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## INTRODUCTION

In response to stress, the hypothalamic–pituitary–adrenal (HPA) axis is activated. Parvocellular neurons, located in the paraventricular nucleus of the hypothalamus release corticotrophin-releasing factor (CRF), which is transported to the anterior pituitary where it stimulates the corticotropes by binding to type I CRF receptors (1). In response to CRF, these cells release the prohormone pro-opiomelanocortin (POMC) (2). Through posttranscriptional modifications, the inert POMC is converted to biologically active peptides, including adrenocorticotrophic hormone (ACTH) (3). ACTH enters the systemic circulation, binds to specific receptors located on the surface of adrenocortical cells, and stimulates the production of adrenocorticosteroid hormones, including cortisol, aldosterone, and adrenal androgens (4). Steroid hormones are produced from the same precursor, cholesterol, by a set of cytochrome P450 steroid hydroxylases (CYP11A1, CYP11B1 and CYP11B2, CYP17 and CYP21) and the steroid dehydrogenase 3 $\beta$ HSD (5). The enzymes are differentially expressed in the three zones of the adrenal cortex (zona glomerulosa, zona fasciculata, and zona reticularis) giving rise to zone-specific hormone production. In humans, the primary source of cholesterol for steroid hormone production is low density lipoprotein (LDL), which is imported via the LDL receptor (LDLR) from the blood stream. Once cholesterol enters the cell, hormone-sensitive lipase (HSL) converts it to free cholesterol substrate (6). Free cholesterol is then delivered to the inner mitochondrial membrane by the actions of steroidogenic acute regulatory protein (StAR) and cholesterol-binding proteins. CYP11A1 (or P450 cholesterol side chain cleavage)

catalyses the first and rate-limiting enzymatic step in the biosynthesis of all steroid hormones, the conversion of cholesterol to pregnenolone (7, 8). Pregnenolone can be further converted into different hormone intermediates in the endoplasmic reticulum and the final production of cortisol and aldosterone occurs in mitochondria within the zona fasciculata and zona glomerulosa, respectively (5).

In the adrenal cortex, ACTH coordinates the biosynthesis of steroid hormones via the second messenger cAMP. In response to ACTH binding to its receptor, conformational changes induce the release of G-proteins, which then activate membrane bound adenylyl cyclases (ACs). Upon activation, ACs generates cAMP from ATP (Figure 1A). cAMP relays ACTH-mediated functions via

the activation of the serine–threonine kinase cAMP-dependent protein kinase A (PKA) or the exchange proteins directly activated by cAMP (EPAC1 and 2 also named cAMP-regulated guanine nucleotide exchange factors (cAMP-GEFs) I and II). Following cAMP activation, PKA and EPAC transmit signals differently. PKA phosphorylates numerous substrates, while EPACs act as guanine exchange factors (GEFs) catalyzing the conversion of the small GTPases Rap1 and Rap2 from an inactive (GDP-bound) to active form [guanine triphosphate (GTP)-bound] (9, 10). While cAMP signaling by PKA in steroidogenic cells has been intensely investigated, the roles of EPAC are only beginning to emerge. This review summarizes our current knowledge of EPAC2 in tissues of the hypothalamus–pituitary–adrenal axis.

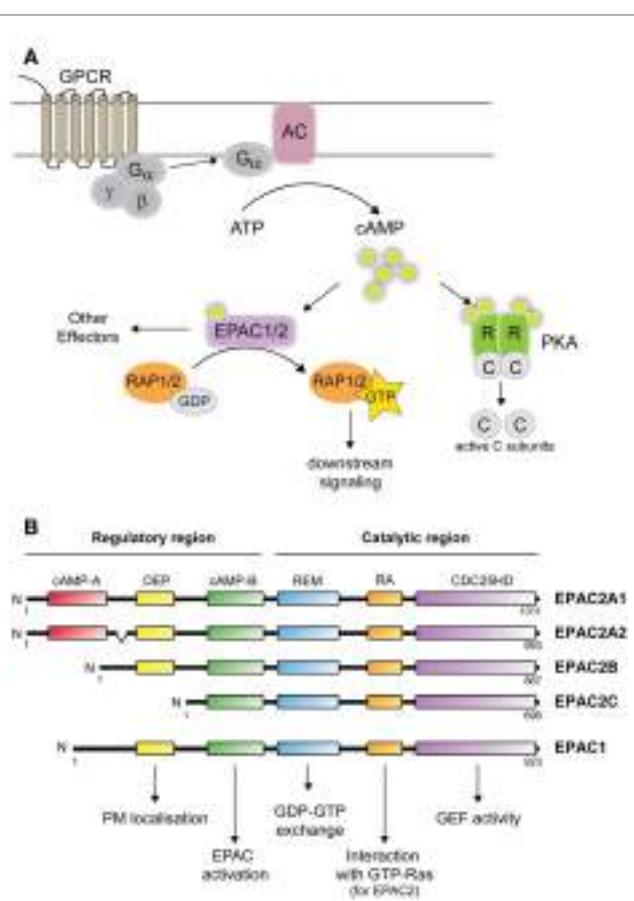
## STRUCTURE AND FUNCTION OF EPAC ISOFORMS

The identification of EPACs was reported in 1998 by two separate groups using different approaches. EPAC1 was discovered by de Rooij and colleagues in a database search for proteins containing cAMP-binding domains with the ultimate goal to explain the PKA-independent cAMP-induced activation of Rap1 (9). The same year, Kawasaki et al. identified both EPAC1 and EPAC2 in a screen for brain-specific genes with cAMP-binding motifs (11). In recent years, it has become evident that the EPAC proteins play essential roles in many biological processes, in some where EPAC and PKA collaborate to achieve a common biological response and in some where the two cAMP effectors have separate functions [reviewed in Ref. (12–14)].

## Expression of EPAC Isoforms

The EPAC proteins are encoded by two different genes: *RAPGEF3* (EPAC1) and *RAPGEF4* (EPAC2), which both give rise to multiple transcripts. Three transcripts are produced from *RAPGEF3*, but only variant 1, encoding EPAC1, has been studied (13). EPAC1 is relatively ubiquitously expressed (9, 11). Specific parts of the brain, thyroid gland, proximal tubules of the kidney, ovary, and skeletal muscle express the highest levels of EPAC1, but lower levels of EPAC1 have been found in virtually all tissues examined, as well as in hematopoietic cells (15). Studies on murine tissues suggest that EPAC1, as well as EPAC2, mRNA expression is also regulated at different stages of development in embryos and after birth (16).

At the transcript level, four different isoforms arise from *RAPGEF4*, termed EPAC2A1, EPAC2A2, EPAC2B, and EPAC2C (Figure 1B) (17–20). The EPAC2A2 transcript might be specifically expressed in the brain (20), but the existence of a corresponding protein is yet to be demonstrated. Thus, potential biological roles for this isoform remain unknown. By contrast, the three other mRNAs are known to give rise to the proteins EPAC2A, EPAC2B, and EPAC2C. EPAC2A was the initial EPAC2 isoform identified (11), and is expressed predominantly in brain (with high levels in the cerebral cortex, hippocampus, habenula, cerebellum, and hypothalamus), pituitary, and endocrine pancreas (11, 17, 20). The EPAC2B isoform was identified by the group of Dr. Seino during their efforts in developing an EPAC2 knockout



**FIGURE 1 | cAMP-mediated signaling and EPAC1 and 2 isoforms.** (A) cAMP signaling: following ligand binding of G-protein-coupled receptor, the membrane bound adenylyl cyclase (AC) is activated, and generate cAMP from ATP. cAMP subsequently activates PKA and/or EPAC1/2. Binding of cAMP to PKA causes the release of the catalytic subunits, which phosphorylate a variety of targets. Binding of cAMP to EPAC leads to guanosine diphosphate (GDP) to guanine triphosphate (GTP) exchange on Rap1 or Rap2. (B) Domain structure illustration of EPAC1 and EPAC2 isoforms consisting of: cAMP-binding domains A and B, disheveled, Egl-10, pleckstrin (DEP) domain, Ras-exchange motif (REM), Ras association (RA) domain, and CDC25-homology domain (HD). The protein structure of EPAC2A2 is shown in accordance to EPAC2A1. Functional roles of each domain are also indicated. PM, plasma membrane; GEF, guanine nucleotide exchange factor activity.

model (19). While confirming deletion of EPAC2A, they discovered the presence of a shorter transcript in the adrenal gland that lacked the N-terminal cAMP-binding domain [(19); **Figure 1B**]. Until now, EPAC2B expression has only been demonstrated in the adrenal gland (19–21), in the Leydig cell-derived cell line MA10 (21) and in endocrine pancreas (20). The physiological roles of EPAC2B appear to diverge from those of EPAC2A since EPAC2B is not able to substitute for EPAC2A in cellular assays monitoring insulin secretion (19). The shortest EPAC2 isoform, EPAC2C, was also identified by the group of Dr. Seino (17, 18) and has so far only been found in the liver, presumably solely in hepatocytes (18, 20). The strict tissue-specific expression of the different EPAC2 isoforms is controlled, at least in part, by DNA methylation. Detailed analyses of the EPAC2 gene have led to the identification of alternative promoters for the different isoforms (18, 20) and bisulfite sequencing demonstrated that the methylation status of the different promoters nearly perfectly mimics their activity and the expression pattern of the corresponding isoform (20).

## Structure and Activation of EPAC Proteins

EPACs are multidomain proteins consisting of two main parts, i.e., an N-terminal regulatory region and a C-terminal catalytic region (**Figure 1B**). The regulatory region is built up by a cAMP-binding domain and a disheveled, Egl-10, pleckstrin (DEP) domain. EPAC2A contains two cAMP-binding domains, cAMP-A and cAMP-B, while EPAC1 has only one such domain. The domain structure of EPAC2B is similar to EPAC1 and lacks the first cAMP-A binding domain, while EPAC2C lacks both the cAMP-A and DEP domains (18, 19). The N-terminal cAMP-A domain in EPAC2A binds cAMP with low affinity and is not believed to be important for cAMP-induced activation (10). Instead, this domain appears to be important for the localization of EPAC2A near the plasma membrane (19). The DEP domain, by its ability to interact with phosphatidic acid, is also important in targeting EPAC to the plasma membrane upon activation by cAMP (22). The catalytic region consists of a CDC25 homology domain (CDC25HD) that catalyzes Rap1 activation, a Ras-exchange motif (REM) domain, and a Ras association (RA) domain (23). The regulatory regions of EPAC1 and EPAC2 function as inhibitors of the C-terminal GEF domain in the absence of cAMP. Binding of cAMP induces a conformational change that opens the catalytic CDC25HD domain from auto-inhibitory restraints and thereby permits GTP loading of Rap (10, 24–26). Both EPAC1 and EPAC2 contain potential RA domains, but only EPAC2 has been shown to interact with Ras-GTP, which contributes in recruiting EPAC2 to the plasma membrane. The enrichment of EPAC2 on the membrane through Ras binding is crucial for EPAC2-mediated Rap1 activation (27, 28). In addition to the plasma membrane, other subcellular localizations have been observed, such as the perinuclear region, nuclear membranes, and mitochondria for EPAC1 [reviewed in Ref. (29)] and the Golgi apparatus and the nucleus for EPAC2B (21).

## Physiological Roles of EPAC

EPACs regulate a multitude of cAMP-mediated cellular processes in many different tissues [extensively reviewed in Ref. (23)], including the formation of cell-cell adhesion (24, 30–33), cell

proliferation (34, 35), differentiation (36, 37), cell survival (38), ion channels regulation (39–41), and Ca<sup>2+</sup>-mediated signaling (23, 42, 43). The development of EPAC knockout mice models has led to a better insight into the biological functions of these proteins. In spite of the involvement of EPAC in multiple cellular pathways, mice lacking EPAC1, EPAC2, or both EPAC1 and EPAC2 do not show gross developmental or reproductive abnormalities. However recent studies have revealed that EPAC1<sup>−/−</sup> and EPAC2<sup>−/−</sup> mice display various phenotypes in response to stress or other challenges. For example, mice lacking EPAC1 or EPAC2 exhibit impaired glucose tolerance and dysfunctional insulin secretion after glucose challenge when compared with their wild-type littermates (44–46). Double knockout mice of both EPACs in the forebrain showed defects in long-term potentiation, spatial learning, and social interactions (47), whereas knocking out only EPAC2 is sufficient to induce social interactions impairment (48). Loss of EPAC2 also causes defects in memory retrieval in a fear condition paradigm (49). Interestingly, single nucleotide polymorphisms within the gene encoding EPAC2 have been linked to autism. Screening of 48 autistic individuals for mutations in the RAPGEF4 gene showed that four rare missense mutations may be a cause of autism (50). In spine synapses, these mutations alter the protein function of EPAC2 by affecting its Rap-GEF activity, the synaptic protein distribution and spine morphology (51). Activation of EPAC2 results in shrinkage of dendritic spine size as well as increased motility and turnover of the spines, thereby contributing to the plasticity of brain circuits (51, 52). Mice lacking EPAC1 or EPAC2 also present with phenotypes in the heart. Deletion of EPAC1 causes a mild decrease in basal cardiac functions, but more interestingly protects mice hearts from various stressors, such as arrhythmogenic stress (53). However, partly in contrast to this study, deletion of EPAC1 was reported to have no effect in cardiac function (54). Instead, loss of EPAC2 was shown to protect against β-adrenergic receptor-dependent arrhythmia (54). Most of the biological functions aforementioned have been attributed retrospectively to EPAC1 and EPAC2A due to their expression pattern. The roles of EPAC2B and EPAC2C, which were discovered later, are overall less studied. The potential roles of EPAC2B are discussed in chapter 2. In the liver, EPAC2C has been shown to suppress apoptosis and iNOS expression and activity in hepatocytes (55). EPAC2C may also control bile acid-stimulated canalicular formation in the liver (56).

## EPAC IN THE HPA AXIS

EPAC2A is expressed in the hypothalamus and pituitary gland and EPAC2B in the adrenal gland. cAMP is known to be an essential regulator at all levels in the HPA axis (57) and, here, we review how EPAC2 is emerging to contribute to cAMP-mediated actions.

## EPAC in the Hypothalamus and the Pituitary

In response to various stress factors, the expression of CRF is stimulated in the PVN of the hypothalamus. CRF synthesis is dependent in part upon the neuropeptide pituitary adenylate

cyclase-activating polypeptide (PACAP) and a subsequent elevation of cAMP (57, 58). So far, only PKA-dependent signaling has been reported to relay the stimulatory effect of cAMP on CRF expression. PKA inhibition was indeed shown to prevent binding of cAMP response element (CRE)-binding protein (CREB) on the CRF gene promoter and to inhibit transcriptional activation (59). EPAC has not been studied in PACAP-mediated actions in the PVN. However, EPAC was shown to mediate the effects of PACAP on long-term depression of synaptic transmission in the hippocampus through Rap in murine hippocampal slices (60), and in mice deleted for both *Epac1* and *Epac2* (47). These studies may, therefore, suggest a potential contribution of EPAC in PACAP-responsive PVN. In the suprachiasmatic nuclei of the hypothalamus, EPAC has been associated with leptin signaling (61) and the regulation of factors involved in setting circadian rhythms in Ref. (62). In noradrenergic neurons isolated from locus coeruleus (LC) in culture, EPAC, but not PKA, was shown to be involved in mediating the actions of cAMP (63). Thus, upon CRF binding to type-1 CRF receptor, LC neurons differentiate into norepinephrine-producing neurons via the activation of cAMP-EPAC-ERK/MAPK pathway, by potentiating brain-derived neurotrophic factor-stimulated synaptic plasticity via tyrosine kinase B signaling (63). Since LC neurons innervate PVN neurons, EPAC may, hence, indirectly stimulate the secretion of CRF from the hypothalamus [reviewed in Ref. (64)].

EPAC2A is also the dominant isoform expressed in the pituitary (19), but we still have very limited information about the potential roles of EPAC2 in this gland. Experiments in AtT20 pituitary cells demonstrated that EPAC, presumably EPAC2A, acts as a mediator of CRF<sub>1</sub>-induced signaling in corticotropes (65) and in HEK-293 overexpressing CRH-R2 $\beta$  cells (66). Upon activation of the CRH receptor, EPAC2A is involved in cAMP-mediated induction of ERK signaling (65), a pathway previously reported to induce POMC transcription in a PKA-independent manner (67). In addition, CRF receptor activation via Gq is known to signal to phospholipase C $\epsilon$  (PLC $\epsilon$ ) and, hence, inositol (1,4,5) triphosphate (IP3) to induce calcium stores mobilization that contributes to ACTH secretion (1, 2, 68). Considering that the activation of PLC $\epsilon$  by EPAC-Rap2 has been reported to activate Ca $^{2+}$  release and the secretion of hormones in different tissues (23), EPAC may also contribute to the secretion of ACTH in corticotropes.

## EPAC in Adrenal Physiology

### Role of EPAC versus PKA in Steroidogenesis

In the adrenal cortex, PKA is undoubtedly the major mediator by which cAMP regulates steroidogenesis (69–71). Once PKA is activated, both an acute and a chronic response occur, which contribute to increased steroid hormone synthesis. During the acute response, PKA phosphorylates HSL, which converts cholesterol esters to free cholesterol. This rapid response also involves an increase in StAR, which facilitates the movement of cholesterol to the inner mitochondrial membrane where the rate-limiting enzyme CYP11A1 resides (72). The chronic response corresponds to the transcriptional activation of all the other steroidogenic enzymes (73, 74). In a study by Schimmer et al.,

using microarray technology to investigate the effects of ACTH in mouse adrenal Y1 cells, the involvement of PKA was shown to account for up to 60% of the effects of ACTH on transcription, while only 6% could be assigned to PKC (70). This study clearly validated the dominant role of PKA in steroidogenesis, but left about 34% of the ACTH effects to be independent of PKA and PKC. In addition, another study had pointed to the importance of cAMP signaling, mediated independently of PKA, for aldosterone production in the adrenal zona glomerulosa (75). These findings suggest a role for EPAC in the regulation of adrenal function. The specific expression of the EPAC2B isoform in steroidogenic cells (19, 21) also points to roles for EPAC-dependent signaling in these cells. We, therefore, systematically assessed the involvement of PKA versus EPAC in steroidogenesis using cell permeable cAMP analogs specific for PKA and EPAC1/2 (N6-benzoyl-cAMP and 8-p-chlorophenylthio-2-O-methyl-cAMP) in adrenocortical cell lines (21). Our study demonstrated that PKA, and not EPAC2B, is the essential cAMP-induced regulator of factors involved in steroid hormone production (such as StAR, CYP11A1, and CYP17) as well as for the biosynthesis of cortisol and aldosterone. The role of EPAC2 was also studied in bovine zona fasciculata, expressing high levels of EPAC2 mRNA (76), using the same EPAC-specific cAMP analog (77). Although this analog induced cortisol biosynthesis, a non-hydrolyzable EPAC activator had no effect. The study concluded that metabolites of the hydrolyzable EPAC-specific analog induced the increase in cortisol observed in a cAMP-independent manner. Although seemingly opposite results were obtained with the same EPAC activating compound, these two studies indicate that EPAC is not important for cortisol production. cAMP rapidly induces the transcription factor nerve growth factor-induced clone B (NGFI-B), a regulator of several steroid hydroxylase genes (78–80). In adrenocortical cells in culture, we also found that NGFI-B-induction by cAMP is mediated by PKA and not by EPAC (21). Current investigations on HPA axis regulation in mouse knockout models will provide insights into the potential roles for EPAC1/2 in this neuroendocrine system.

### EPAC2B Contributes to Cytoskeletal Remodeling in the Adrenal Cortex

In adrenocortical cells in culture, cAMP characteristically induces changes in cell shape and a concomitant reorganization of F-actin microfilaments [reviewed in Ref. (81)]. Furthermore, cytoskeletal reorganization has been shown to contribute to steroidogenic hormone production by allowing the correct positioning of lipid droplets, the ER and mitochondria where cholesterol and its metabolites are transported and metabolized (82–84). While using PKA- and EPAC-specific agonists to study their effect on steroidogenesis in adrenocortical cell lines, we observed that the activation of both cAMP effectors contributed to cell rounding and the reorganization of F-actin fibers (21). Considering that PKA activation contributes to the regulation and expression of many enzymes necessary for steroidogenesis, the additional effect mediated by PKA on F-actin remodeling would, hence, correlate well with enzymatic outputs. By contrast, the effects of EPAC on F-actin remodeling are not correlated to steroidogenesis and may, therefore, contribute to other aspects of adrenal physiology. In

line with this, we also found that activation of EPAC2B induced a marked decrease in migration (21). This finding implies that EPAC2B plays a role in cell motility and this suggests wider implications, such as adrenal cancer cell invasion. Although EPAC2 has so far not been implicated in cancer development, several studies have demonstrated roles, albeit contradictory, for EPAC1 in cell migration and metastasis (85). The molecular mechanisms implicated include, at least, an increase of  $\text{Ca}^{2+}$  release mediated by PLC-IP3 promoting actin remodelling and cell migration of melanoma cells (86) as well as integrin activation important for cell migration and metastasis of pancreatic cancer cells (87). EPAC2B may, hence, act in the same way as EPAC1 in the adrenal gland.

## CONCLUSION

Since the discovery of EPACs in 1998, our understanding of cAMP-induced signaling and its roles in physiological processes has changed dramatically. Important initial *in vitro* studies on EPAC paved the way for current phenotypic analyses of genetic mouse models lacking EPAC in single or double knockouts. Based on these gene knockout models an important picture has emerged, namely that although deletion of EPAC does not cause gross defects in mice kept at standard protected conditions in the

animal facility, exposure to stressful situations provoke significant phenotypes. EPAC2 is expressed along the HPA axis, and it is interesting to note that whereas the hypothalamus and pituitary specifically express EPAC2A, the adrenal cortex expresses solely the EPAC2B isoform. While EPAC has been shown to mediate potential roles in the hypothalamus, putative functions in the PVN are yet to be determined. At the hypophyseal level, EPAC2A has been implicated in the regulation of POMC expression, and in the adrenal cortex, EPAC2B affects the migration of adrenocortical cells in culture. The generation of spatial and temporal conditional gene knockout models is now required to pinpoint the specific roles of the different EPAC isoforms during development and adult life. Moreover, the ongoing efforts to develop isoform-specific agonists and antagonists hold great promise for insights into isoform-specific functions. Such compounds will also be important potential new drugs to treat diseases in which EPAC plays a role. Several studies do indeed suggest that EPACs are promising drug targets (88), giving hope that small molecules targeting EPACs will serve as useful treatments in the future.

## AUTHOR CONTRIBUTIONS

AEL, RA, and MB wrote parts of the manuscript. AL coordinated editing.

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# Signaling Interactions in the Adrenal Cortex

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The major physiological stimuli of aldosterone secretion are angiotensin II (All) and extracellular K<sup>+</sup>, whereas cortisol production is primarily regulated by corticotropin (ACTH) in fasciculata cells. All triggers Ca<sup>2+</sup> release from internal stores that is followed by store-operated and voltage-dependent Ca<sup>2+</sup> entry, whereas K<sup>+</sup>-evoked depolarization activates voltage-dependent Ca<sup>2+</sup> channels. ACTH acts primarily through the formation of cAMP and subsequent protein phosphorylation by protein kinase A. Both Ca<sup>2+</sup> and cAMP facilitate the transfer of cholesterol to mitochondrial inner membrane. The cytosolic Ca<sup>2+</sup> signal is transferred into the mitochondrial matrix and enhances pyridine nucleotide reduction. Increased formation of NADH results in increased ATP production, whereas that of NADPH supports steroid production. In reality, the control of adrenocortical function is a lot more sophisticated with second messengers crosstalk and mutually modifying each other's pathways. Cytosolic Ca<sup>2+</sup> and cGMP are both capable of modifying cAMP metabolism, while cAMP may enhance Ca<sup>2+</sup> release and voltage-activated Ca<sup>2+</sup> channel activity. Besides, mitochondrial Ca<sup>2+</sup> signal brings about cAMP formation within the organelle and this further enhances aldosterone production. Maintained aldosterone and cortisol secretion are optimized by the concurrent actions of Ca<sup>2+</sup> and cAMP, as exemplified by the apparent synergism of Ca<sup>2+</sup> influx (inducing cAMP formation) and Ca<sup>2+</sup> release during response to All. Thus, cross-actions of parallel signal transducing pathways are not mere intracellular curiosities but rather substantial phenomena, which fine-tune the biological response. Our review focuses on these functionally relevant interactions between the Ca<sup>2+</sup> and the cyclic nucleotide signal transducing pathways hitherto described in the adrenal cortex.

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## INTRODUCTION

The adrenal cortex contains three zones, of which *glomerulosa* secretes the mineralocorticoid aldosterone, *fasciculata* synthesizes the glucocorticoid cortisol (or corticosterone in rodents), whereas the *reticularis* produces androgens [reviewed in Ref. (1)]. Aldosterone, acting on the distal nephron, augments Na<sup>+</sup> reabsorption as well as K<sup>+</sup> and H<sup>+</sup> excretion. Through changes in sodium balance, it influences the extracellular fluid space and blood pressure, and its importance in cardiovascular, renal, and inflammatory diseases has also been recognized (2–4). Cortisol, among other things, controls intermediary metabolism, modulates immune responses, and is essential for the resistance

of the organism to noxious stimuli. Adrenal androgens exert important anabolic effects in females and have substantial clinical significance in adrenal pathologies.

Sodium and/or fluid depletion, hemodynamic changes, and hyperkalemia stimulate aldosterone secretion. When fluid loss is severe, ACTH synergizes with angiotensin II (AII) in stimulating glomerulosa cells. During hypervolemia, atrial natriuretic peptide (ANP) inhibits aldosterone secretion [for reviews, see Ref. (5, 6)]. Cortisol production is governed by ACTH. The regulation of ACTH secretion and the signaling in zona *reticularis* (7) are beyond the scope of this review.

## CLASSICAL SIGNALING PATHWAYS IN THE ADRENAL CORTEX

### Signaling Pathways in Glomerulosa Cells

The major signaling pathways of ACTH, K<sup>+</sup>, and AII, termed “classical” here have been described in several reviews [e.g., Ref. (5, 6, 8, 9)] and are only briefly summarized below.

ACTH binds to the melanocortin-type receptor MC2R, which activates adenylyl cyclase (AC) *via* the heterotrimeric G-protein G<sub>s</sub> (10, 11), and subsequent cAMP formation activates protein kinase A (PKA). PKA then phosphorylates and induces the hormone-sensitive lipase (previously “cholesterol ester hydrolase”) (12) as well as the steroidogenic acute regulatory protein (StAR), the protein transporting cholesterol into the mitochondria (13, 14). As a result of these, the steroid precursor cholesterol is released from lipid droplets and transported to side-chain cleavage by CYP11A1, located in the inner mitochondrial membrane. This causes the stimulation of adrenal steroidogenesis.

Extracellular K<sup>+</sup> and AII act by generating cytosolic Ca<sup>2+</sup> signal. Depolarization induced by physiological elevations of [K<sup>+</sup>] activates T-type voltage-dependent Ca<sup>2+</sup> channels the current of which was detected in rat (15–17), bovine (16, 18, 19), and human glomerulosa cells (20). Concomitant cell swelling evoked by K<sup>+</sup> also enhances this T-type current (21, 22).

The unique sensitivity of glomerulosa cells to K<sup>+</sup> (6, 23, 24) may be attributed to their high permeability to K<sup>+</sup> (19, 25–28) and the function of the T-type channel Ca<sub>v</sub>3.2. The channel's subunit  $\alpha_{1H}$  is expressed in rat, murine, and bovine glomerulosa cell (29, 30). In view of the very negative membrane potential of isolated glomerulosa cells (27, 31), basal Ca<sup>2+</sup> influx was attributed to a steady-state window current (19, 32). The control of Ca<sub>v</sub>3.2 in glomerulosa cells has recently been analyzed in murine adrenal slices (30), in which cells had a mean resting potential of -82 mV. Spontaneous membrane potential oscillations generated by Ca<sub>v</sub>3.2 were observed between -87 and -75 mV. Increasing [K<sup>+</sup>] up to 5 mM depolarized the membrane and increased oscillation frequency and peak amplitudes, whereas the increased frequency upon AII stimulation was most probably due to a G<sub>i</sub>-mediated shift in the voltage dependence of channel activation toward more negative potentials (33). In either cases, the ensuing Ca<sup>2+</sup> signal (*via* CaMKII and p42/44 MAP kinase) acts on hormone-sensitive lipase (34) and StAR (13, 35, 36) [similarly to the actions of PKA (37)].

Angiotensin II stimulates aldosterone secretion after binding to AT<sub>1</sub> receptors (AT1Rs) (38–40). Acting *via* the G-protein G<sub>q</sub> and phospholipase C<sub>β</sub> it induces the formation of inositol 1,4,5-trisphosphate (IP<sub>3</sub>) (41–43) which, through specific receptors [IP<sub>3</sub>Rs (44–46)] generates Ca<sup>2+</sup> signal. Out of the three receptor isoforms expressed in glomerulosa cells (47), the dominant IP<sub>3</sub>R1 exhibits the greatest affinity for IP<sub>3</sub>. The initial Ca<sup>2+</sup> release is followed by Ca<sup>2+</sup> influx (48, 49) through store-operated (50, 51) and later *via* T-type Ca<sup>2+</sup> channels (18, 33) [but see Ref. (52)]. In isolated rat glomerulosa cells, AII-induced T-type current is activated by depolarization (19) brought about by the inhibition of the Na<sup>+</sup>/K<sup>+</sup> pump (53) and by the two-pore domain K<sup>+</sup> channel TASK (25, 54). In murine cells maintained *in situ*, T-current is enhanced by a G<sub>i</sub>-mediated increase in the frequency of oscillating action potentials (30, 33).

Angiotensin II inhibits L-type current (55) and thus attenuates Ca<sup>2+</sup> signals evoked by high [K<sup>+</sup>] (56, 57). This effect of the peptide is mediated by the G-protein G<sub>i</sub> expressed in glomerulosa cells (55, 58).

Due to space limits, this review does not deal with diacylglycerol – protein kinase C (PKC), lipoxygenase, and MAPK pathways [reviewed, e.g., in Ref. (6)].

### Signaling in Fasciculata Cells

The physiological stimulus of glucocorticoid synthesis and secretion by fasciculata cells is ACTH, acting *via* MC2R receptors and cAMP. The mode of cAMP action is identical to that described above for glomerulosa cell [for review, see Ref. (59)].

ACTH action on fasciculata cells requires Ca<sup>2+</sup>. As observed already in the 70s, ACTH induces membrane potential changes (60) due mainly to Ca<sup>2+</sup> influx (61). Both T-type (Ca<sub>v</sub>3.2) and L-type (Ca<sub>v</sub>1.3 and a non-identified) isoforms were characterized in bovine fasciculata cells, and their participation in ACTH- and AII-stimulated cortisol secretion was demonstrated (62). We are not aware of data on Ca<sup>2+</sup> channels in rat and native human fasciculata cells; however, the observation that rat fasciculata cells were unresponsive to 13 mM K<sup>+</sup> (23) indicates the lack of T-type Ca<sup>2+</sup> channels. The resting membrane potential in bovine fasciculata cells is set by the background K<sup>+</sup> channel bTREK-1 (63), whereas the TASK-3 background K<sup>+</sup> channel, characteristic for rat glomerulosa cell (26), is undetectable in bovine fasciculata cells (64).

Albeit AT1R is expressed in human, bovine, and ovine fasciculata cells (65–67) data whether AII *alone* stimulates cortisol secretion are conflicting (62, 68–70). Rat *fasciculata* cells do not express detectable amounts of AT1R (71–73) [but see Ref. (74)] or inositol 1,4,5-trisphosphate receptor (IP<sub>3</sub>R) mRNA (47) and, accordingly, AII does not stimulate steroid production in these cells (23, 74, 75).

The expression of AT1R in fasciculata cells and the stimulation of cortisol secretion by the peptide raise the question whether AII plays any role in the control of cortisol secretion in man. In lack of comprehensive studies, we hypothesize that in stress situations, stimulation of fasciculata cells by AII may contribute to the stimulatory action of ACTH. On the other hand, in case of long-term high AII levels, cortisol secretion is maintained at resting level by the feed-back control of ACTH secretion.

The human adrenocortical cancer-derived H295R cell, a widely used model for studying steroid production, does not express either MC2R receptors (76) or Ca<sub>v</sub>3.2 T-type Ca<sup>2+</sup> channels (30) [but see Ref. (77)]. Not surprisingly, these cells are insensitive to ACTH and respond to K<sup>+</sup> at supraphysiological concentrations only (78).

## INTERACTION OF SIGNALING PATHWAYS IN ADRENOCORTICAL CELLS

### (Auto)Regulation of Ca<sup>2+</sup> Metabolism by Ca<sup>2+</sup>

The formation, metabolism, and the action of IP<sub>3</sub> all depend on cytosolic [Ca<sup>2+</sup>] ([Ca<sup>2+</sup>]<sub>c</sub>). Phospholipase C<sub>β</sub>, generating IP<sub>3</sub> from PIP<sub>2</sub> (79) and the IP<sub>3</sub> metabolizing IP<sub>3</sub>-3 kinase are both activated by Ca<sup>2+</sup> (80–82). High [Ca<sup>2+</sup>]<sub>c</sub> may reduce IP<sub>3</sub> binding (83), whereas elevation of [Ca<sup>2+</sup>]<sub>c</sub> up to ~300 nM increases the sensitivity of IP<sub>3</sub>R1 to IP<sub>3</sub> [reviewed in Ref. (6)]. These characteristics play an important role in the oscillatory pattern of Ca<sup>2+</sup> release. IP<sub>3</sub>R phosphorylation by PKA, PKC, or CaMKII enhances Ca<sup>2+</sup> release, while calcineurin decreases this phosphorylation state (84). Also, calcium–calmodulin activates the plasmalemmal Ca ATPase (85) and inhibits the Na<sup>+</sup>/K<sup>+</sup> ATPase (86, 87), the latter resulting in depolarization and Ca<sup>2+</sup> influx through T-type channels (see Signaling Pathways in Glomerulosa Cells).

### Effects of Ca<sup>2+</sup> on Cytosolic cAMP

Early reports on K<sup>+</sup>-evoked cAMP formation suggested a role for Ca<sup>2+</sup> in the activation of AC (88, 89). Maintained secretagogue effect of ACTH in rat glomerulosa (90) and bovine fasciculata cells is also Ca<sup>2+</sup>-dependent (62) with calcium–calmodulin affecting primarily the formation of cAMP (91). In fact, in bovine cells, the effect of ACTH on cAMP formation correlates to extracellular [Ca<sup>2+</sup>] (92), and ACTH-induced cAMP formation is potentiated by AII in the presence of Ca<sup>2+</sup> only (69).

Nevertheless, conflicting data were reported concerning the effect of AII on cAMP formation in bovine adrenocortical cells (69, 93–95). Reduced cAMP formation was reported in AII-stimulated rat glomerulosa cells (96, 97), whereas enhanced cAMP formation was observed in the human H295R cell (98). In this respect, the Ca<sup>2+</sup> sensitivity of different transmembrane adenylyl cyclase isoforms (99, 100) should be considered. The Ca<sup>2+</sup>-activatable isoform AC1 is expressed in human glomerulosa and fasciculata cells (99); the Ca<sup>2+</sup>/calmodulin-activatable AC3 was found in human (99), rat (101), and bovine (69) glomerulosa cells. Ca<sup>2+</sup>-inhibited isoforms (AC5 and AC6) were detected in human (99) and rat glomerulosa cells (102). It should also be kept in mind that Ca<sup>2+</sup>-activatable AC isoforms are more responsive to store-operated Ca<sup>2+</sup> entry than to Ca<sup>2+</sup> release. This phenomenon is due to the colocalization of Ca<sup>2+</sup> activatable AC isoforms and store-operated Ca<sup>2+</sup> channels in plasmalemmal lipid rafts (103) and may account for the delayed cAMP response to AII (98).

After the description of G<sub>i</sub> in rat glomerulosa cells (58), the reported inhibition of AC by AII was attributed to this inhibitory G-protein (55, 95). Summarizing, the cell-type differences in the

effect AII on AC may be attributed to G<sub>i</sub> density and the ratio of the various AC isoforms.

The Ca<sup>2+</sup>-modified signaling pathways are summarized in Figure 1.

### Effects of cAMP on Ca<sup>2+</sup> Signaling

ACTH or cell-permeable cAMP analogs may induce a sustained Ca<sup>2+</sup> signal after a lag time of a few minutes as was shown in rat (104), bovine (92), and human glomerulosa cells (105), as well as in H295R cells (106). Several molecular interactions may warrant such an effect. PKA phosphorylates L-type Ca<sup>2+</sup> channels (105, 107) [but see Ref. (108)], the ensuing Ca<sup>2+</sup> current activates phospholipase C<sub>β</sub>, and the generated IP<sub>3</sub> induces Ca<sup>2+</sup> release from the endoplasmic reticulum (ER). In addition, PKA also phosphorylates and activates IP<sub>3</sub>R1 [reviewed in Ref. (6)]. In fact, ACTH evokes a small phosphoinositide response (109) and PKC activation (110) in rat glomerulosa cells. By the same token, 8Br-cAMP enhanced AII-induced IP<sub>3</sub> formation in bovine cells (111). Taken together, cAMP and its downstream effectors may enhance both Ca<sup>2+</sup> influx and Ca<sup>2+</sup> release in the adrenals.

The cAMP-modified signaling pathways are summarized in Figure 2.

### Signaling Modulation by cGMP

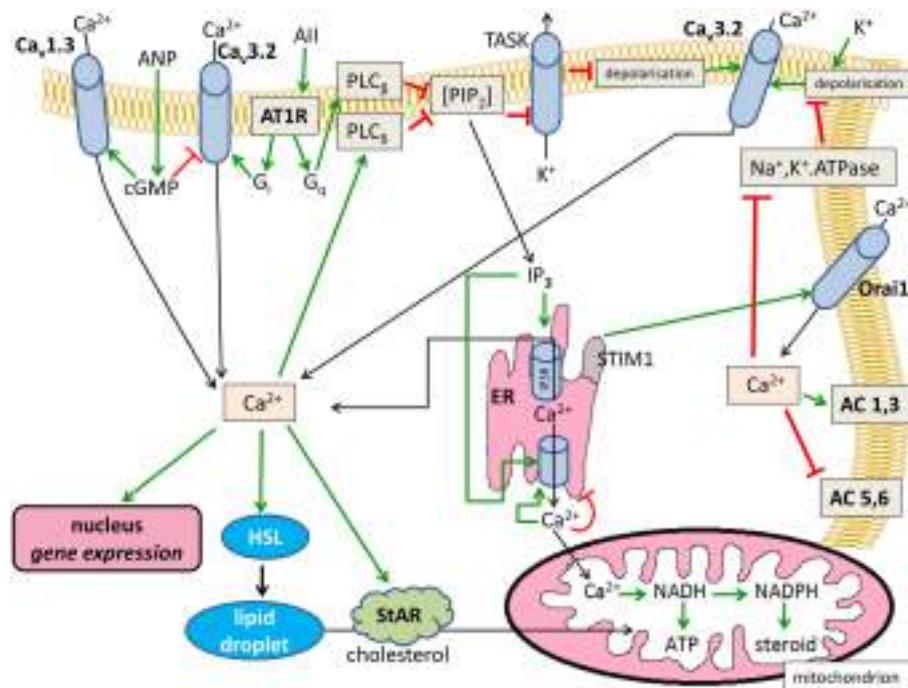
Cyclic GMP is formed after the activation of ANP receptors. The nucleotide reduces T-type Ca<sup>2+</sup> current (112) and inhibits the AC (113). In addition, cGMP activates PDE2A (114–116), one of phosphodiesterase isozymes identified in adrenocortical cells [reviewed by Vezzosi and Bertherat (117)]. By these actions, ANP reduces basal and stimulated aldosterone secretion (112).

### Synergistic Effects of Ca<sup>2+</sup> Release and Ca<sup>2+</sup> Influx

Moderate hyperkalemia increases the sensitivity and the maximal aldosterone response to AII (118–120). Potassium (4–8 mM) potentiates the secretory response to thapsigargin (evoking net Ca<sup>2+</sup> release from the ER), whereas the secretory effects of thapsigargin and AII (300 pM) are additive only (119). When net Ca<sup>2+</sup> release was induced with Ni<sup>2+</sup>, an inhibitor of microsomal Ca<sup>2+</sup> uptake, the aldosterone response to physiological concentrations of K<sup>+</sup> was again potentiated (121). These observations indicate that Ca<sup>2+</sup> release and influx act in synergism on aldosterone secretion.

The synergism between Ca<sup>2+</sup> release and influx may be explained by the formation of microdomains. Increased subplasmalemmal [Ca<sup>2+</sup>] (formed around the orifice of Ca<sup>2+</sup> channels) may activate e.g., Ca<sup>2+</sup>-dependent AC isoforms and may induce specific gene expression (122, 123). On the other hand, Ca<sup>2+</sup> release into the perinuclear space may turn on Ca<sup>2+</sup>-dependent nuclear genes and enhance NAD(P)H formation in ER-vicinal mitochondria (see Ca<sup>2+</sup> Signal and Mitochondrial Function). In addition, the reduction in exchangeable Ca<sup>2+</sup> pool during exposure to AII (124) may be counterbalanced by concomitant Ca<sup>2+</sup> influx.

Angiotensin II-induced initial IP<sub>3</sub> peak is followed by sustained suprabasal IP<sub>3</sub> formation (41). Li<sup>+</sup> inhibits the resynthesis of phosphoinositides and precludes the maintained formation of IP<sub>3</sub>, and thus attenuates the post-initial phase of AII-induced



**FIGURE 1 | Effects of  $\text{Ca}^{2+}$  on cytosolic cAMP in glomerulosa cells.** Positive modulations are shown with green arrows and negative effects are shown with red blunted arrows. Black arrows indicate substance transport.  $\text{Ca}_v1.3$ , L-type voltage-dependent  $\text{Ca}^{2+}$  channel;  $\text{Ca}_v3.2$ , T-type voltage-dependent  $\text{Ca}^{2+}$  channel; ANP, atrial natriuretic peptide; AII, angiotensin II; AT1R, angiotensin II receptor type 1;  $G_s$  and  $G_q$ , heterotrimeric G-proteins;  $\text{PIP}_2$ , phosphatidyl inositol 1,4,5-trisphosphate; TASK, KCNK3 or KCNK9-type  $K^+$  channel; ER, endoplasmic reticulum;  $\text{IP}_3$ , inositol 1,4,5-trisphosphate; IP3R,  $\text{IP}_3$  receptor; HSL, hormone-sensitive lipase; StAR, steroidogenic acute regulatory protein.

(but not ACTH-induced) aldosterone output of glomerulosa cells (125). This indicates that sustained suprabasal  $\text{IP}_3$  formation,  $\text{Ca}^{2+}$  release and, probably, store-operated  $\text{Ca}^{2+}$  entry all support long-lasting aldosterone secretion.

## EFFECTS OF CONVERGENT SIGNALING ON GENE EXPRESSION

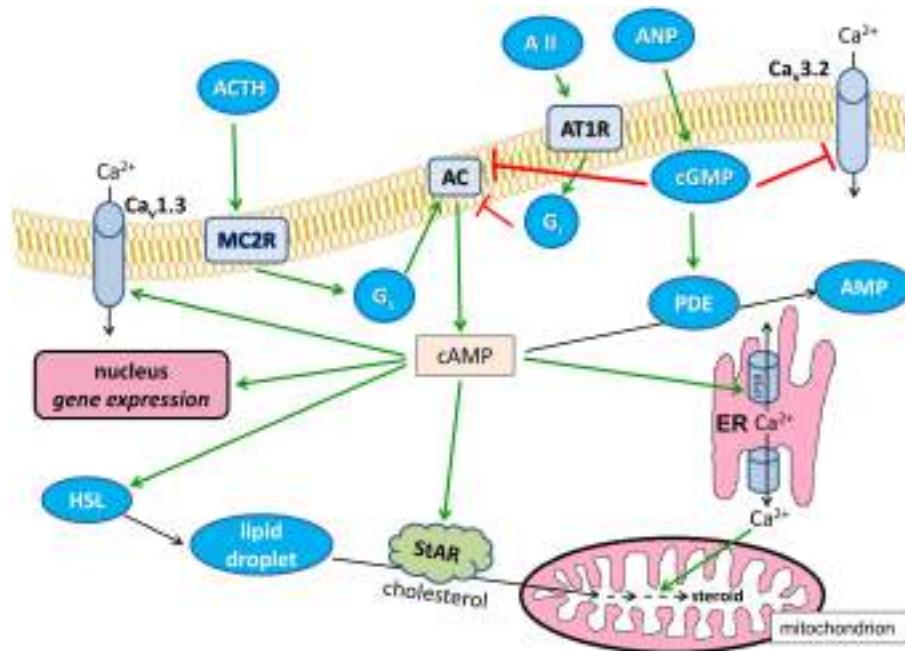
Both  $\text{Ca}^{2+}$  and cyclic nucleotide signaling affect the transcriptome of adrenocortical cells (126, 127). Complex transcriptional or epigenetic (128) changes during adrenal zonation, remodeling, and neoplastic transformation are beyond the scope of this study [for review, see, e.g., Ref. (129, 130)]. Instead, we focus on instances where gene expression is modulated by parallel signal transducing pathways. One illustrative example of such an interplay involves the transcriptional regulation of hormone-sensitive lipase and StAR, both of which are induced by the cAMP-PKA (12–14) and by the  $\text{Ca}^{2+}$  pathway (13, 34–36). Along similar lines,  $\text{Ca}^{2+}$  and cAMP, through overlapping *cis* regulatory elements, synergistically induce the transcription of aldosterone synthase (CYP11B2) (131) and (as observed in non-adrenal cells) the mitochondrial  $\text{Ca}^{2+}$  uniporter (MCU, see below) (132). Transcription of type I  $3\beta$ -hydroxysteroid dehydrogenase (HSD3B1), expressed predominantly in the human zona glomerulosa (133), can be induced by AII but not by  $K^+$  (134). A plausible explanation is that only AII [in part *via* PKC (135,

136)] recruits the nuclear receptor subfamily 4 (NGFI-B), which is also necessary for the induction of HSD3B1. In contrast, both AII and  $K^+$  induce CYP11B2 expression *via* the  $\text{Ca}^{2+}/\text{CaMK}$  and MAP kinase pathways (137).

In vascular smooth muscle cells, both  $G_s$ - and  $G_q$ -initiated signaling dampen the expression of AT1R through mRNA destabilization (138). Interestingly, to carry out this mRNA degradation, the pathways partially converge on PKA (139). An effect closely reminiscent of such a convergence was observed in H295R cells where forskolin, db-cAMP, and AII all brought about a rapid drop in AT1R message levels. [Nevertheless, long-term AT1R repression was induced with forskolin/db-cAMP only (140).]

## $\text{Ca}^{2+}$ SIGNAL AND MITOCHONDRIAL FUNCTION

Calcium activates three dehydrogenases in suspended or homogenized mitochondria (141).  $\text{Ca}^{2+}$ -dependent mitochondrial NADH and NADPH (NAD(P)H) formation in living cells was first demonstrated in  $K^+$ -stimulated glomerulosa cells (142). Similar response to AII and vasopressin was also reported (143, 144). The significance of increased NADH and ensuing ATP production (145) in any biological response is obvious, whereas NADPH is a cofactor of steroid biosynthesis (1). Noteworthy, the spatial and temporal pattern of AII-induced cytosolic  $\text{Ca}^{2+}$  signal depends on mitochondrial metabolism (78).



**FIGURE 2 | Effects of cAMP on Ca<sup>2+</sup> signaling in glomerulosa cells.** Positive modulations are shown with green arrows and negative effects are shown with red blunted arrows. Black arrows indicate substance transport. Ca<sub>v</sub>1.3, L-type voltage-dependent Ca<sup>2+</sup> channel; Ca<sub>v</sub>3.2, T-type voltage-dependent Ca<sup>2+</sup> channel; ANP, atrial natriuretic peptide; AII, angiotensin II; AT1R, angiotensin II receptor type 1; G<sub>i</sub> and G<sub>o</sub>, heterotrimeric G-proteins; AC, transmembrane adenylyl cyclase; ER, endoplasmic reticulum; IP<sub>3</sub>R, IP<sub>3</sub> receptor; HSL, hormone-sensitive lipase; StAR, steroidogenic acute regulatory protein; ACTH, corticotropin; MC2R, melanocortin receptor type 2; PDE, cAMP phosphodiesterase; AMP, adenosine monophosphate.

The primary event in the mitochondrial response to a cytosolic Ca<sup>2+</sup> signal is the transfer of the ion into the mitochondrial matrix (146–149). Ca<sup>2+</sup> transport occurs through the MCU complex, the velocity of which is a sigmoid function of [Ca<sup>2+</sup>]<sub>c</sub> due to the allosteric control of the MCU channel by the regulatory subunits MICU1 and MICU2 [reviewed in Ref. (150)]. IP<sub>3</sub>-induced Ca<sup>2+</sup> release from the ER generates high-Ca<sup>2+</sup> microdomains between the ER and mitochondria and allows for mitochondrial Ca<sup>2+</sup> uptake by the low-Ca<sup>2+</sup>-affinity MCU (151). However, the mitochondria of glomerulosa cells are uniquely sensitive to Ca<sup>2+</sup> (152) and influx-induced low-Ca<sup>2+</sup> signals are also effective in elevating mitochondrial [Ca<sup>2+</sup>] ([Ca<sup>2+</sup>]<sub>m</sub>) (153). This responsiveness may be essential for maintained aldosterone secretion in response to long-lasting hyperkalemia, characterized by small elevation of [Ca<sup>2+</sup>].

Increased [Ca<sup>2+</sup>]<sub>m</sub> and the ensuing NAD(P)H formation play an essential role in the stimulation of aldosterone production. Targeted mitochondrial expression of a Ca<sup>2+</sup> binding protein reduces both [Ca<sup>2+</sup>]<sub>m</sub> and NAD(P)H and ensuing aldosterone production in response to AII (154). The opposite, increased mitochondrial Ca<sup>2+</sup> uptake after the knockdown of p38 MAPK or the silencing of mitochondrial protein OPA1 results in increased NAD(P)H formation and enhanced aldosterone production (155).

A recently recognized and biologically significant action of Ca<sup>2+</sup> signaling is the formation of cAMP in mitochondria. In addition to the nine isoforms of transmembrane AC, a soluble

isoform (sAC) was prepared from testis (156). Its activity is not influenced by forskolin or G-proteins but increased by bicarbonate (157) and Ca<sup>2+</sup> (158). The expression of sAC in the mitochondrial matrix together with a degrading mechanism sensitive to phosphodiesterase 2A inhibitors were recently described in HeLa cells (159, 160). The activity of intramitochondrial sAC increased in response to mitochondrial Ca<sup>2+</sup> signal in HeLa and CHO cells and in rat cardiomyocytes (161). Importantly, mitochondrial cAMP (mt-cAMP) supported ATP formation (160, 161).

The sAC is also expressed in H295R adrenocortical cells, and it is found in the particulate fraction predominantly. In these cells, AII-induced mitochondrial Ca<sup>2+</sup> signal increased the formation of mt-cAMP, and this response was enhanced by the PDE2A inhibitor EHNA. Mitochondrial cAMP signaling was attenuated with the sAC inhibitor 2-OH-estradiol, after silencing of the sAC gene and by the buffering of mitochondrial Ca<sup>2+</sup> by S100G protein. All these maneuvers also attenuated aldosterone production, showing the cell-type-specific significance of mt-cAMP for the first time (98).

## CONCLUSION

Adrenocortical steroid production is under the control of both Ca<sup>2+</sup> signaling and cyclic nucleotide metabolism. Importantly, these intracellular pathways are rarely, or probably never, independent. As postulated by Berridge in 1975 (162), cAMP and Ca<sup>2+</sup> signaling may be antagonistic or synergistic in nature

and, as hopefully accentuated by this review, the adrenal cortex is no exception to this rule. As shown in **Figures 1** and **2**, the aforementioned signal transducing pathways have the potential to interact at a number of points and levels of signaling. However, it needs to be stressed that these potential interactions are not enforced all at once but, instead, may be limited temporally and spatially (e.g., to signaling microdomains). Also, significant variance in the expression pattern and intensity of the relevant signaling molecules is to be expected depending on species and on the stimuli the organism is concurrently exposed to.

In spite of the interspecies differences and of the incongruences in some experimental data, it is probably safe to conclude that an adequate biological response necessitates the intricate interplay of parallel signaling pathways. That is to say that, e.g., in glomerulosa cells, sustained aldosterone response evoked by long-lasting tonic stimuli will be satisfactory only if the  $\text{Ca}^{2+}$  release/influx is accompanied by the sufficient formation of cAMP. Albeit the secretagogues AII and  $\text{K}^+$  invoke predominantly  $\text{Ca}^{2+}$  signaling

and the effects of ACTH are mediated chiefly by cAMP, the increase of both factors at the same time may potentiate the final response. Thus, the separate intracellular pathways need not be activated to the same extent but nevertheless have to be recruited simultaneously for a sufficient steroid production to follow.

## AUTHOR CONTRIBUTIONS

AS and GS compiled the literary data and wrote the manuscript. Data on the control of expression have been discussed with LH.

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# T-Type Calcium Channel: A Privileged Gate for Calcium Entry and Control of Adrenal Steroidogenesis

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Intracellular calcium plays a crucial role in modulating a variety of functions such as muscle contraction, hormone secretion, gene expression, or cell growth. Calcium signaling has been however shown to be more complex than initially thought. Indeed, it is confined within cell microdomains, and different calcium channels are associated with different functions, as shown by various channelopathies. Sporadic mutations on voltage-operated L-type calcium channels in adrenal glomerulosa cells have been shown recently to be the second most prevalent genetic abnormalities present in human aldosterone-producing adenoma. The observed modification of the threshold of activation of the mutated channels not only provides an explanation for this gain of function but also reminds us on the importance of maintaining adequate electrophysiological characteristics to make channels able to exert specific cellular functions. Indeed, the contribution to steroid production of the various calcium channels expressed in adrenocortical cells is not equal, and the reason has been investigated for a long time. Given the very negative resting potential of these cells, and the small membrane depolarization induced by their physiological agonists, low threshold T-type calcium channels are particularly well suited for responding under these conditions and conveying calcium into the cell, at the right place for controlling steroidogenesis. In contrast, high threshold L-type channels are normally activated by much stronger cell depolarizations. The fact that dihydropyridine calcium antagonists, specific for L-type channels, are poorly efficient for reducing aldosterone secretion either *in vivo* or *in vitro*, strongly supports the view that these two types of channels differently affect steroid biosynthesis. Whether a similar analysis is transposable to fasciculata cells and cortisol secretion is one of the questions addressed in the present review. No similar mutations on L-type or T-type channels have been described yet to affect cortisol secretion or to be linked to the development of Cushing syndrome, but several evidences suggest that the function of T channels is also crucial in fasciculata cells. Putative molecular mechanisms and cellular structural organization making T channels a privileged entry for the “steroidogenic calcium” are also discussed.

**Keywords:** adrenal cortex, steroidogenesis, aldosterone, cortisol, ACTH, calcium signaling, electrophysiology, T-type calcium channels

**Abbreviations:** APA, aldosterone-producing adenoma; BK, broad conductance calcium-activated potassium channel; CPA, cortisol-producing adenoma;  $I_0$ , maximal current;  $I_{stb}$ , steady-state current; ISH, *in situ* hybridization; PA, primary aldosteronism; PMA, phorbol 12 myristate 13-acetate ester; SK, small conductance calcium-activated potassium channel; StAR, steroidogenic acute regulatory protein; ZF, zona fasciculata; ZG, zona glomerulosa.

## INTRODUCTION

Voltage-operated calcium channels play a crucial role in signal transduction of many excitable and non-excitable cell types (1). While a rapid modulation of their activity by hormone-stimulated kinases and/or G proteins has been recognized for a long time (2, 3), a control of their expression levels in the cell has been also described (4).

Among these channels, low threshold-activated, T-type (for transient and tiny current) calcium channels appear distinct concerning their electrophysiological and molecular properties. Although their existence has been shown early by studies of their voltage-dependence, kinetics, and single channel conductance (5–7), their cloning and their molecular characterization has been delayed, in part because of a lack of specific pharmacological tools and because they share less structural homology with the rest of the voltage-operated calcium channels. Thus, in the light of their molecular structure (8, 9), T-type calcium channels have been proposed to play specific physiological roles within the cells, and it has been suggested that they could be involved, depending on the levels of their expression, in the development of several diseases, such as hypertension, cardiac failure, epilepsy, or cancer (4).

## Electrophysiological Properties of T-Type Calcium Channels

An important and discriminating property of T channels is their ability to activate upon small depolarization of the membrane, allowing a surge of calcium entry into excitable cells at the beginning of an action potential (when the electrochemical gradient is highly favorable for cation entry), as well as in only slightly depolarized non-excitable cells, like isolated adrenal cortex steroidogenic cells. Their rapid, voltage-dependent inactivation and their slow deactivation (Figure 1A) make their gating characteristics distinct from those of other channels. This feature not only allows isolating specifically the corresponding currents thanks to defined voltage patch clamp protocols but also is responsible for the specific role played by these channels in the modulation of cardiac and neuronal cell excitability (10, 11). In cells devoid of action potential, such as isolated adrenal glomerulosa cells in culture, a sustained calcium entry within a permissive window of voltage is possible thanks to a significant overlap between the activation and inactivation potential ranges of the channel (12).

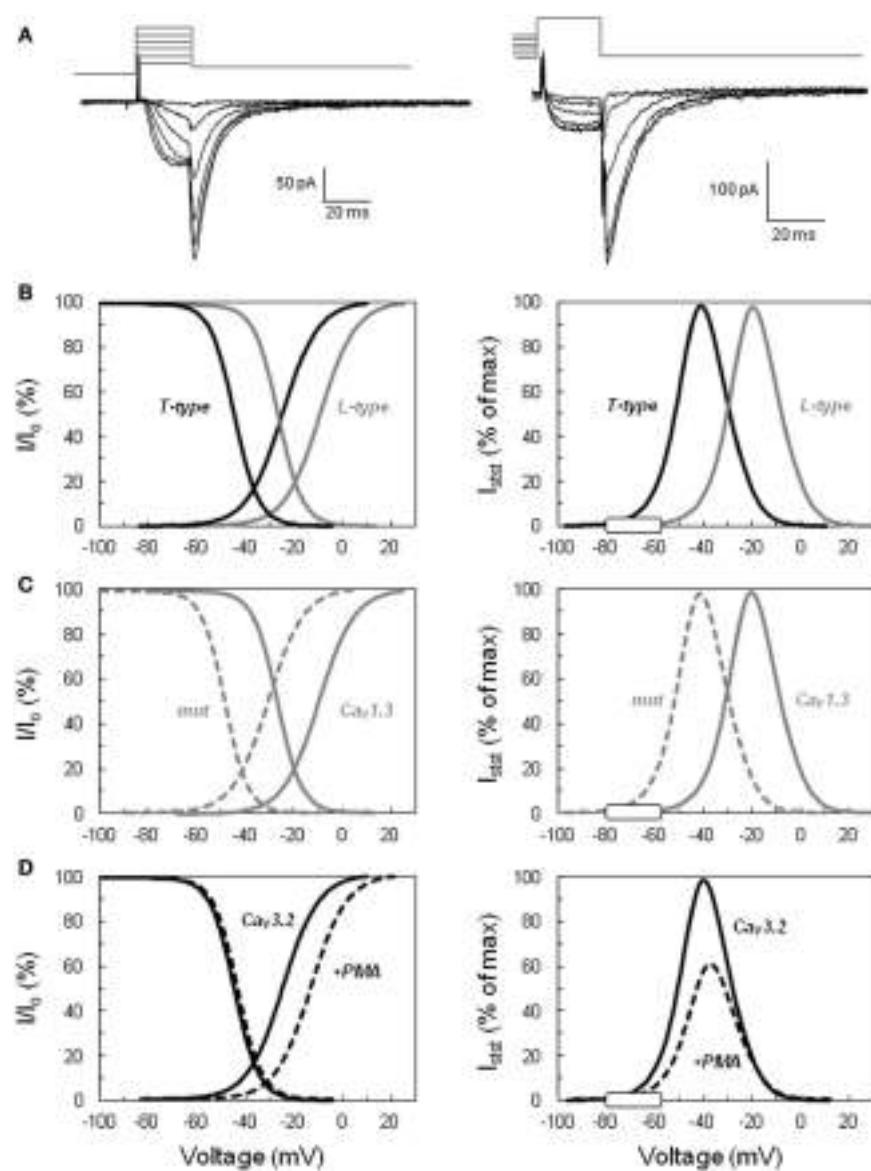
Indeed, voltage-operated calcium channels are classically characterized by their activation curve, reflecting the probability of channel opening at various membrane potentials (and, therefore, the proportion of channels activating at each voltage), and their steady-state inactivation curve, showing the proportion of channels remaining available (not inactivated) at different voltages (Figure 1B). These curves are produced experimentally by measuring currents under specific protocols, normalizing them and mathematically fitting data to Boltzmann's equation. Examination of these curves reveals, for each type of channel, the presence of a *permissive window of voltage*, in which activation and inactivation overlap. A *sustained* flux of calcium through the channels is theoretically possible in this window, where a

significant proportion of channels are already activated but not yet completely inactivated. This window delimits the range of voltages over which a steady-state current can flow through the channels, and the relative amplitude of this current can be calculated as a function of voltage using Ohm's law (15). It is important to realize at this point that, because only a small fraction of channels is open at any time in this mode (upon slight membrane depolarization), the current amplitude is tiny as compared to the maximal current observed within the same cell when all channels open together during a putative action potential or upon a strong depolarization. However, because the channel activation is *sustained* for minutes (due to lack of complete inactivation), calcium accumulated within the cell during this period is huge in comparison to the amount entering during a single action potential that leads the cell to voltages less favorable for calcium influx.

Low threshold T-type calcium channels activate (and inactivate) at lower voltages than high-threshold L-type calcium channels, and, as a consequence, also present their permissive window at lower voltages. In fact, any channel modification (through phosphorylation, binding of G protein or genetic mutation) affecting its activation and/or inactivation curves will result in a marked change of the properties of the steady state current. Indeed, not only the position of the window will be shifted under these conditions but also the maximal amplitude of the steady-state current, which depends on both the extent of the overlap of the activation and inactivation curves and on the electrochemical gradient for calcium entry.

The resting potential of glomerulosa cells from different species has been measured to be between  $-86$  and  $-73$  mV (16–19), values that are at the left edge of the T channel window (see Figure 1B, right panel), but farther from that of L-type channel. Values reported for fasciculata cells are between  $-76$  and  $-66$  mV (16, 20, 21), showing that fasciculata cells are slightly depolarized (by 8–10 mV) as compared to glomerulosa cells under resting conditions. Moreover, depolarization of the cells by *physiological concentrations* of agonists like AngII, ACTH, or potassium (see below) has been determined to be maximally 10–20 mV (18–20, 22), which is sufficient for increasing the steady-state current through T channels by several folds. Increasing extracellular potassium, progressively from low to supra-physiological concentrations, has been shown to increase aldosterone secretion in parallel to the size of the predicted T-channel steady-state calcium current (12).

Whether adrenal cortical cells are naturally excitable (i.e., able to generate action potentials) has been debated. Indeed, when cell to cell contacts are preserved, several authors observed low frequency action potentials in both resting and stimulated glomerulosa and fasciculata cells. *Isolated* glomerulosa cells have been conventionally considered as non-excitable because their membrane potential rests close to the equilibrium potential for potassium (16) and remains negative to  $-60$  mV upon stimulation with AngII or physiological concentrations of potassium. In contrast, some mouse zona glomerulosa cells *within adrenal slices* spontaneously generate membrane potential oscillations of low periodicity (0.44 Hz), as shown under whole cell current clamp conditions (17). Similar properties of rat, rabbit, and cat glomerulosa, and fasciculata cells had been previously reported



**FIGURE 1 | Activation and inactivation of voltage-operated calcium channels and steady-state “window” currents. (A)** Examples of slowly deactivating (T-type) Ba<sup>2+</sup> currents recorded in the whole cell configuration of the patch clamp technique. Left. Voltage protocol for determining the activation curve: tail currents were evoked by repolarizing the cell to -65 mV after a short period (20 ms) of depolarization at various voltages (-45 to +5 mV for this selection of traces) from a holding potential of -90 mV. Right. Voltage protocol for determining the steady-state inactivation curve: tail currents were elicited in the same cell at -65 mV, but after steady-state inactivation of the channels for 10 s at various holding potentials (here from -80 to -30 mV) and 20 ms activation at +20 mV. Current amplitude upon cell repolarization was then determined by fitting tail currents to an exponential function (the time constant was approximately 7 ms). **(B)** Comparison of low (T-type) versus high (L-type) threshold voltage-operated calcium channels. Left panel shows normalized activation and inactivation curves determined for T-type channels using the same type of protocol as shown in panel A. Tail current amplitudes were plotted as a function of the test voltage, fitted to Boltzmann's equation, and normalized to the maximal current ( $I_{max}$ ). Curves for L-type channels were similarly defined from L-current amplitudes determined with a different voltage protocol, including the inactivation of T currents. Right panel displays the calculated normalized steady-state current ( $I_{stat}$ ) expected through T-type and L-type channels within their respective permissive window of voltage. The theoretical steady-state currents were obtained from the activation and inactivation curves according to Ohm's law and expressed as a percentage of the maximal current. The white rectangle on the voltage axis indicates the range of membrane potentials reached in naive glomerulosa cells and in cells stimulated with physiological concentrations of angiotensin II or extracellular potassium. **(C)** Effect of the Ile770Met mutation described in the CACNA1D L-type calcium channel (13) on the channel activation, inactivation, and steady-state current. Curves have been determined as in **(B)** for the wild-type channel (continuous line, Ca<sub>v</sub>1.3) and for the mutant channel (dotted line, mut) and show the significant shift of the channel permissive window toward lower voltages. **(D)** Effect of PKC activation on CACNA1H T channel activation, inactivation, and steady-state current. Curves have been determined as in **(B)** for the naive channel (continuous line, Ca<sub>v</sub>3.2) and for the channel in glomerulosa cells treated with the PKC activator phorbol 12 myristate 13-acetate ester (dotted line, +PMA) and show the significant reduction of the amplitude of the maximal steady-state current with the slight shift of the permissive window toward higher voltages. The graphs of this figure have been constructed based on data available in Ref. (14, 13).

(16, 18, 23). Electrical activity has generally to be induced in silent cells by depolarization with secretagogues (AngII, ACTH, or potassium) or by injecting depolarizing current in the current clamp mode (16, 18, 20), which is coherent with thresholds for triggering the action potential observed above  $-64$  mV (17). Spontaneous or evoked voltage oscillations appear insensitive to inhibition by tetrodotoxin or nifedipine, but completely abolished by low nickel concentrations, suggesting that they are supported by calcium currents carried by low threshold T-type calcium channels (Cav3.2) (17, 20). Moreover, the frequency of the electrical oscillator is regulated positively by potassium and AngII (17), providing an additional way to these agonists for controlling steroid production.

Indeed, the presence of action potentials on top of the mechanism previously described as being responsible for the steady state calcium entry through T-type channels is expected to affect calcium signaling and therefore steroidogenesis. However, the exact role and influence of the spontaneous or evoked action potentials on steroid production (mostly measured experimentally from isolated cells, devoid of action potentials) have not been precisely and specifically quantified yet, probably given the strong relationship existing between this electrical activity and calcium entry. For example, it is not known whether a low but sustained entry of calcium is not more efficient for stimulating and maintaining steroid production than pulsatile peaks of high calcium with intermittent periods of channel inactivation.

## Calcium Channel Mutations in Primary Aldosteronism

Aldosterone secretion from adrenal glomerulosa cells is under the control of AngII and extracellular potassium (24, 25), and indirectly regulates blood pressure through stimulation of renal sodium reabsorption. A dysregulation of aldosterone production may lead to systemic hypertension and hypokalemia (26). Adrenal aldosterone-producing adenomas (APA) constitutively produce aldosterone (27) and are a common cause of severe hypertension. About 5–10% of patients referred to hypertension clinics have APA (28, 29), which are typically benign and well circumscribed, and their removal cures or ameliorates hypertension. The principal genetic cause of APA development [present in 40% of these tumors (30)] has been attributed to recurrent mutations in the KCNJ5 potassium channel, affecting its ion selectivity filter and therefore responsible for a sustained depolarization of the glomerulosa cell with the subsequent activation of low voltage-operated calcium channels (31). Other mutations associated to the presence of APA were identified by whole exome sequencing. Somatic mutations affected two members of the P-type ATPase gene family, ATP1A1 (the  $\alpha 1$  subunit of the Na/K-ATPase) and ATP2B3 (the plasma membrane calcium ATPase), highlighting again the importance of maintaining a strongly negative resting potential and a low cytosolic calcium concentration in adrenal glomerulosa cells for maintaining a low basal aldosterone production (32).

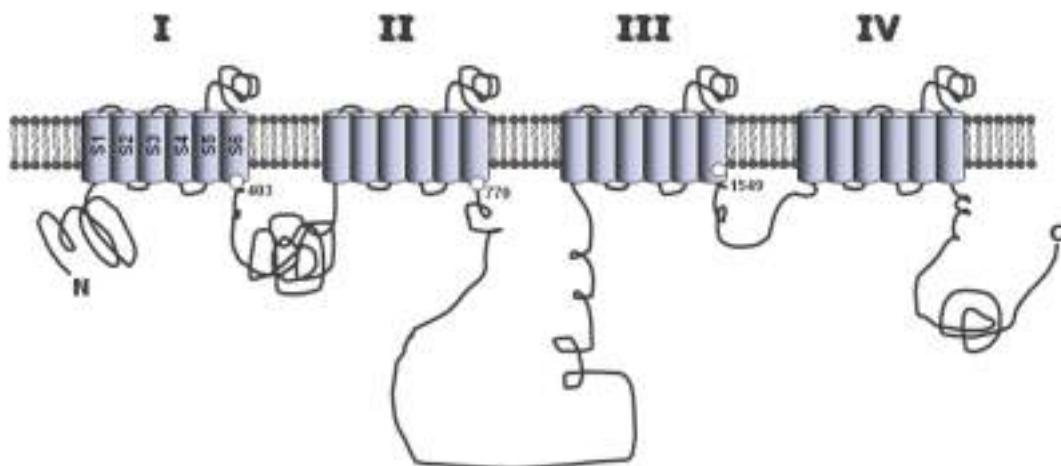
More recently, one additional gene, coding for a calcium channel, was identified with somatic and germline mutations present in other APA tumors without KCNJ5 mutation (13).

CACNA1D encodes the  $\alpha 1$  (pore-forming) subunit of Cav1.3, a high-threshold L-type calcium channel. CACNA1D mutations were identified in 9% of APA and represent the second most frequent cause of the disease (30). In contrast to patients with KCNJ5 mutations who are more frequently females, CACNA1D carriers are mostly males. Paradoxically, APA are generally composed of large cells with the morphological appearance of fasciculata cells (ZF-like cells) but still express markers of glomerulosa cells, including the enzymes required for aldosterone biosynthesis (33). While KCNJ5 mutations were associated with large ZF-like APA, CACNA1D mutations are present in small ZG-like APA, suggesting that the cellular composition influences the adenoma size (32, 34). Whether the resting potential of these cells, expected to be less negative in KCNJ5 mutant APAs (like in fasciculata cells) than in normal glomerulosa or in CACNA1D mutant cells, is involved in the determination of cell size and morphology remains to be demonstrated, which is probably a difficult task given the reported heterogeneity of the lesions.

Mutations identified in CACNA1D and associated with APA were affecting two conserved amino acids of the L-type Cav1.3 channel, Gly403 and Ile770, both located near the cytosolic end of S6 transmembrane segments in domains I and II (Figure 2), regions implicated in channel gating (13). To assess the effect of these mutations on channel function, wild type and mutant channels were expressed in HEK293T cells and their electrophysiological properties investigated with the patch clamp technique. Compared to wt channels, both mutants showed maximum current amplitude at less depolarized potentials, as well as a significant leftward shift (by 20 mV) of their activation and steady-state inactivation curves (Figure 1C). As a consequence, the permissive window of the channel is also shifted to more negative voltages, corresponding to membrane potentials reached upon physiological stimulations. In other words, these gain-of-function mutations of the channel, by shifting its threshold of activation, make the Cav1.3 channel “T-like.”

Recently, using whole exome sequencing in subjects with unexplained primary aldosteronism (PA) diagnosed at a young age (<10 years), a new mutation has been identified that affect a T-type calcium channel (CACNA1H). Interestingly, the mutation (M1549V) is located within the S6 segment of domain III (Figure 2). Electrophysiology demonstrates that this variant causes a 10 times reduction of the rate of channel inactivation and a modest shift of the activation curve to more hyperpolarized potentials, two effects inferred to produce increased calcium influx and aldosterone hypersecretion (35).

Clearly, these observations highlight the importance of low threshold T-type calcium channels in the control of aldosterone biosynthesis, and they recently reactivated the general interest for a comprehensive understanding of the functional specificity of these channels in steroidogenesis. This review aims at recapitulating the arguments in favor of a privileged functional role for T channels in the control of steroid production, at comparing their involvement in aldosterone and cortisol secretion, and, finally, at discussing whether similar mutations on calcium channels, as observed in PA patients, are susceptible to occur in fasciculata cells and to be responsible of some forms of Cushing syndrome.



**FIGURE 2 | Common structure of the alpha 1 subunit of voltage-operated calcium channels.** The main, pore-forming  $\alpha_1$  subunits of the various voltage-operated calcium channels share a common general structure, with four homologous repeats (I–IV), each composed of six hydrophobic, putative membrane-spanning alpha helix domains (S1–S6). The three large loops connecting repeats together, as well as the N- and C-terminal extremities are located in the cytosol. The positions of Gly403 and Ile770 mutated in CACNA1D (13), and of Met1549 in CACNA1H (35), are indicated at the end of the S6 segments in repeats I, II, and III, respectively.

## STRUCTURE, EXPRESSION, AND PROPERTIES OF T-TYPE CALCIUM CHANNELS IN ADRENAL CORTEX

Although most studies of low-threshold T-type calcium current expression have focused on the analysis of neuronal and cardiac tissues, the presence of similar currents has been demonstrated throughout the body in both excitable and non-excitable cells, including adrenal glomerulosa (36–44) and fasciculata cells (45–48) from various species.

Three different genes code for the various T channels that are all composed of a single, large  $\alpha_1$  subunit (49). According to the proposed nomenclature of voltage-gated calcium channels (50),  $\alpha_1G$ ,  $\alpha_1H$ , and  $\alpha_1I$  T channel isoforms have been renamed  $\text{Ca}_v3.1$ ,  $\text{Ca}_v3.2$ , and  $\text{Ca}_v3.3$ , respectively (Table 1).

The expression in *Xenopus* oocytes or HEK293 cells of each of the three cloned  $\alpha_1$  subunits coding for T channels generates typical low threshold transient currents with classical properties associated with T channels (51–54). Currents induced by  $\text{Ca}_v3.1$  and  $\text{Ca}_v3.2$  channels are nearly identical but can be easily distinguished by the higher sensitivity of  $\text{Ca}_v3.2$  to inhibition by nickel (55).

As for high threshold calcium channels and sodium channels (56, 57), the  $\alpha_1$  subunits of T channels consist of four homologous repeats (I–IV), each being composed of six transmembrane segments (S1–S6), including a highly conserved pore loop (between S5 and S6) and a distinct voltage sensor (S4) (Figure 2). In contrast, more divergence between isoform sequences is found within the large intracellular loops, particularly that linking domains II and III, which also contains a site for alternative splicing in  $\alpha_1G$  (58). Interestingly, this variable region of the channel is responsible for the interaction of several calcium channels with specific cell proteins [such as the ryanodine receptor binding to the skeletal muscle L-type channel (59), or syntaxin binding to the neuronal

**TABLE 1 | Nomenclature of T-type calcium channels.**

Channel name	Protein isoform	Gene name
$\text{Ca}_v3.1$	$\alpha_1G$	CACNA1G
$\text{Ca}_v3.2$	$\alpha_1H$	CACNA1H
$\text{Ca}_v3.3$	$\alpha_1I$	CACNA1I

N-type channel (60)], and could therefore participate to the establishment of specific functions for each channel isoform within the cell (see below).

The T channel family presents only 28% sequence identity with other voltage-gated calcium channels, while L ( $\text{Ca}_v1.x$ ) and non-L ( $\text{Ca}_v2.x$ ) high threshold channel families share more than 50% of their sequence. Replacement of two out of the four glutamate residues generally present in each P (pore-forming) region of the channel by aspartate seems to be responsible for the specific ion selectivity and permeation of T channels (61). Like for other calcium channels, molecular diversity among T channels is increased by alternative splicing of the mRNA of each of the three isotypes, and the properties of some of the 24 putative  $\text{Ca}_v3.1$  splice variants have been described by several groups (58, 62–64).

Physiological roles for T-type channels have been proposed in several tissues, including pacing of the heart (10), smooth muscle contraction (65), pain neurotransmission (5), fertilization (66), or adrenal steroid biosynthesis (38, 46). The tissue-selective expression of these channels and their isoforms has been determined at the molecular level by Northern blot analysis, *in situ* hybridization (ISH), RT-PCR, as well as by immunohistochemistry (IH) in human and other species.  $\text{Ca}_v3.1$  has been shown to be expressed predominantly in brain as well as in heart, while  $\text{Ca}_v3.2$  is mostly found in kidney and liver, but can be also observed in adrenal cortical cells.

The analysis of the expression pattern of the various T channel isoforms revealed that mRNA coding for each of the three  $\alpha_1$  subunits is detectable in the adrenal gland, although in varying quantities. The molecular identity of the channels has been determined more specifically in the adrenal cortex, showing that the predominant subtype expressed in both glomerulosa and fasciculata cells from various species is Cav3.2 (Table 2), a finding in agreement with the high sensitivity to nickel of the T currents recorded in these cells (67). In several studies, a stronger expression of the channel was reported to be present in glomerulosa cells as compared to fasciculata cells (35, 67, 68). Similarly, Cav3.2 has been found to be the main isoform expressed in the human adrenocortical cell line H295R (69), but Cav3.1 could be induced in the same cells by changing culture conditions (70).

Indeed, besides short-term modulation of T channels by hormones through the transduction mechanisms described below, a slower and more sustained regulation of channel activity results from the control of the expression of the  $\alpha_1$  subunit. Such a regulation of T channel expression has been observed during the development of various organs, suggesting that these channels could play important roles at specific stages of the fetal life. The density of T channels has been shown to be relatively elevated in H295R cells, a cell line derived from a human adrenocarcinoma, as compared to corresponding normal tissue. This is reminiscent of the many pathological situations where re-expression of fetal genes has been observed during the evolution of oncologic diseases. Interestingly, aldosterone, which is produced by H295R cells, has been shown to significantly increase the T current amplitude, as well as the  $\alpha_1H$  transcript levels (69). Because T channels support steroidogenesis in various cells, including H295R cells, this autocrine action of aldosterone on channel expression represents a positive feedback loop that could help tumor cells to maintain differentiated functions like steroidogenesis.

Treatment of isolated rat (47) or bovine (71) fasciculata cells with ACTH for 3–6 days has been shown to induce the expression of CACNA1H mRNA and to markedly increase the amplitude of the T-type calcium currents, a response efficiently blocked by cycloheximide, an inhibitor of protein synthesis. ACTH treatment did not affect L-type current expression in these cells. Interestingly, the putative role of cAMP in this response to ACTH has been challenged by the use of distinct cellular messenger analogs and metabolites mimicking ACTH action without PKA activation (71, 73), and by the fact that ACTH-induced T current up-regulation was not affected by the PKA antagonist H89 (47).

Recently, the relative expression of four distinct calcium channels (Cav1.2, 1.3, 2.2, and 3.2) has been analyzed at the mRNA and protein levels in human normal adrenal cortex, as

well as in aldosterone (APA) and in cortisol-producing adenoma (CPA). While RT-PCR results showed that Cav3.2 (CACNA1H) was the second most expressed channel in APA (after Cav1.3), Cav3.2 was clearly dominant in CPA (68), which is consistent with the prevalence of the T-current in fasciculata cell, as shown by electrophysiological recordings (45, 47). This also suggests a much stronger expression of T channels in CPA than in normal fasciculata cells. Moreover, in APA, only the T subunit Cav3.2 was positively correlated among patients with individual peripheral blood aldosterone levels (68). In CPA cases, however, this correlation was not observed with cortisol levels.

The electrophysiological properties of endogenous T-type calcium channels have been extensively investigated with the patch clamp technique in rat (19, 47, 74), bovine (15, 45, 74–76), and human (43, 72) glomerulosa and fasciculata cells (Table 3), and compared to those of recombinant Cav3.2 expressed in 293 cells (77). The threshold of current activation, the peak current voltage, and the half-maximal activation and steady-state inactivation ( $V_{1/2}$ ) were all within the range of voltages expected for low threshold T-type calcium channels. In other studies, using slow ramp depolarization, T current activation threshold in rat or bovine glomerulosa cells was determined to be at around  $-70$  mV and that for L-type current at  $-55$  mV (14, 78).

Interestingly, in spite of the fact that the same channel isoform as in glomerulosa cells (Cav3.2) is apparently expressed in zona fasciculata (71, 72), what has been confirmed by the high sensitivity of the current to inhibition by nickel in these cells, all parameters appear slightly shifted toward more depolarized potentials in fasciculata cells. The reason for this difference is unknown and could be in part related to the selection of the reviewed literature.

The functional relevance of these observations, as previously mentioned, resides in the fact that, in the absence of action potentials and upon limited cell depolarization induced by low physiological concentrations of potassium or AngII, the membrane potential is expected to rapidly reach the region of the T channel permissive window, allowing calcium influx, but not that of L channels. As a consequence, T-type channels appear to be the main contributors to *sustained* calcium influx in response to AngII or physiological concentrations of potassium in glomerulosa cells, because the resting potential of these cells is highly negative, close to the potassium inversion potential ( $-80$  mV), and the agonists do not depolarize the cell enough to reach the threshold of L-type channel activation (Figure 1B).

In contrast, it is probable that upon strong cell depolarization induced by supra-physiological concentrations of agonists, or

**TABLE 3 | Electrophysiological properties of T channels in adrenocortical cells.**

Cell/tissue	Species	$\alpha_1G/CaV3.1$	$\alpha_1H/CaV3.2$	Glomerulosa	Fasciculata	Reference
Adrenal zona glomerulosa	Bovine	–	Cloning/ISH (67)	–80 to $-60$ (mV)	–70 to $-50$	(15, 19, 43, 45, 47, 74–76)
Adrenal zona glomerulosa	Rat	–	ISH (67)	–40 to $-30$ (mV)	–30 to $-10$	(19, 43, 45, 47, 72, 74–76)
Adrenal zona fasciculata	Bovine		Northern blot (71)	–47 to $-23$ ( $V_{1/2}$ mV)	–50 to $-17$	(15, 19, 45, 47, 72, 76)
Adrenal zona fasciculata	Human		Northern blot (72)			
Adrenal cortex	Human		IH (35, 68)			
H295R cells	Human	RT-PCR (70)	RT-PCR (69, 70)	–74 to $-50$ ( $V_{1/2}$ mV)	–65 to $-43$	(15, 19, 45, 47, 72, 74, 76)

**TABLE 2 | Adrenal cortex expression of T-type calcium channel isoatypes.**

Cell/tissue	Species	$\alpha_1G/CaV3.1$	$\alpha_1H/CaV3.2$
Adrenal zona glomerulosa	Bovine	–	Cloning/ISH (67)
Adrenal zona glomerulosa	Rat	–	ISH (67)
Adrenal zona fasciculata	Bovine		Northern blot (71)
Adrenal zona fasciculata	Human		Northern blot (72)
Adrenal cortex	Human		IH (35, 68)
H295R cells	Human	RT-PCR (70)	RT-PCR (69, 70)

under particular conditions, T channels discharge altogether, generating pulses of action potentials able to secondly activate high threshold L-type channels (75). However, the relative contribution to steroidogenesis of calcium entering the cell through L-type channels in this mode appears quite limited, given the poor efficiency of dihydropyridines or other specific L-type channel calcium antagonists for inhibiting aldosterone or cortisol secretion, *in vivo* or *in vitro* (see below).

## PHARMACOLOGICAL DEMONSTRATION OF T-CHANNEL FUNCTION IN STEROIDOGENESIS

Adrenocortical T-type currents have been implicated early in the control of steroid biosynthesis (14, 38, 46, 79, 80), and this specific role for T channels has been extensively reviewed in the literature (12, 24, 81, 82). A direct demonstration of the crucial role played by these channels involved their inhibition by pharmacological agents. However, in spite of strong efforts to develop or isolate highly selective calcium antagonists during the last decades, only a few of the characterized pharmacological compounds appeared to preferentially affect T-type channels. Some of these drugs have been recently tested on heterologously expressed recombinant channels and sometimes display weak channel subtype selectivity. The reported potency ( $IC_{50}$ ) of these compounds on the various T channel isoforms is indicated in **Table 4**.

Inorganic polyvalent cations were among the first chemicals used to block T-type currents and their efficacy varies depending on the tissue examined, reflecting different channel isoform expression patterns (96). For example, nickel revealed to be useful for discriminating between  $Ca_v3.2$  ( $IC_{50} < 15 \mu M$ ) and  $Ca_v3.1$  ( $IC_{50} > 250 \mu M$ ). In contrast, the relative efficacy of zinc to inhibit T currents in various neuronal tissues (97, 98) suggests that this cation could have a higher affinity for  $Ca_v3.1$ .

Mibepradil, a derivative of verapamil initially developed as a promising antihypertensive drug (99), and kurtoxin, a purified scorpion toxin (92), have been shown to preferentially inhibit T currents than high voltage-activated currents. Unfortunately, at the same concentrations, mibepradil also attenuates potassium currents (100), and kurtoxin interacts with sodium channels (92).

therefore limiting their use for determining endogenous T channel function.

Dihydropyridines are widely used as antihypertensive drugs and are generally considered as specific L-type channel antagonists. However, some of these molecules also efficiently affect T channel activity. Indeed, while all these compounds inhibit L channels with similar potency (with  $IC_{50}$  around  $0.1 \mu M$ ), nifedipine and nicaldipine are also efficient T channel antagonists, while nimodipine and nifedipine are much more useful for discriminating between L and T currents (101). As expected, dihydropyridine efficacy varies from one cell type to the other (96). Interestingly, while their binding affinity on L channels is clearly voltage-dependent, this dependence is much less pronounced in their interaction with T channels (102), suggesting that the selectivity of these drugs should also vary from one cell type to the other in part because of the difference in their resting potential.

Many other substances, such as succinimide derivatives, phenytoin, pimozide, flunarizine, zonisamide, barbiturates, some anesthetics, and benzodiazepines, employed in clinic essentially for treating neurological or psychiatric disorders, have been shown to also affect T channel activity in non-neuronal cells (96). However, it is noteworthy that none of the above mentioned pharmacological agents is absolutely selective for T-type channels and most of them also affect other types of ionic channels, particularly high threshold calcium channels, when employed at slightly higher concentrations.

The role of T channels in the control of steroidogenesis has been thoroughly investigated by comparing the effects of different classes of calcium antagonists. Aldosterone secretion by adrenal glomerulosa cells, either *in vivo*, or *in vitro* in response to AngII or potassium, is efficiently inhibited by several T channel blockers, like tetrodotoxin (103, 104), mibepradil (19, 105), zonisamide (14), peripheral-type benzodiazepines (106), nickel (104), efonidipine (107, 108), or nicardipine (109), but not by highly selective L channel antagonists (14, 79, 107, 108). The relative lack of effect *in vivo* of several dihydropyridine antihypertensive drugs on circulating aldosterone concentrations in human has been previously discussed (110). In a recent meta-analysis (111), T channel antagonists appeared more efficient than L channel blockers for reducing aldosterone secretion and improving renal function, but not for decreasing blood pressure.

**TABLE 4 | Pharmacology of T-type calcium channel isoatypes.**

Agent (type/class)	$Ca_v3.1$	$Ca_v3.2$	$Ca_v3.3$	$Ca_v1.x$	Reference
Nickel (inorganic ion)	250–470	<b>5.4–13.0</b>	$IC_{50} (\mu M)$	>200	(53, 55, 83–86)
Mibepradil (antihypertensive)	<b>0.12–1.20</b>	1.0–1.4	1.5	>12	(51, 53, 84, 87–90)
Phenytoin (anticonvulsant)	75–140	8–192	?	>360	(83, 91)
Propofol (anesthetic)	20	27	?		(91)
Pentobarbital (barbiturate)	310	345	?	>600	(91)
Kurtoxin (scorpion toxin)	<b>0.015</b>	0.061	?	>10	(92)
Pimozide (neuroleptic)	0.035	0.054	0.030		(93)
Flunarizine (neuroleptic)	<b>0.53 (K<sub>D</sub>)</b>	3.55 (K <sub>D</sub> )	0.84 (K <sub>D</sub> )		(93)
Anandamide (cannabinoid)	4.15	<b>0.33</b>	1.10		(94)
Amiloride (diuretics)		156–167			(53, 86)
Curcumin (condiment)		20			(95)
TTA-P2 (antinociceptive)		0.35		>3	(75)

*Bold* indicates the channel isoform with the highest sensitivity to the most discriminant drugs.

An extensive pharmacological characterization of both T-type and L-type channels appears particularly relevant in cases of APA, not only for defining the treatment of hyperaldosteronism but also for diagnostic purposes. Indeed, if the cause of the disease is linked to a mutation on the potassium channel (KCNJ5), leading to sustained cell depolarization and calcium influx through T-type channels, most dihydropyridines will be expected to be quite inefficient for preventing aldosterone hypersecretion. In contrast, if a mutation on the L-type channel subunit  $\alpha 1D$  (CACNA1D) is causal (see above), and the mutant channel retains the same sensitivity to this class of calcium antagonists as the wild-type channel, the drugs will probably reduce aldosterone secretion. This hypothesis has been recently supported by the observation that a patient identified as affected by a *de novo* germline CACNA1D mutation responded particularly well to treatment with the dihydropyridine amlodipine, which normalized blood pressure and resolved ventricular hypertrophy in this patient (13).

Adrenocorticotrophic hormone-induced cortisol secretion from bovine adrenal fasciculata cells has been similarly shown to be inhibited by low nickel concentrations (45). A parallel inhibition of T-type currents in these cells with an  $IC_{50}$  of 20  $\mu M$  strongly suggests that the Cav3.2 channel isoform is also mostly responsible for the steroidogenic response in fasciculata cells (45, 47). This was confirmed by the observation that mibepradil also inhibited both T-type calcium current and cortisol secretion induced by ACTH in bovine fasciculata cells with  $IC_{50}$ 's of 1.0 and 3.5  $\mu M$ , respectively (76). Curcumin directly modulates the activity of several types of ion channels, including Cav3.2. In addition to a slight stimulatory effect on cortisol production from naive fasciculata cells, attributed to cell depolarization in response to potassium current inhibition, this compound has been shown to markedly reduce in parallel the large cortisol responses to both ACTH or AngII, and the Cav3.2 current (95). Similar results were obtained recently with the organic compound TT-P2, a recently developed potent and quite selective antagonist of T-type calcium channels (75).

The relative lack of selectivity of most of these calcium antagonists has been partially circumvented by combining the information collected with several unrelated compounds and comparing their individual action on both T currents and steroid production in a comprehensive way (112). Indeed, the degree of aldosterone inhibition by a large series of calcium antagonists has been shown

to be strongly correlated to the extent of T current inhibition, but not to that of L current inhibition (see below).

## MODULATION OF T-CHANNEL ACTIVITY BY HORMONES

The unique signaling function of T channels suggests that their activity must be rapidly modulated by various hormones and extracellular modulators. While such a control of channel activity has been extensively described for high voltage-activated calcium channels (113, 114), much less information is available concerning T channels.

Nevertheless, rapid changes of T channel activity, positive or negative, have been reported in response to various agonists in several tissues, including adrenal cortical cells (Table 5).

For example, the effect of angiotensin II (AngII) on several different calcium channels has been thoroughly reviewed (126). Basically, the hormone can affect the activity of voltage-operated calcium channels either indirectly, by inducing cell depolarization, or directly, by modifying the channel intrinsic electrophysiological properties.

## Modulation of Channel Activity through Membrane Depolarization

A modulation by AngII of the glomerulosa cell membrane potential has been known for a long time (22). Indeed, application of AngII on isolated rat glomerulosa cells induces a biphasic response: a brief hyperpolarization phase followed by a sustained and reversible decrease of membrane conductance accompanied by a depolarization from the resting potential, estimated in these cells to be around  $-80$  mV. This observation was confirmed by means of fluorescent probes for measuring membrane potential (127), or the patch clamp technique in the perforated patch configuration (19). As previously discussed, at low physiological concentrations, AngII did not evoke action potentials in naive glomerulosa cells, but only shifted the voltage by 10–20 mV. The hormone-induced cell depolarization observed in rat, bovine, and human glomerulosa cells was always due to the inhibition of potassium permeability (128), but some differences between species were seen in the characteristics of the potassium currents involved. Four distinct types

**TABLE 5 |** T-type calcium channel modulation by hormones in adrenal cortex.

Agonists	Tissue/cell type (species)	Effect	Cellular messenger	Reference
AngII	Adrenal glomerulosa (bovine)	+	Gi protein	(38, 42, 115)
AngII	Adrenal glomerulosa (bovine)	+	CaMKII	(116–118)
AngII	Adrenal glomerulosa (bovine)	-	PKC	(15, 119, 120)
ANP	Adrenal glomerulosa (bovine)	-	cGMP?	(40, 41, 121)
Dopamine	Adrenal glomerulosa (rat)	-	G prot ( $\beta\gamma$ )/PKA	(122, 123)
Serotonin	Adrenal glomerulosa (rat)	+	Gs/PKA	(124)
Endozepine	Adrenal (frog)	+	PKA	(125)
ACTH/AngII	Adrenal fasciculata (human)	+	n/a	(72)
ACTH/AngII	Adrenal fasciculata (bovine)	+	n/a	(46, 75)
ACTH/VIP	Adrenal fasciculata (rat)	+	n/a	(47)
Aldosterone	H295R (human)	+	Gene expression	(69)

of potassium channels were identified in rat and bovine glomerulosa cells (129), but AngII induced a substantial inhibition of only inward rectifier and delayed rectifier potassium channel activities in these species (130). This action was reversible and blocked by the AT1 receptor antagonist losartan (131, 132). In contrast, AngII has been shown to also inhibit a charybdotoxin-sensitive current in rat glomerulosa cells (133), suggesting that a large conductance calcium-activated (BK) potassium channel is also modulated by the hormone.

The modulation of potassium permeability by AngII has been also investigated in bovine glomerulosa cells by measuring the efflux of  $^{86}\text{Rb}$  or  $^{43}\text{K}$  (134–136). The authors found that after a transient activation that was sensitive to apamin, a blocker of calcium-sensitive (SK) potassium channels, the efflux was then inhibited in a concentration-dependent manner by AngII, and that changes in potassium conductance reflect changes in membrane potential. Thus, the transient hyperpolarization would be due to the activation of calcium-activated potassium channels responding to the transient elevation of cytosolic free calcium concentration ( $[\text{Ca}^{2+}]_c$ ) occurring upon calcium release from intracellular stores, while other types of potassium channels, such as the delayed rectifier, are later inhibited in a sustained fashion and are responsible for the depolarization phase. The latter effect of AngII on the potassium permeability being mimicked by diacylglycerol, a role for PKC in this process has been suggested (137). The functional link between potassium conductance modulation and aldosterone secretion was also supported by the demonstration that AngII-induced steroidogenesis is markedly affected by potassium ionophores like valinomycin (138), or by various potassium channel antagonists (139) or agonists (140), and, more recently, by the demonstration of the role of the KCNJ5 channel in maintaining a very negative resting potential within these cells and therefore a low basal aldosterone production (31).

Similar results have been obtained with adrenal fasciculata cells. Indeed, in bovine cells, AngII induces a biphasic response corresponding to a transient hyperpolarization followed by a sustained depolarization (141). The hyperpolarization phase appears to be due to the activation of calcium-dependent potassium currents, but also to chloride currents, the resting potential of fasciculata cells being less negative than that of glomerulosa cells. The identity of the conductance responsible for the fasciculata cell depolarization upon AngII challenge is less clearly defined. A cholera toxin-sensitive potassium current has been proposed to maintain the resting potential of bovine fasciculata cells and to be reduced by AngII and ACTH (142). However other mechanisms have been also suggested, such as the activation of a non-selective cationic conductance, the capacitative calcium influx triggered by calcium release from the stores, or the stimulation of the electrogenic  $3\text{Na}/1\text{Ca}$  exchanger by elevated  $[\text{Ca}^{2+}]_c$ .

It should be repeated here that the cell depolarization evoked by hormones like AngII or ACTH is sustained but very limited in amplitude. As previously discussed, the consequence therefore is a selective activation of low threshold T-type calcium channels, particularly in cells displaying a very negative resting potential. These particular conditions also explain the exquisite sensitivity

of glomerulosa cells to small variations of extracellular potassium, within its physiological range of concentrations.

However, it is important to remember that isolating glomerulosa and fasciculata cells from the adrenal cortex could prevent the occurrence of spontaneous or evoked electrical activity. The presence of action potentials in these cells would allow bringing the membrane depolarization transiently up to voltage values corresponding to L-channel activation and T-channel inactivation and, therefore, the relative contribution of low and high voltage channels in the steroidogenic response to secretagogues could have been wrongly estimated in isolated cells devoid of AP. Nevertheless, because T channels are apparently involved in the triggering and the propagation of the putative action potentials required for L channel activation (17), their function remains crucial even in cells able to work in an “excitable mode.”

## Modulation of Channel Activity through Protein Modification

The modulation of calcium channels by G proteins has been recognized for a long time as one on the main mechanisms employed by hormones to control the influx of calcium into their target cells (3, 114, 143). In most cases, G proteins that interact with calcium channels are sensitive to pertussis toxin treatment. This toxin, which efficiently blocks the activation of both  $\text{G}_i$  and  $\text{G}_o$  proteins, therefore represents a valuable tool for determining the intracellular signaling pathway leading to channel modulation. Moreover, the interaction between the receptor, the G protein and the channel is generally considered to be restricted to a small area of the membrane because it can be observed in the excised patch configuration of the patch-clamp technique, in contrast to other mechanisms involving diffusible molecules acting on channels located at distance from the receptor. It is important to realize that, depending on the mechanism involved, a given receptor can influence calcium influx very locally or broadly within the cell, a distinction that can have physiological consequences depending on the structural and functional organization of the cell (see below).

The modulation of T-type channels by AngII in bovine adrenal glomerulosa cells has been the object of some controversy. Indeed, early observations suggest that, in these cells, AngII increases the amplitude of the slowly deactivating and rapidly inactivating calcium currents, linked to the T-type channel activity (38). Further studies revealed that AngII, in fact, induces a shift of the channel activation curve toward more negative potential values (making the channel more prone to opening upon limited cell depolarization), an effect requiring the presence of GTP in the patch pipette and mimicked by the addition of GTP $\gamma$ S (42). These results, as well as the fact that AngII action is prevented by pertussis toxin treatment or introduction of a monoclonal antibody generated against recombinant  $\text{G}\alpha_i$  (115), are in favor of the involvement of a  $\text{G}_i$  protein in the activation of T channels. However, a direct interaction between the G protein and the channel is questioned by the observation that the hormone can increase the single channel opening probability in the cell attached configuration (42). Indeed, the latter observation strongly suggests that a rapidly diffusible

second messenger is generated by AngII and responsible for the modulation of T channels. In fact the possibility that, in adrenal glomerulosa cells, AngII modulates T channel activity by more than one single mechanism has been raised by the following observation: although activation of CaMKII by the rise of intracellular calcium also shifts the activation curve of T channels to more negative potentials, as demonstrated with pharmacological inhibitors of the kinase (116), these inhibitors only minimally reduced AngII action (115).

In contrast, in another series of experiments, AngII has been shown to markedly *reduce* T channel activity in bovine glomerulosa cells through a PKC-mediated shift of the channel activation curve to more *positive* values (15). This inhibition of T channels was mimicked by PMA (**Figure 1D**) and correlated with the reduction of the potassium-induced aldosterone production observed in the presence of the same agent. The functional link between the T channel activity and aldosterone production evoked by extracellular potassium was further demonstrated by the observation that PMA did not affect either L-type channel activity (120) or steroidogenesis triggered with a calcium ionophore (14). This paradoxical inhibitory action of AngII is somehow balanced by the cell depolarization evoked by the hormone and can have some physiological relevance. Indeed, aldosterone production induced by extracellular potassium, which is highly dependent on the activity of T-type channels, is reduced in a PKC-dependent manner by AngII in rat glomerulosa cells, putatively to prevent overstimulation in the presence of both agonists (144).

The multiplicity of the mechanisms involved by a same hormone like AngII to modulate T currents, sometimes in opposite directions, could reflect the presence of various T channel isoforms within the cell under specific culture conditions. It would be therefore relevant to determine whether the expression of the various channel isoforms is changing during development or upon specific pathological states, in order to better predict the hormone action on calcium influx.

Several other physiological modulators of steroidogenesis and/or their intracellular messengers have been reported to affect T channel activity in adrenocortical cells (**Table 5**). Moreover, activation of ectopic receptors for serotonin or GIP, illegitimately expressed in human fasciculata cells, and causing Cushing syndrome through stimulation of cAMP in ACTH-independent macronodular adrenal hyperplasia, has been also reported to enhance T channel activity through a PKA-dependent mechanism (145).

Finally, it is noteworthy that channel modulation, being through phosphorylation or binding to G proteins, not only affects the electrophysiological properties of the channel but could also modify channel interaction with cellular proteins involved in the calcium signal transduction.

## PUTATIVE MECHANISMS CONFERRING FUNCTIONAL SPECIFICITY TO T-CHANNELS IN STEROIDOGENESIS

As previously discussed, a close correlation has been observed in many instances between the production of aldosterone and the

activity of T channels (but not L channels) upon modulation with potassium (12, 38), AngII (144), ANP (40), or several pharmacological agents affecting PKC (15, 144). Similarly, the steroidogenic response of glomerulosa cells to prolactin (103) or serotonin (124) has been shown to rely, at least partially, on the modulation of T channels, as well as the inhibition of aldosterone secretion by dopamine (122, 123, 146). The production of glucocorticoids by fasciculata cells was also dependent on T-type calcium channels when stimulated either by ACTH (46) or by an endozepine triakontetraneuropeptide (125).

Because a specific role in the modulation of steroid biosynthesis and secretion has been attributed to T-type channels in adrenal cortical cells, the challenge then consisted in understanding how their molecular and electrophysiological characteristics and how the structural organization of the cell confer a functional advantage to these channels.

In a few experiments, both T-type and L-type calcium currents have been simultaneously determined under various discriminating pharmacological conditions, and this concomitantly with measurement of cytosolic calcium fluctuations in the same single cell (14). Potassium-induced cytosolic calcium elevation and aldosterone production in response to the same agents were also determined in parallel. Steroidogenesis appeared systematically linked to T channel (but not to L channel) activity while cytosolic calcium fluctuations depended principally on L current amplitude (112). This finding suggests dissociation between the cytosolic calcium signaling evoked by extracellular potassium and the rate-limiting steps of aldosterone biosynthesis, known to occur within the mitochondria (147, 148). According to our working hypothesis, in order to exert their preferential stimulatory action on steroid biosynthesis, T channels have to fulfill at least two prerequisites: (a) maintain a sustained influx of calcium into the cell, and (b) direct a part of this calcium toward the mitochondria.

The first point is addressed by considering the particular electrophysiological properties of T channels. Indeed, how can transient (T-type) calcium currents, which inactivate within a fraction of a second in excitable cells, support activation of steroidogenesis in response to potassium for minutes or even hours? As previously discussed, upon slight depolarization of the cell, and in the absence of action potentials, a sustained entry of calcium within a permissive window of voltage is possible due to the significant overlap between the activation and inactivation potential ranges of the channel (12, 15, 40). This steady-state current is tiny and barely detectable with the conventional patch-clamp technique, but, because it is *sustained*, in the long run it allows a large amount of calcium to enter and accumulate within the cell, at least sufficiently for activating steroidogenesis. The resting potential of the rat and bovine glomerulosa cells has been estimated to be around  $-80$  mV, a value close to the low voltage edge of this window. As a consequence, a small depolarization of the membrane, induced by a slight increase of extracellular potassium in the physiological range, may increase by several folds the steady-state influx of calcium through T channels (**Figure 1B**). While L-type channels also display a similar window of voltage, this is located at much more positive potentials, rarely reached under physiological conditions (19).

In addition to the establishment of a permissive window of voltage in which calcium can enter the cells near the resting potential, the specific function of T channels is probably also conferred by their localization within the cell membrane. Indeed, it should be noted here that, at supraphysiological cell depolarization, even in the absence of regenerative electrical activity, other types of calcium channels are simultaneously activated in the same cells, leading to an apparent redundancy of the calcium entry pathways. However, only a part of the functions attributed to T channels is actually shared by these other channels, which sometimes even exert opposite effects on steroidogenesis (79). This fact suggests that the cell is able to decipher the calcium signal specifically resulting from T channel activation. This is theoretically possible for two reasons: (1) the signal is confined due to the compartmentalization of calcium within the cell, and (2) there is a strict organization of the transduction mechanisms at the molecular level (149). In other words, the close environment of the channel (the proximity of specific calcium-binding protein and/or cellular organelles) dictates the function of calcium entering the cell at this precise location.

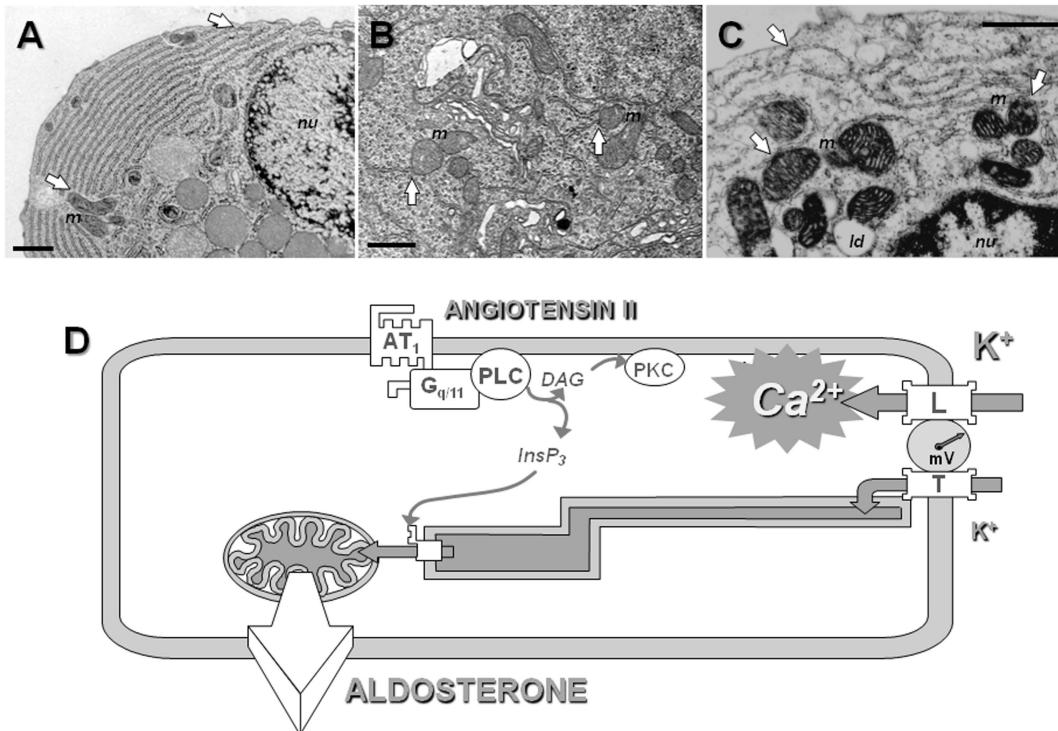
Mitochondria play a particular role in intracellular calcium homeostasis. Indeed, because of the very negative potential of their inner membrane (around  $-180$  mV), and the presence of a specific calcium uniporter, they avidly take up calcium when its concentration rises in the cytosol. Mitochondrial calcium influx is rapidly balanced by an equivalent efflux of calcium out of the organelle, which is dependent on sodium exchange, but only up to a given cytosolic concentration called the “mitochondrial set-point” (147). Above this set-point, fluxes are no more balanced and calcium rapidly accumulates within the mitochondrial matrix. This allows the cytosolic calcium concentration to come back down to this critical set-point. This property of mitochondria makes them very efficient cytosolic buffers, able to attenuate large calcium transients within the cell. At the same time, the mitochondria are also a target for calcium, particularly in steroidogenic cells. Indeed, steroids are synthesized by successive oxidations of a common precursor, cholesterol. Some important enzymatic steps in this process require molecular oxygen and, for this reason, must occur within the mitochondria. The rate limiting step in steroid biosynthesis is indeed the intramitochondrial conversion of cholesterol to pregnenolone, which requires the transfer of the substrate from the cytosol across the double membrane into the organelle. This early step in steroidogenesis is controlled by cytosolic and mitochondrial calcium levels as shown by several independent experimental observations: (a) rising ambient calcium stimulates aldosterone production in permeabilized glomerulosa cells and this response is prevented by the addition of ruthenium red, a blocker of the mitochondrial calcium uniporter (148), (b) intracellular calcium stimulates both intramitochondrial cholesterol transfer (150) and the expression of the steroidogenic acute regulatory (StAR) protein (151), which is required for efficient cholesterol transport into the mitochondria, and (c) cytosolic calcium fluctuations evoked by AngII are relayed and amplified within the mitochondrial matrix (152).

Interestingly, when mitochondrial calcium fluctuations induced by AngII were recorded at the *single organelle* levels with a GFP-derived probe (153), all mitochondria of the same cell did not respond simultaneously to the hormone, but calcium hot spots appeared in different regions of the cell, at different time points, and in an apparent stochastic manner (112). This observation suggests that the mitochondrial calcium response does not result from calcium uptake upon diffusion of calcium across the entire cytosol, but more probably upon local calcium release from intracellular stores located in proximity of the organelles.

Because calcium stimulates early, rate limiting steps of steroidogenesis occurring within the mitochondria, and because calcium entering the cell through T channels is not detected within the cytosol of bovine adrenal glomerulosa cells (14), we have proposed that calcium is directly conveyed from the plasma membrane to the mitochondria *via* the lumen of the endoplasmic reticulum, which would act as a sort of “*intracellular calcium pipeline*” (112, 149) (Figure 3).

This functional model is structurally supported by the distribution of the reticulum endoplasmic and the mitochondria within glomerulosa cells (and other cell types), with the presence of many “quasi synaptic” physical contacts observed between these organelles, as shown by electron microscopy, as well as by a close apposition of the reticulum with the plasma membrane in many points at the cell periphery (Figures 3A–C). This model therefore predicts specific interactions between T channels embedded within the plasma membrane and some components of the reticulum (Figure 3D). This proposal has been supported by the observation that disrupting physically the interaction between the reticulum and the cell membrane impairs the transduction of calcium signal elicited by potassium to the mitochondria, and therefore steroidogenesis in H295R cells (154). It is noteworthy that this organization of calcium influx, involving the local transfer of calcium entering the cell directly into the endoplasmic reticulum, is somehow reminiscent of the early “capacitative” model proposed by Putney (155), before revisiting it for taking into account results obtained with thapsigargin (156, 157), and it is quite intriguing that the  $\alpha_1\text{H}$  isoform of T channels, which is the most abundant in glomerulosa cells, has been proposed to mediate a capacitative calcium entry in non-excitable cancer cells (158).

The application of the pipeline model to the control of cortisol within fasciculata cells has not yet been specifically evaluated. The dichotomy between T-type and L-type channel functions is probably less obvious in these cells, where ACTH-induced steroid secretion is more efficiently inhibited by nifedipine and potentiated by Bay K8644, consistent with the involvement of L-type channels in the steroidogenic response (159, 160), in spite of the relatively low levels of expression of these channels reported in bovine fasciculata cells (45). However, species differences have been reported in term of dihydropyridine efficiency for reducing cortisol secretion (160). Clearly, additional investigations are still required for clarifying the functional relationship existing between calcium channels and limiting steps of cortisol biosynthesis, and therefore more specifically defining the role of each calcium channel.



**FIGURE 3 | The concept of intracellular calcium pipeline: a model for explaining the selective transport of calcium from the T channels into the mitochondria.** Electron microscopy reveals the presence of close apposition in many places of the endoplasmic reticulum (ER) with the plasma membrane or the mitochondria (white arrows), within various cell types, including rat parotid cells (**A**), rat spinal cord neurons (**B**), or bovine adrenal glomerulosa cells (**C**). Scale = 1  $\mu$ m; *m* indicates mitochondria, *nu* nucleus and *ld* lipid droplets. See Ref. (112) for additional information. (**D**). A hypothetical model for the cellular transport of calcium into mitochondria. At the pipeline filling site, T-type calcium channels and, to a lesser extent, L-type channels are activated upon cell depolarization by potassium or angiotensin II. Several experimental data suggest that calcium entering through T-type channels could be selectively pumped into the lumen of the ER, while calcium entering through L-type channels would be poured into the cytosol. At the pipeline delivery site, InsP<sub>3</sub> receptors are maintained in proximity of the mitochondria within “quasi synaptic” structures. Calcium released upon activation of the InsP<sub>3</sub> receptors, due to calcium overloading of the ER and/or to InsP<sub>3</sub> production by AT<sub>1</sub> receptor-activated PLC, is rapidly internalized into the very negatively charged matrix, through the mitochondrial inner membrane calcium uniporter. Intramitochondrial calcium elevation then stimulates limiting steps of aldosterone biosynthesis. AT<sub>1</sub>, angiotensin II receptor, type 1; G<sub>q/11</sub>, heterotrimeric G protein of the q/11 family; PLC, phospholipase C  $\beta$ ; PKC, protein kinase C; InsP<sub>3</sub>, inositol 1,4,5-trisphosphate.

## CONCLUSION

Two properties of T-type calcium channels appear to make them particularly suited for stimulating steroidogenesis: (1) their ability to activate and stay open upon slight depolarization of the cell, and (2) their capacity to convey calcium entering the cell directly to the mitochondria, where regulated steps of steroid biosynthesis occur.

The first property will be particularly favorable in cells displaying a very negative resting potential like glomerulosa cells, because small depolarization induced by physiological concentrations of agonists cannot reach the threshold for activation of L-type channels in these cells. The presence of these channels also renders the cells very sensitive to small changes of the extracellular potassium concentration, which participates, through the modulation of aldosterone secretion, to the maintenance of a normal kalemia. The fact that fasciculata cells have apparently a less negative resting potential probably could explain why steroidogenesis is globally less dependent on T-type channels in these cells and also partly involves L-type channel activity.

Because of calcium toxicity and polyvalence of calcium function, a strong confinement of this cation within the cell is necessary. The functional specificity of some channels is therefore also partly linked to their ability to transport calcium precisely to its site of action. In the case of steroidogenic cells, the mitochondrion is a main target for calcium. Because of the distance between the plasma membrane and these organelles in glomerulosa cells, the availability of intracellular calcium traffic through the lumen of the endoplasmic reticulum presents some advantages. Our working hypothesis is that T-type calcium channels are particularly efficient for stimulating aldosterone biosynthesis in glomerulosa cells because of their ability to load the endoplasmic reticulum with calcium. If the calcium pipeline model is true, one can predict that molecular structures must be maintained at both extremities and that T channels must interact physically with specific proteins. It is therefore conceivable that some mutations of the channel itself (or of the putative protein interacting with the channel) could prevent this interaction and therefore drastically reduce the ability of the channel to modulate

steroidogenesis in spite of the fact that its electrophysiological properties are preserved. However, to our knowledge, such a loss of function mutation responsible for a hypoadrenocorticism has not been described.

The gain of function mutations on CACHA1D observed in APA cells could, theoretically, also affect cortisol secretion from fasciculata cells. Indeed, Cav1.3 channels have been recently shown to be expressed in fasciculata cells and to participate to ACTH- and AngII-stimulated steroidogenic response (75). Similar mutations, shifting the channel permissive window to more negative voltages, would therefore be expected to increase cortisol secretion in an agonist-independent manner. Even if such mutations have not been formally demonstrated, the predicted high sensitivity of these tumors to inhibition by dihydropyridines would be worth of testing. Indeed, an important issue is whether the mutation status may influence the diagnosis, the treatment options, or the therapeutic outcome (32). Moreover, given the

ubiquitous role played by calcium in cell biology and the prevalence of the diseases in which a dysfunction of calcium channels has been involved, a detailed understanding of the mechanisms conferring functional specificity to a given class of channels, like T-type channels, provides relevant information for developing strategies in order to get more efficient pharmacological tools in order to treat diseases resulting from calcium deregulation.

## AUTHOR CONTRIBUTIONS

MR is responsible for the complete elaboration, writing, and correction of the present manuscript.

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# Aldo-Keto Reductases 1B in Adrenal Cortex Physiology

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Aldose reductase (AKR1B) proteins are monomeric enzymes, belonging to the aldo-keto reductase (AKR) superfamily. They perform oxidoreduction of carbonyl groups from a wide variety of substrates, such as aliphatic and aromatic aldehydes or ketones. Due to the involvement of human aldose reductases in pathologies, such as diabetic complications and cancer, AKR1B subgroup enzymatic properties have been extensively characterized. However, the issue of AKR1B function in non-pathologic conditions remains poorly resolved. Adrenal activities generated large amount of harmful aldehydes from lipid peroxidation and steroidogenesis, including 4-hydroxynonenal (4-HNE) and isocaproaldehyde (4-methylpentanal), which can both be reduced by AKR1B proteins. More recently, some AKR1B isoforms have been shown to be endowed with prostaglandin F synthase (PGFS) activity, suggesting that, in addition to possible scavenger function, they could instigate paracrine signals. Interestingly, the adrenal gland is one of the major sites for human and murine AKR1B expression, suggesting that their detoxifying/signaling activity could be specifically required for the correct handling of adrenal function. Moreover, chronic effects of ACTH result in a coordinated regulation of genes encoding the steroidogenic enzymes and some AKR1B isoforms. This review presents the molecular mechanisms accounting for the adrenal-specific expression of some AKR1B genes. Using data from recent mouse genetic models, we will try to connect their enzymatic properties and regulation with adrenal functions.

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## INTRODUCTION

The pituitary adrenocorticotrophic hormone is the main regulator of adrenal steroidogenesis acting through the cAMP-dependent protein kinase (PKA) signaling pathway. The fixation of ACTH second messenger, cAMP, on PKA regulatory subunits, leads to the release of catalytic subunits that in turn phosphorylate several targets, including transcription factors, such as the CCAAT enhancer-binding protein (C/EBP) and the cAMP response element-binding protein (CREB). Acting coordinately with tissue-specific factors such as the steroidogenic factor 1 (SF1), they stimulate expression of genes encoding enzymes and proteins involved in cholesterol metabolism, mobilization, and transport. In the adrenal cortex, steroidogenesis activation results in the generation of large amount of lipid aldehydes i.e., isocaproaldehyde (4-methylpentanal) produced by the CYP11A1 cholesterol side-chain cleavage and 4-hydroxynonenal (4-HNE), whose harmfulness has to be supported by coordinately regulated detoxifying enzymes. We and other groups have previously observed that the adrenal gland is one of the main sites of expression of both murine and human AKR1B proteins

(1–3). Aldose reductases are cytosolic monomeric enzymes, belonging to the aldo-keto reductase (AKR) superfamily. This superfamily encompasses more than 150 NAD(P)(H)-dependent oxidoreductases distributed in all prokaryotic and eukaryotic kingdoms, including yeast, plant, invertebrates, and vertebrates. They catalyze the reduction of carbonyl groups from a wide variety of substrates, such as aliphatic and aromatic aldehydes, ketones, keto prostaglandins, ketosteroids, and xenobiotics. Based on sequence identity, these proteins are divided in 15 families termed AKR1–AKR15, each family having less than 40% amino acid sequence identity with the others (4–6).

Among the AKR1 family, the aldose reductase subgroup-designated AKR family 1 member B (AKR1B) is one of the most characterized because of its involvement in human diseases, such as diabetic complications resulting from the ability of the former AKR family 1 member B1 (AKR1B1) to reduce glucose into sorbitol in a NADPH + H<sup>+</sup>-dependent manner during hyperglycemia. In addition to glucose conversion, AKR1B proteins display multiple other activities, including reduction of aldehyde group of by-products derived from lipid peroxidation or steroid synthesis, retinoids, xenobiotics, and prostaglandins (1, 7–9). The AKR1B subfamily includes proteins sharing a high degree of similarity (i.e., more than 65% of identity; **Table 1**). They are organized in two subgroups based on their ability to reduce glucose: aldose reductases (AR; AKR1B1–6) and aldose reductase-like proteins (ARLP; Akr1b7–19), respectively (4, 10–12). Their structure, enzymatic properties, and substrate specificities have been the subject of many studies (1, 5, 7, 13–17), emphasizing that in addition to their high percentage of identity, they also display redundant substrate specificities and overlapping expression patterns. These potential redundancies, then, complicate study of their distinct biological functions in specific physiological or pathological processes. Analysis of murine genetic models and identification of the mechanisms regulating their expression are the necessary steps to complete our understanding in AKR1Bs biological function.

This review will provide an updated integrative view on specific regulations of human and murine aldose reductase genes with enzymatic and functional data in the adrenal gland

physiology [further information on AKR1Bs in other endocrine functions is reviewed in Ref. (18)]. Since several studies allowed identification of some murine and human aldose reductase genes as orthologs, common features will be presented for each corresponding pair, and individual isoform specificities will be discussed.

## HUMAN AND MURINE AKR1B GENE SYNOPSIS

### Human AKR1B Genes

Three human AKR1B genes organized in tandem on chromosome 7q33–35 have been identified (**Table 2**; **Figure 1**): *AKR1B1* [human aldose reductase (19)], *AKR1B10* [also designated as HSI reductase: human small intestine reductase (1, 7)], and *AKR1B15* (20). *AKR1B1* seems to be ubiquitously expressed, whereas *AKR1B10* expression was only reported in small intestine, colon, liver, thymus, and adrenal gland (1, 7). *AKR1B15* gene was recently characterized and identified as closely related to the *AKR1B1* and *AKR1B10* cluster on chromosome 7 (**Figure 1**). *AKR1B15* undergoes alternative splicing, giving rise to two protein isoforms, designated as *AKR1B15.1* and *AKR1B15.2*, expressed in thyroid gland and testis, respectively, and both in adipose tissue and placenta. *AKR1B15.1* transcript encodes a putative protein sharing 68 and 91% sequence identity with *AKR1B1* and *AKR1B10*, respectively (21). Both *AKR1B15* transcripts were absent from human adrenal (20).

### Murine Akr1b Genes

Four murine *Akr1b* genes have been described: *Akr1b3* (murine aldose reductase), *Akr1b7* [previously named MVDP: mouse vas deferens protein (22)], *Akr1b8* [previously named FR-1: fibroblast growth factor (FGF)-related protein (23)], and *Akr1b16* (21) (**Table 2**). Murine aldose reductase genes are located on chromosome 6 (locus 6B1), and their tandem arrangement suggests (as for the three human *AKR1Bs*) that these four genes arise from an ancestral gene duplication event (10, 12) (**Figure 1**). Several studies had allowed identification of murine *Akr1b3*, *Akr1b8* as the orthologs of the human *AKR1B1* and *AKR1B10*, respectively. However, this phylogenetic analysis between human and mouse AR has some limits and will be commented below. *AKR1B1*, *Akr1b3*, and *Akr1b16* are rather ubiquitously expressed (11, 21), whereas *Akr1b7* and *Akr1b8* exhibit a restricted tissue distribution. Indeed, *Akr1b7* is detected in vas deferens, adrenal glands, gonads, intestine, white adipose tissue, eye, liver, and kidney (2, 22, 24–26) and *Akr1b8* in testis, heart, adrenal glands, intestine, and liver (2, 11, 23).

## AKR1B IN ADRENALS: BETWEEN DETOXIFICATION AND PARACRINE SIGNALING

### *Akr1b3/AKR1B1: Expression Pattern and Relevant Functions*

In studies using murine adrenal cell lines (Y1 adrenocortical cells and MPC862L chromaffin cells), we found that *Akr1b3* protein

**TABLE 1 | Comparison of protein sequence identity (%) between human (h) and murine (m) AKR1B proteins.**

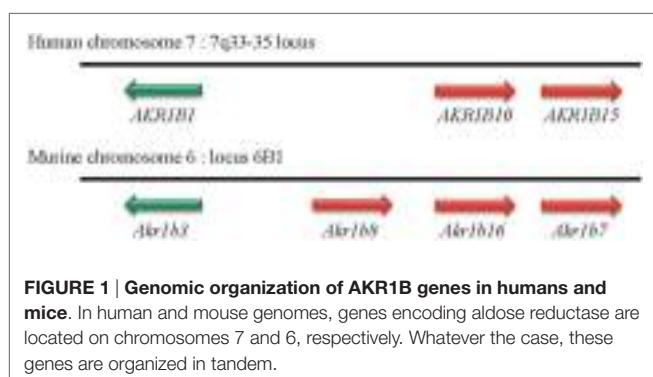
	AKR1	Aldose reductases			Aldose reductase-like proteins			
		B1	b3	b7	b8	B10	B15	b16
Aldose reductases	B1 (h)	100	85.8	71.6	70.7	71	65.1	70.7
	b3 (m)	85.8	100	69.7	69.4	70.7	65.4	70.3
Aldose reductase-like proteins	b7 (m)	71.8	69.7	100	82.3	79.8	72.1	84.8
	b8 (m)	70.7	69.4	82.3	100	82.3	74.9	82.6
	B10 (h)	65.1	70.7	79.8	82.3	100	86	82.9
	B15 (h)	65.1	65.4	72.1	74.9	86	100	76.2
	b16 (m)	70.7	70.3	84.8	82.6	82.9	76.2	100

The protein sequences were aligned using the Clustal Omega program. The amino acid sequences used to achieve this multiple alignment correspond to the accession numbers listed in **Table 2**.

The Gray shade highlights the necessary 100% identity between 2 identical protein sequences.

**TABLE 2 | Human and murine members of the aldo-keto reductase B1 subgroup (AKR1B).**

Symbol	Common associated protein designation	Species	ARN	Protein
AKR1B1	Aldose reductase	Human	NM_001628	NP_001619
Ark1b3	Aldose reductase	Mouse	NM_009658	NP_033788
Akr1b7	Mouse vas deferens protein (MVDP)	Mouse	NM_009731	NP_033861
Akr1b8	Fibroblast growth factor-regulated protein 1 (FR-1)	Mouse	NM_008012	NP_032038
AKR1B10	Small intestine reductase (HSI)	Human	NM_020299	NP_064695
AKR1B15	Aldose reductase (putative)	Human	NM_001080538	NP_001074007
Akr1b16	Aldose reductase (putative)	Mouse	NM_172398	NP_765986



accumulates in both adrenal cortex and medulla. Moreover, *in vivo* and *ex vivo* hormonal manipulations demonstrated that unlike the other murine Akr1b7 and Akr1b8 isoforms, Akr1b3 is expressed in the whole gland (27). Finally, cAMP stimulation failed to modulate *Akr1b3* expression in Y1 cell line, confirming that *Akr1b3* was insensitive to ACTH signaling (28) (Table 3).

Considering their enzymatic properties and expression levels in murine adrenal cortex, Akr1b7 and Akr1b8 are considered as the main isocapraldehyde reductase and 4-HNE reductase, respectively, while Akr1b3 could rather participate in the elimination of these toxic compounds in basal physiological conditions (27–29). Moreover, Akr1b3 also displays 9-,11-endoperoxide reductase activity that, when coupled to COX-1 (cyclooxygenase type 1), allows prostaglandin F2 $\alpha$  (PGF $2\alpha$ ) synthesis in adrenal cortex and medulla (see below).

Despite all these evidences upon Akr1b3 involvement in both lipid aldehyde detoxification and PGF $2\alpha$  synthesis, *in vivo* *Akr1b3* gene invalidation (*Akr1b3*<sup>-/-</sup> mice) did not highlight any phenotype related to adrenal gland (30, 31). The lack of adrenal dysfunction in *Akr1b3*<sup>-/-</sup> mice may result from the redundancy of enzymatic properties carried by the other murine isoforms expressed in the gland (Akr1b7 and Akr1b8) that could then compensate Akr1b3 loss.

**TABLE 3 | Localization and regulation of AKR1B in adrenal gland.**

Isoforms	Localization	Analyses	Control by ACTH/cAMP	Transcriptional regulators	Reference
<b>Human</b>					
AKR1B1	Cortex	IHC, RNA master blot	+	n.d.	(1, 27)
AKR1B10	Adrenal <sup>a</sup>	RNA master blot	n.d.	n.d.	(1)
AKR1B15	n.d.	n.d.	n.d.	n.d.	–
<b>Mouse</b>					
Akr1b3	Cortex and medulla	WB	No	No	(27, 28)
Akr1b7	Cortex	NB, WB, IHC, ISH	+	Sp1, C/EBP $\beta$ , SF1	(2, 27, 28, 55, 58, 67)
Akr1b8	Cortex	WB, ISH	No	No	(2, 27, 28)
Akr1b16	n.d.	n.d.	n.d.	n.d.	–

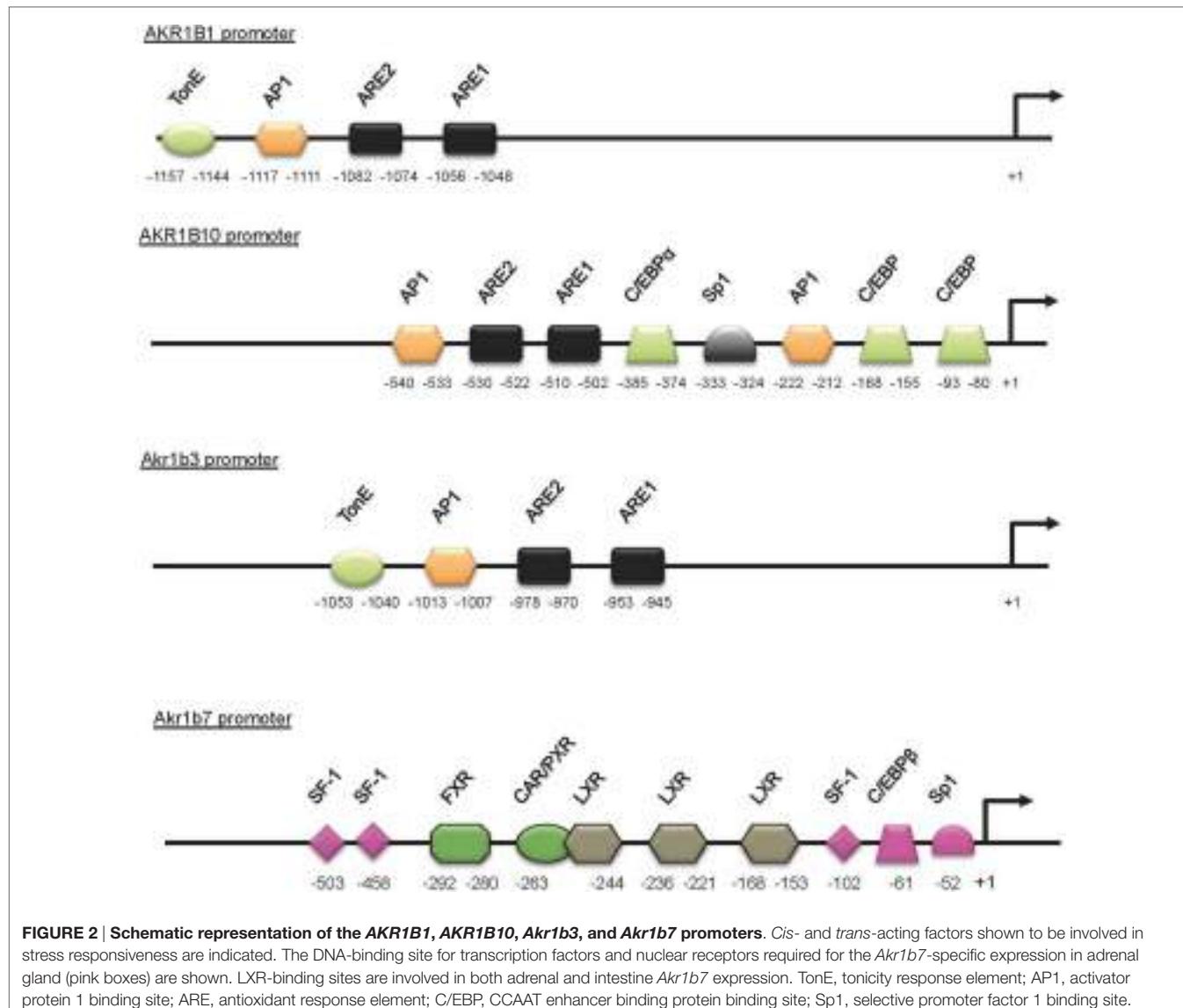
<sup>a</sup>Intra-adrenal tissue localization was not specified.

n.d., not determined; NB, Northern Blot; WB, Western Blot; RT-PCR, reverse transcription-polymerase chain reaction; ISH, *in situ* hybridization; IHC, immunohistochemistry; n.d., not determined.

In human adrenal gland, AKR1B1 transcripts have been initially detected using RNA master blot (1) (Table 3). Thereafter, using immunohistochemistry, we confirmed those results and demonstrated that AKR1B1 expression pattern is restricted to the cortex of adrenal gland (27). Treatment of the human adrenocortical tumor cells NC1-H295 with forskolin (adenylyl cyclase inducer) allowed us to suggest that similar to the murine isoform Akr1b7, AKR1B1 expression was sensitive to ACTH (32) (Table 3). The molecular mechanisms and *cis*-acting elements responsible for ACTH/cAMP responsiveness of AKR1B1 gene have not been investigated to date (Figure 2). Analysis of AKR1B1 expression in stress-related disorders was not explored to date. Moreover, analysis of adrenal samples from Cushing's disease (ACTH-producing pituitary tumor) revealed unchanged mRNA levels of AKR1B1 gene (32).

Based on its enzymatic properties, AKR1B1 has long been considered as the sole isocapraldehyde reductase in the human adrenal gland (33) (Figure 3A; Table 4). Interestingly, unlike murine Akr1b7 isoform, NADPH-dependent isocapraldehyde reductase activity carried by AKR1B1 was inhibited by tolrestat, a potent and specific aldose reductase inhibitor belonging to the carboxylic acids group of AR inhibitors (13, 29, 33). We demonstrated that AKR1B1 was also able to convert PGH $_2$  into PGF $2\alpha$  (34) (Figure 3C; Table 4). This 9-,11-endoperoxide reductase activity is also strictly NADPH-dependent and inhibited by tolrestat.

We observed that in the human adrenal gland, AKR1B1 and the cAMP-inducible COX-2 isoform were co-localized in steroidogenic cortical cells (27) (Table 3). Then, we can consider that human adrenal cortex has the potential to produce PGF $2\alpha$  in response to ACTH surge. The shared properties of human AKR1B1 and mouse Akr1b7, such as hormonal regulation and reductase activity toward common substrates, prompted us to postulate that they can be considered as functional orthologs



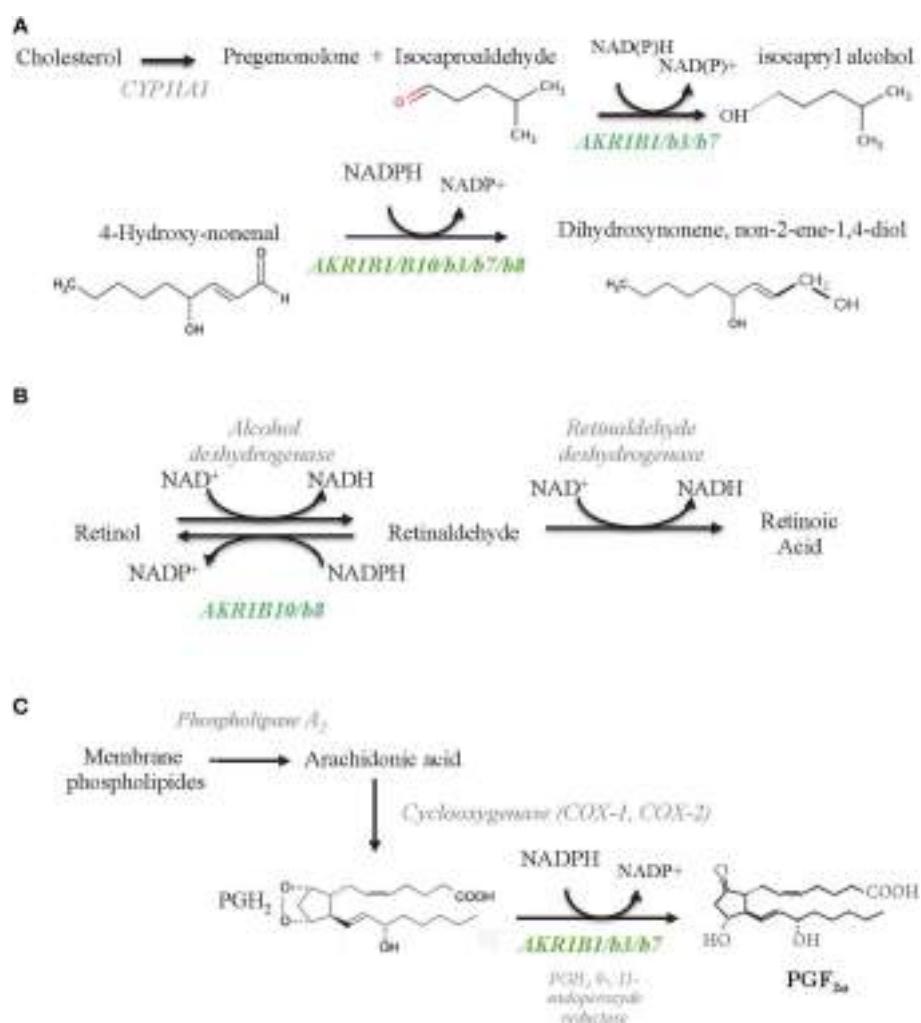
**FIGURE 2 | Schematic representation of the AKR1B1, AKR1B10, Akr1b3, and Akr1b7 promoters.** Cis- and trans-acting factors shown to be involved in stress responsiveness are indicated. The DNA-binding site for transcription factors and nuclear receptors required for the *Akr1b7*-specific expression in adrenal gland (pink boxes) are shown. LXR-binding sites are involved in both adrenal and intestine *Akr1b7* expression. TonE, tonicity response element; AP1, activator protein 1 binding site; ARE, antioxidant response element; C/EBP, CCAAT enhancer binding protein binding site; Sp1, selective promoter factor 1 binding site.

at least in the adrenal cortex (32). Moreover, increased levels of AKR1B1 transcripts in human were observed in adrenocortical adenomas harboring glucocorticoid autonomous hypersecretion (32). The possibility that AKR1B1 prostaglandin F synthase (PGFS) activity could participate in an intra-adrenal feedback loop between endocrine activities of cortex and medulla in human adrenal gland remains to be explored.

Given the high expression of AKR1B1 in the adrenal cortex, we evaluated alterations in its expression in association with human adrenal disorders. The relative abundance of AKR1B1 mRNA was decreased in adrenocortical carcinomas (ACC) when compared to benign tumors, Cushing's hyperplasia, or normal adrenals (32). These data were reinforced by de Reyniès et al., who demonstrated that decreased AKR1B1 expression was associated with malignancy using an unsupervised clustering analysis of the human adrenal tumors transcriptome (35). This identified AKR1B1 as a potential negative marker for adrenocortical malignancy.

## Akr1b8/AKR1B10: Phylogeny, Regulation, and Enzymatic Specificities

*Akr1b8*mRNA was initially detected in both fetal and adult murine adrenal cortex but remained undetected in the medulla by *in situ* hybridization (2). In fibroblasts as well as in adrenocortical Y1 cell line, *Akr1b8* was previously shown to be controlled by the FGF and phorbol myristate acetate (PMA) (23, 28). On the contrary, dexamethasone-induced ACTH suppression did not influence *Akr1b8* mRNA and protein accumulation (27), suggesting that its biological function did not seem to be related to the ACTH-dependent steroidogenic activity present in adrenocortical cells (Table 3). Interestingly, in digestive tract organs, such as liver and small intestine, *Akr1b8* gene was recently showed to be a target of NF-E2-related factor2 (Nrf2), which mediates transcriptional response to oxidative stress by binding to antioxidant response element (ARE) sites (36). As expected, *Akr1b8* expression was downregulated in digestive tract from Nrf2 knockout mice.



**FIGURE 3 | Schematic diagrams of enzymatic reactions illustrating AKR1B involvement toward lipid aldehyde detoxification (A), retinoids (B), and prostaglandins (C) metabolism.** All the indicated substrates and enzymatic activities are supposed to coexist in the adrenal glands, but their relative importance in adrenal function remains unknown so far. AKR1B isoforms endowed with each of these activities are indicated in green.

**TABLE 4 | Kinetic constants of AKR1B toward isocapraldehyde, 4-hydroxy-nonenal, retinaldehyde, and prostaglandin H<sub>2</sub>.**

Substrates	Isocapraldehyde		4-hydroxy-nonenal		All-trans-retinaldehyde		Prostaglandin H <sub>2</sub>	
	K <sub>m</sub> (μM)	k <sub>cat</sub> (s <sup>-1</sup> )	K <sub>m</sub> (μM)	k <sub>cat</sub> (s <sup>-1</sup> )	K <sub>m</sub> (mM)	k <sub>cat</sub> (min <sup>-1</sup> )	K <sub>m</sub> (μM)	V <sub>max</sub> (nmol/min/mg)
<b>Human</b>								
AKR1B1	1 <sup>a</sup>	0.66 <sup>a</sup>	716 <sup>d</sup>	0.84 <sup>d</sup>	1.1 <sup>e</sup>	0.35 <sup>e</sup>	1.9 <sup>f</sup>	44 <sup>f</sup>
AKR1B10	330 <sup>b</sup>	0.72 <sup>b</sup>	31 <sup>d</sup>	2.01 <sup>d</sup>	0.6 <sup>e</sup>	27 <sup>e</sup>	No activity <sup>f</sup>	
AKR1B15	n.d.		2.2 <sup>g</sup>	0.08 <sup>g</sup>	1 <sup>g</sup>	5.4 <sup>g</sup>	n.d.	
<b>Mouse</b>								
Akr1b3	62 <sup>c</sup>	1.3 <sup>c</sup>	665 <sup>g</sup>	0.82 <sup>d</sup>	1.0 <sup>e</sup>	0.52 <sup>e</sup>	26 <sup>f</sup>	26 <sup>f</sup>
Akr1b7	320 <sup>c</sup>	0.38 <sup>c</sup>	256 <sup>g</sup>	0.1 <sup>d</sup>	0.5 <sup>e</sup>	0.02 <sup>e</sup>	53.4 <sup>f</sup>	53.4 <sup>f</sup>
Akr1b8	71 <sup>c</sup>	0.03 <sup>c</sup>	230 <sup>g</sup>	3.18 <sup>d</sup>	2.1 <sup>e</sup>	0.05 <sup>e</sup>	No activity <sup>f</sup>	
Akr1b16	n.d.		n.d.		n.d.		n.d.	

n.d., not determined.

Reference: <sup>a</sup>(33); <sup>b</sup>(40); <sup>c</sup>(28); <sup>d</sup>(11); <sup>e</sup>(12); <sup>f</sup>(34); <sup>g</sup>(14).

However, no adrenal phenotype was described in these mice, and neither oxidative stress nor Nrf2 have been involved in adrenal Akr1b8 expression so far.

Previous studies demonstrated that some AKR1B proteins showed efficient catalytic activity for the reduction of the abundant and highly reactive lipid-derived aldehyde 4-hydroxy-2-nonenal

and the phospholipid aldehydes (17). In particular, Akr1b8 displays the most efficient phospholipid aldehyde and HNE-reductase activity in mouse tissues (17, 28, 37) (**Figure 3; Table 4**). All these enzymatic data suggest that in the adrenal cortex, Akr1b8 isoform could be devoted to detoxify aldehyde lipids abundantly accumulated in this tissue (38). Furthermore, expression of an antisense RNA directed against *Akr1b7* suppressed isocaproaldehyde reductase activity in adrenocortical Y1 cells without any alteration of Akr1b8 protein accumulation (29). In view of its enzymatic features and constitutive expression, Akr1b8 is unlikely to be the principal isocaproaldehyde reductase in the adrenocortical gland (28). *Akr1b8* gene disruption in mice led to reduced lipid synthesis and diminished proliferation of colonic epithelial cells but had no evident effect on general appearance, body weight, and reproduction. However, *in vivo* adrenal Akr1b8 physiological role remains to be examined since *Akr1b8* gene disruption first report did not notice evident effect on the adrenal physiology (39).

Whether *Akr1b8* and human *AKR1B10* gene can be considered as ortholog is still a matter of debate since they share high sequence identity, and proteins display several close structural and enzymatic properties (11) (**Tables 1 and 4**). In contrast to *Akr1b8*, *AKR1B10* gene expression is not controlled by FGF (12). Although *AKR1B10* mRNA was initially detected in adrenal glands using a human RNA Master Blot, to date, there is no more information available on its *in situ* localization and transcriptional control in this organ (1). Moreover, *AKR1B10* gene expression pattern only partially overlaps that of *Akr1b8*, since *AKR1B10* transcripts are absent from heart, lung, or testis (7, 11).

Comparative studies demonstrated that AKR1B10 exhibits higher 4-HNE-reductase activity than AKR1B1, while lower than the murine *Akr1b8* (11, 16). *Ex vivo* studies revealed that both human AKR1B1 and -B10 also share the ability to reduce isocaproaldehyde (1, 40). Nevertheless, in a comparative enzymatic study, Hara and colleagues showed that AKR1B1 had a more effective isocaproaldehyde reductase activity than AKR1B10, suggesting that in human steroidogenic organs, the latter was unlikely to play a major role in the detoxification of steroidogenic by-products (41).

The AKR superfamily has been added as a novel group of cytosolic enzymes that could contribute to retinoid-redox conversion. Based on their cofactor specificity (NADPH), AKR work in the reductive direction (42). Retinol (vitamin A) and its derivatives, retinaldehyde and retinoic acid (RA), are essential for the growth and maintenance of many tissues. RA is a key molecule in the development of different vertebrate organs by promoting cell differentiation and apoptosis. The control of retinaldehyde levels is essential in the regulation of RA synthesis and therefore of its signaling role. Once synthesized from β-carotene through the β-carotene 15,15' monooxygenase 1 (BCO1), retinaldehyde has two alternative fates, its irreversible oxidation to RA (metabolism fate) by the aldehyde dehydrogenases (ADH) or its reduction back to retinol (storage fate) by the retinaldehyde reductase activity of AKR (43) (**Figure 3B; Table 4**). Comparative *in vitro* enzymatic studies on murine and human AKRs have fairly evidenced that among AKR1B proteins, AKR1B10 is so far the only retinaldehyde

reductase with the highest  $k_{cat}$  value for the retinaldehyde reduction (11, 12, 44). *Ex vivo* AKR1B10 overexpression in different cell systems demonstrated its contribution in increasing retinol production (45, 46). In rodent, previous data evidenced that normal adrenal gland may function as an important site of retinoic acid synthesis involving class I- and IV-ADH, thus furthering retinaldehyde metabolism rather than its storage through the AKR activity (47). According to its expression, whether the well-established retinaldehyde reductase activity of AKR1B10 is operated in normal human adrenal physiology remains to be explored.

*AKR1B10* expression was initially characterized in hepatocellular carcinoma and subsequently found to be altered by tumorigenesis process in several other organs (7, 48–50). Moreover, *AKR1B10* expression was associated with smoker's non-small cell lung carcinomas (48) and was suggested to be involved in drug resistance (51). A putative mechanism by which the activity of AKR1B enzymes could promote tumor growth is the conversion of retinaldehyde to retinol resulting in RA deprivation and blockage of its differentiating effect, promoting cell proliferation and fostering tumorigenesis (43). Furthermore, recent studies have shown that in breast cancer cells, AKR1B10 associates with acetyl-CoA carboxylase-alpha (ACCA), the rate-limiting enzyme of *de novo* synthesis of long-chain fatty acids, and blocks its ubiquitination and proteasome degradation. Long-chain fatty acids are the building blocks of biomembranes and the precursor of lipid second messengers, playing a critical role in cell growth and proliferation (52). The AKR1B10-mediated regulation on ACCA stability represents a novel regulatory mechanism, in which AKR1B10 promotes cell survival *via* modulating lipid synthesis, mitochondrial function, oxidative stress, and carbonyl levels (53).

Adrenocortical carcinomas are very aggressive and rare malignant tumors with poor prognosis (54). Microarray analysis was used to seek molecular predictors of malignancy and survival in a large cohort of unilateral adrenocortical tumors (<http://www.ebi.ac.uk/arrayexpress>, experiment E-TABM-311). Unsupervised clustering analysis allowed robust discrimination of malignant and benign tumors. On the basis of this analysis, *AKR1B10* expression was not found to be associated with the ACC group (35).

## Akr1b7: Expression Profile, Detoxification Function, and Paracrine Action

High levels of *Akr1b7* transcripts were initially observed by *in situ* hybridization in fetal and adult murine adrenal cortex but were undetectable in the medulla (2). We confirmed these results by immunohistochemistry experiments, which allowed us to further restrict major *Akr1b7* expression to the *zona fasciculata* (55). *In vivo*, ACTH suppression with dexamethasone treatment resulted in a marked decrease of *Akr1b7* mRNA levels that were restored when the treated mice were injected with exogenous ACTH. This ACTH/cAMP-induced *Akr1b7* transcription was blocked by a PKA inhibitor (H89) in the murine adrenocortical ATC and Y1 cell lines (55, 56).

In the adrenal gland, basal and ACTH-induced expressions of *Akr1b7* gene depend on three SF1 response element (SFRE) and

on other *cis*-elements located in the upstream promoter region (**Figure 3**). Using transgenic mice and transfection experiments, we characterized a cryptic SFRE 102 bp upstream of the transcription start site that supports basal expression of *Akr1b7* in the adrenal cortex. Among the two other SFREs identified further upstream, the site at  $-458$  was a *bona fide* SFRE, essential for both basal and cAMP-stimulated promoter activity. The last SFRE, an Sp1, and C/EBP $\beta$  binding sites, respectively, localized at positions  $-503$ ,  $-52$ , and  $-61$  are all involved in cAMP responsiveness (57, 58).

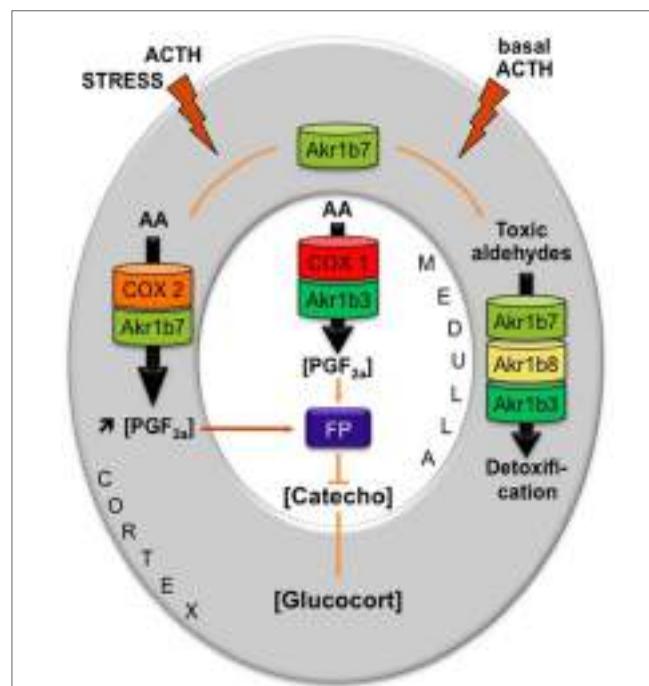
AKR1Bs are capable to handle the large amount of isocaproaldehyde, a toxic by-product coming from the cholesterol side-chain cleavage during the initial step of steroid biosynthesis. Furthermore, isocaproaldehyde accumulation decreased viability of Y1 cells (29). Although *Akr1b3*, *Akr1b7*, and *Akr1b8* all were able to reduce isocaproaldehyde, the two former seemed to be the more efficient reductases for this substrate (28). However, the silencing of *Akr1b7* gene was sufficient to abolish the cAMP-induced isocaproaldehyde reductase activity in Y1 cells. Therefore, *Akr1b7* was the main enzyme in charge of isocaproaldehyde detoxification in the adrenal cortex (29). Altogether, these data showed that in the adrenal cortex, ACTH not only controls expression of enzymes synthesizing steroids but also of proteins scavenging toxic by-products derived from steroidogenesis.

Madore et al. established that the bovine 20 $\alpha$ -hydroxysteroid dehydrogenase, AKR1B5 was responsible for PGF $_{2\alpha}$  synthesis in the endometrium (59). Thereafter, we demonstrated by *ex vivo* studies, that AKR1B1, *Akr1b3*, and *Akr1b7* were also able to reduce PGH $_2$  into PGF $_{2\alpha}$ , whereas *Akr1b8* and AKR1B10 were devoid of this PGF $_{2\alpha}$  synthase activity (**Table 4**). Due to their recent discovery, this 9-,11-endoperoxide reductase activity has not been investigated yet for *Akr1b16* and AKR1B15. Moreover, their enzymatic constants suggested that AKR1B1, *Akr1b3*, and *Akr1b7* had a higher 9-,11-endoperoxide reductase activity than the other PGF synthases already described (34). Prostaglandins are paracrine/autocrine signal molecules produced from a common precursor, PGH $_2$ , which is derived from arachidonic acid by COX-1 or COX-2. Unlike COX-1, which is a constitutively expressed enzyme, COX-2 is not expressed in most organs under basal conditions but can be stimulated by inflammation and various mitogenic factors (60). Following these observations, we carefully examined the PGF $_{2\alpha}$  biosynthetic pathway in the adrenal gland (27).

Prostaglandin F2 $\alpha$  was produced by both cortical (steroidogenic cells) and medullary (chromaffin cells) tissue of the adrenal gland. In primary adrenocortical cell culture, PGF $_{2\alpha}$  release was induced 2.5-fold by ACTH treatment. This secretion was correlated with ACTH responsiveness of both COX-2 and *Akr1b7*. Using *ex vivo* gain- and loss-of-function strategies, we demonstrated the pivotal role of *Akr1b7* in ACTH-induced PGF $_{2\alpha}$  release, and it is functionally coupled with COX-2. In the adrenal medulla in which *Akr1b7* was not expressed, PGF $_{2\alpha}$  was produced from the coordinated activities of *Akr1b3* and COX-1. Adrenal expression of PGF $_{2\alpha}$ -specific receptor (FP) was restricted to the chromaffin cells, suggesting that both autocrine and paracrine mechanisms (within the medulla and between steroidogenic and medulla cells, respectively) were relaying PGF $_{2\alpha}$  action. In agreement with this

hypothesis, we demonstrated that PGF $_{2\alpha}$  repressed both basal and glucocorticoid-induced dopamine release in the chromaffin cell line MPC862L. Comparison of the PGF $_{2\alpha}$ -responsiveness of isolated cells and whole adrenal tissue cultures showed that PGF $_{2\alpha}$ -mediated repression of glucocorticoid release is an indirect mechanism relying on a decrease in catecholamine secretion, which in turn decreased cortical steroidogenesis.

These functional data led us to propose an intra-adrenal feedback loop in which adrenal endocrine activities are regulated through the involvement of AKRs [**Figure 4** and Ref. (18)]. Surprisingly, however, the absence of *Akr1b7* *in vivo* did not



**FIGURE 4 |** Proposed model integrating dual functions of aldose reductases in the regulation of mouse adrenal endocrine functions [adapted from Ref. (18)]. AKR1B family is endowed with enzymatic activities that at least ensure two metabolic functions: the production of PGF $_{2\alpha}$  and the detoxification of lipid aldehydes. Arachidonic acid (AA) is metabolized into PGH $_2$ , the precursor of all prostanoids, by COX enzymes and then converted into PGF $_{2\alpha}$  by the 9-,11-endoperoxide reductase activity of PGF synthases of the AKR1B family. AKR1B are also capable to reduce toxic lipid aldehydes resulting from the particularly high prooxidant activities of P450 cytochromes in adrenocortical cells (i.e., isocaproaldehyde and 4-HNE). The mechanism integrating these dual functions was the following: (1) In basal conditions, PGF $_{2\alpha}$  is constitutively secreted by chromaffin cells (by the coupling of COX-2 and *Akr1b3*), thus regulating catecholamine production and also limiting their paracrine action on steroidogenesis. (2) During a stress situation, ACTH transiently induces COX-2 and *Akr1b7* expression, which results in PGF $_{2\alpha}$  production inside the cortex. PGF $_{2\alpha}$  produced in the cortex then represses catecholamine release by the medulla via a paracrine action on its FP receptor. Decreased catecholamine release in turn reduces the effect of ACTH on glucocorticoids production (27). After the stress response has ended, COX-2 returns to undetectable levels. The coupling between *Akr1b7* and COX-2 does not take place. Then, *Akr1b7* together with *Akr1b8* and *Akr1b3* function only as cortical detoxifying enzymes of the harmful aldehydes produced under chronic/basal stimulation of steroidogenesis. Catecho, catecholamine; Glucocort, glucocorticoids.

affect basal adrenocortical function as illustrated by normal glucocorticoid plasma levels in *Akr1b7<sup>-/-</sup>* mice (61). Indeed, these mice displayed an obese phenotype that did not rely on adrenal dysfunction but on the lack of *Akr1b7*-dependent production of PGF<sub>2α</sub> within the stromal vascular adipose tissue (3, 61). Adrenal expression of *Akr1b3* and *b8* is not affected in knockout mice and since they all share redundant enzymatic activities regarding detoxification of lipid aldehydes (see *Akr1b3/AKR1B1: Expression Pattern and Relevant Functions and Akr1b8/AKR1B10: Phylogeny, Regulation, and Enzymatic Specificities*), the remaining isoforms can compensate the absence of *Akr1b7* at least in basal conditions. Importantly, *Akr1b7* is the only one out of the three adrenal isoforms to be ACTH-responsive (27) and also the most abundantly expressed (3). Taken together, these hallmarks would predict that physiological importance of *Akr1b* enzymes in adrenal function should be rather explored under stress conditions during which scavenging capacity of constitutive (and less abundant) isoforms should be exhausted.

## FUTURE DIRECTIONS

Fighting against oxidative stress is a challenging but mandatory task for adrenocortical cells. Indeed, P450 cytochrome systems involved in steroidogenesis, and in particular glucocorticoid production, contribute very significantly to oxidative stress by cellular reactive oxygen species (ROS) production (62). The redox imbalance due to excessive ROS production can cause adrenal damage that may progress to severe insufficiency, including familial glucocorticoid deficiency (FGD). Therefore, adrenal cortex is well supplied in antioxidant defense genes encoding enzymes of the superoxide dismutase (SOD), glutathione peroxidase (GPX), and peroxiredoxin (PRDX) families

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[for review, see Ref. (63)]. Since the precursor works of Feige's group showing the ACTH responsiveness of *SOD2* expression, the expected coordinated regulation of antioxidant enzymatic systems and P450s systems producing prooxidant by-products has been somewhat neglected (64). Accordingly, AKR1B enzymes family may be considered as antioxidant defense genes. Among these, ACTH-responsive ones, e.g., *Akr1b7* and *SOD2* genes, could participate in the adaptive response of antioxidant systems of adrenal cortex under stress conditions. Disturbance in redox homeostasis was the most recently discovered cause of FGD and mutations in *NNT* gene (nicotinamide nucleotide transhydrogenase) account for about 10% of cases (65). *NNT* ensures mitochondrial NADPH supply that is essential to ROS detoxification enzymatic systems. Then, it would be interesting to know whether adrenal-specific deficit in AKR1B enzymes could contribute to cortical damage or adrenal insufficiency in mice carrying a spontaneous *Nnt* mutation (66). This could provide the proof of principle for studying the physiological contribution of AKR1B family in detoxifying function in steroidogenic organs and beyond.

## AUTHOR CONTRIBUTIONS

All authors listed have made substantial, direct, and intellectual contribution to the work and approved it for publication.

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# Adrenocortical Gap Junctions and Their Functions

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Adrenal cortical steroidogenesis and proliferation are thought to be modulated by gap junction-mediated direct cell–cell communication of regulatory molecules between cells. Such communication is regulated by the number of gap junction channels between contacting cells, the rate at which information flows between these channels, and the rate of channel turnover. Knowledge of the factors regulating gap junction-mediated communication and the turnover process are critical to an understanding of adrenal cortical cell functions, including development, hormonal response to adrenocorticotropin, and neoplastic dedifferentiation. Here, we review what is known about gap junctions in the adrenal gland, with particular attention to their role in adrenocortical cell steroidogenesis and proliferation. Information and insight gained from electrophysiological, molecular biological, and imaging (immunocytochemical, freeze fracture, transmission electron microscopic, and live cell) techniques will be provided.

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## INTRODUCTION

The adrenal is a complex gland that is histologically and functionally two tissues, the cortex and medulla, within a connective tissue capsule (1). Not only the two regions of the adrenal gland are from different embryonic origins but also the cells of the medulla are composed of cells that have a neuroendocrine function, while the cells of the cortex are epithelial cells that function in endocrine metabolism. There is evidence that cells of both the cortex and the medulla are regulated by cell–cell communication of regulatory molecules through membrane channels, called gap junctions (2–8). The efficiency of the adrenal gland, as well as other endocrine glands, to respond to stimulation is thought to depend not only on hormone receptor interaction but also on intercellular communication through gap junctions.

Gap junctions in the adrenal gland, as in other tissues, provide low-resistance pathways for the direct intercellular exchange of small molecules (9, 10). In early years, gap junctions and cell communication were mainly studied with electron microscopic (11–13), electrophysiological (14), and fluorescent dye transfer (15, 16) techniques. More recently, however, the proteins (connexins) composing the gap junction pore have been identified, and the tissue distribution of the different connexin family members has been demonstrated (17–20). Further, the molecular details of the

**Abbreviations:** 18-alpha/beta GA, 18-alpha/beta glycercerhetic acid; ACTH, adrenocorticotropin hormone; DbcAMP, dibutyryl cyclic adenosine monophosphate; FSH, follicle-stimulating hormone; GFP, green fluorescent protein; LAMP, lysosomal-associated membrane protein; MAPK, mitogen-activated protein kinase; PKA, protein kinase A; PKC, protein kinase C; RNAi, RNA interference; SBAC, bovine adrenal cortical cell line; SW-13, Scott white human adrenal cortical tumor cell line; TEM, transmission electron microscopy; TPA, 12-O-tetradecanoylphorbol-13-acetate; Y-1, mouse adrenal cortical tumor cell line.

assembly of connexins into functional gap junction channels, the involvement of kinases in this assembly, and the architectural arrangement of connexins into functional pore complexes have been described (21, 22).

In this review, we present a historical summary of gap junctions in the adrenal cortex from their discovery with imaging and electrophysiological techniques to current studies of the connexin types, distribution, abundance, and turnover. The role of gap junctions in the adrenal cortical response to adrenocorticotropin (ACTH) will be discussed, and the fundamental concepts and implication of gap junctions in steroidogenesis, proliferation, and cancer will be analyzed. Finally, gap junction-mediated cross talk between the cells of the adrenal cortex and medulla will be discussed, as it relates to adrenocortical function. We will begin with a brief review of gap junctions in the adrenal cortex.

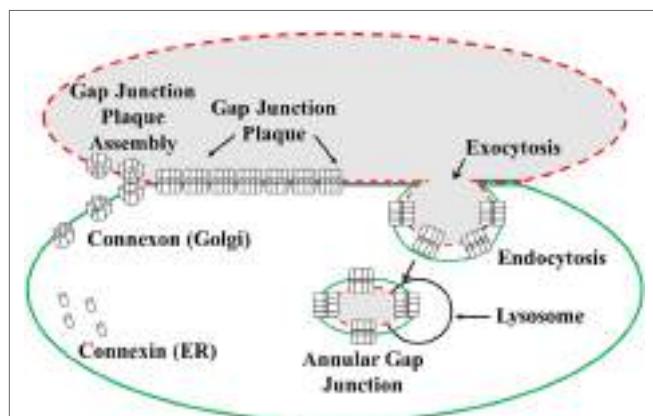
## CHARACTERIZATION OF GAP JUNCTIONS IN THE ADRENAL CORTEX

Gap junctions occur between the membranes of two closely opposed cells and are characterized by the pairing of intramembranous connexin complexes across a 2–4 nm gap (Figures 1 and 2) (23). The first transmission electron microscopic images of gap junction plaques in the adrenal gland were acquired from fetal rat adrenals in 1970 (24) and more extensively from adult glands of a number of animals in 1972 (11, 25). Gap junctions in the adrenal resembled those of other tissues and were positively identified by the presence of the characteristic pentalaminar membrane and the 2–4 nm gap separating the membrane of two

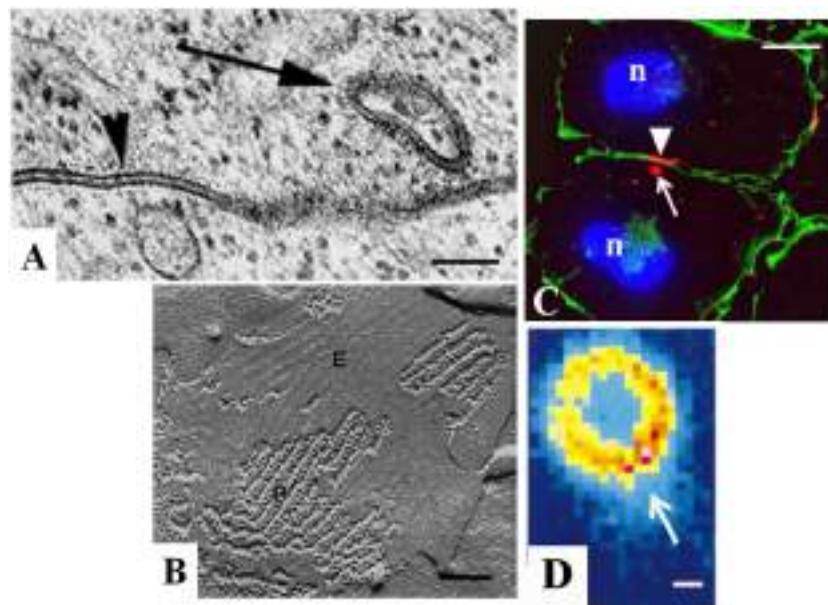
adjacent cells (Figure 2A). The area of membrane covered by gap junction plaques (plaque size) and the packing pattern of adrenal cortical cell gap junction channels could be obtained with freeze-fracture electron microscopy (11). With this technique, the adrenal cell membrane bilayer was split in the hydrophobic plane (26), and the typical protoplasmic (P)-fracture face and extracellular (E)-fracture face distribution of membrane particles was used to identify gap junctions (Figure 2B). Gap junctions could be distinguished by the observed clustering of 8.5 nm particles on the P-fracture face and pits on the E-fracture face (27, 28) (Figure 2B). With freeze-fracture techniques, the size of gap junction plaques was demonstrated in the rat adrenal cortex to be larger and more abundant than those found in most other tissues of the body, especially those plaques in the area of the adrenal cortex near or juxtaposed to the medulla (24). Gap junction plaques have now been described in the adrenal from a vast number of different mammalian species (6, 29–31). An important advantage of freeze-fracture imaging was the capacity to confirm that the adrenal cortical gap junctions were composed of aggregates of intramembranous particles that were paired with one another across the intercellular space. The observation that these paired particles extended across the gap, seen with transmission electron microscopy, provided structural evidence for the existence of channels, which could serve as means for movement of molecules between cells. However, the critical role of gap junctions as channels for communication was established by experiments in which it was demonstrated that hepatocytes and myocardial cells only passed current if gap junctions were observed and if gap junctions were not present, current did not pass between cells (32).

The movement of current was first demonstrated in the adrenal cortex with electrophysiological techniques in experiments, which used adult and fetal rabbit adrenal gland slices (10). Although such studies confirmed that material was capable of communicating between cells in the adrenal, the function of this movement was not addressed in these early studies. In addition, information on the possible size of molecules that could move between the gap junction channels was provided from dye transfer studies in which molecules smaller than 1,000 Da could communicate between cells while larger molecules were excluded (16, 33, 34). Based mainly on the early electrophysiological and dye transfer studies, as well as transmission electron microscopic and freeze-fracture images, it was hypothesized that gap junctions allowed the passage of molecules between adjacent cells, and thus, modulate adrenal cortical cell population growth and hormonal response (8, 35). However, the molecules composing and regulating the gap junction pore and, more important, the definitive role of gap junction-mediated communication in adrenal cortical function remained to be defined.

Our knowledge concerning the role of gap junctions, in general, was greatly enhanced by the isolation and characterization of the connexin proteins that composed gap junction pores. It has become clear that the particles seen with freeze-fracture electron microscopy are the gap junction channels and that each gap junction channel consists of 12 connexin molecules, 6 from one cell docked to 6 in the adjacent cell. The clustering of these channels forms the gap junction plaque (27, 36).



**FIGURE 1 | Illustration of the formation and degradation of gap junction plaques and annular gap junctions.** Connexin proteins synthesized in the endoplasmic reticulum (ER) oligomerize to form connexon complexes. The connexons are transported to the cell surface and inserted into the plasma membrane where they form hemichannels. These hemichannels can dock with hemichannels of an apposing cell and cluster to form a gap junction plaque, characterized by a 2–4 nm gap between the two cell membranes. Gap junction plaques are removed from the cell surface through endoexocytosis, which results in the formation of an annular gap junction. The annular gap junction is then degraded through lysosomal proteolysis [modified from Ref. (130)].



**FIGURE 2 | Characterization of gap junctions in SW-13 adrenocortical tumor cells.** The size, location, and structure of gap junction plaques (arrowheads) and annular gap junctions (arrows) have been determined with (A) transmission electron microscopy, (B) freeze-fracture electron microscopy, (C) immunofluorescence, and (D) confocal microscopy. The protoplasmic (P) and extracellular (E) fracture faces are shown in the freeze-fracture replica of the gap junction plaque in (B). Cell borders are defined by cortical actin (green) in (C). Note the lumen of the annular gap junction revealed with confocal microscopy in (D). n, nucleus. Bars: (A) 100 nm, (B) 60 nm, (C) 10  $\mu$ m, and (D) 0.3  $\mu$ m. [(A) from Ref. (48), (B) from Ref. (130), (C) from Ref. (131), and (D) from Ref. (132)].

The production of antibodies directed at the connexin proteins allowed the detection of gap junction protein types with immunofluorescence and western blot techniques (19, 20, 37). In humans, there are 21 different connexin types that differ by their amino acid sequences and molecular weights (38). Connexin 43 (Cx43) gap junction protein was demonstrated as the major, if not only, connexin gap junction protein type in the adrenal cortex (5, 30, 31, 39, 40). While investigators have also reported Cx26, Cx32, and Cx50 in humans (41), their expression has not been reported in other mammals (3, 6, 7, 42). It should be noted, however, that of the 21 known connexin family members (38), only 6 have been extensively evaluated in the adrenal cortex, and it is possible that others will be detected with further analysis.

With immunofluorescence microscopy, the distribution of Cx43 could be quickly detected and its distribution more reliably compared with adrenal zones than with conventional transmission electron microscopy (5, 30, 40, 43). The Cx43 gap junctions in the adrenal cortex appear as small puncta or longer plaques on the cell surface between contacting cells (Figures 2C,D). The cells of the adrenal cortex are polyhedral in shape, and their three-dimensional relationship to one another has been revealed with serial section transmission electron microscopy (44) and scanning electron microscopy of freeze-cracked adrenal glands (45). Based on the three-dimensional imaging provided by these techniques, gap junctions are thought to form at smooth “contact” sites and on facets on microvilli of cells (45). The facets from two cells were observed to be in close apposition, and it is here as well

as at the smooth sites on the cell body that the gap junctions most likely are formed (45).

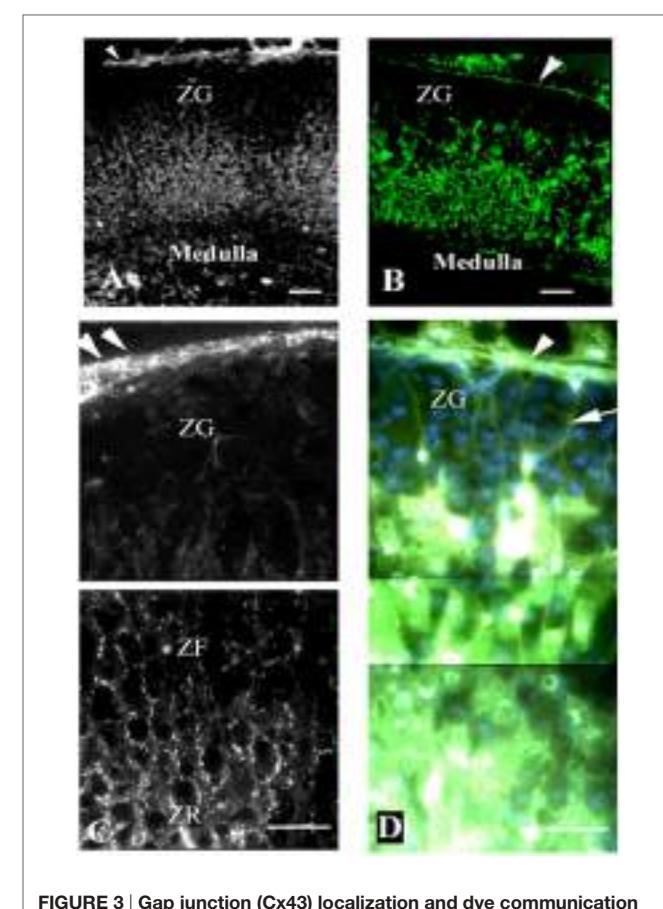
In addition to the typical surface gap junction plaques, cytoplasmic gap junction vesicles have also been reported in the adrenal gland and in adrenal cell cultures with both transmission electron microscopic and immunocytochemical techniques (46–49). It has been confirmed with live cell imaging of cells expressing green fluorescent protein construct (Cx43-GFP) that these annular gap junction vesicles form from a unique process in which the gap junction membrane of one cell is internalized into the cytoplasm of the adjacent cell to form a double-membraned vesicle composed of gap junction protein. This internalization process occurs from the central regions of the gap junction plaques or, in some cases, the entire gap junction plaque membrane is internalized. Once internalized, the annular gap junction vesicle is degraded (50–52), and thus, this is a method for disassembling gap junction plaques and regulating communication. In contrast, gap junction plaque assembly occurs by the addition of new gap junction channels at the gap junction plaque periphery (53). Mechanisms for controlling cell-cell communication are thought to involve both the assembly of gap junction plaques at the cell surface and the disassembly of these plaques by an internalization process that results in annular gap junction vesicle release into the cytoplasm. The capacity to specifically analyze connexin protein distribution within specific compartments is particularly critical for the study of tissues, such as the adrenal, which have cells that express different steroidogenic enzymes and respond differently to stimuli, based on their specific zonal locations.

## GAP JUNCTION DISTRIBUTION IN THE ADRENAL CORTEX

In the human, the adrenal cortex can be divided into three morphologically and functionally distinct zones: the outer most zone, zona glomerulosa, and the inner zones, zona fasciculata (ZF) and zona reticularis (ZR). These zones are composed of cells that express different steroidogenic enzymes and thus produce different steroid hormones. Specifically, the ZF produces glucocorticoids, and the ZR produces androgens. Both of these inner zones secrete hormones in response to ACTH. The outer zone, however, produces aldosterone in response to changes in sodium, potassium, and the peptide hormone, angiotensin II.

Just as the three zones of the adrenal have been demonstrated to be morphologically and functionally distinct, the level of expression of gap junction protein in the three zones of the adrenal has been demonstrated to differ using immunocytochemistry (**Figures 3 and 4**). Specifically, a differential distribution of Cx43 has been reported in the adrenal of human (4, 42) as well as a host of other mammals including rat (3, 5), mouse (40, 54), guinea pig (55), rhesus monkey (40), and cow (4, 8). Specifically, little or no Cx43 gap junction protein was detected between adrenal cells in the zona glomerulosa. In contrast, numerous gap junction plaques were evident at areas of cell-cell contact in the inner cortical areas, such as the ZF and ZR (**Figure 3**). The distribution of gap junctions once speculated from transmission electron and freeze-fracture microscopy (29, 55) could be confirmed and, more importantly quantitated, while simultaneously validating which zone was being viewed. With computer-assisted microspectrofluorometric image analysis, it was demonstrated that there were twice as many gap junctions per area between cells in the ZR than between cells in the ZF (6). While the number of gap junctions differed, there were no significant differences in the average size of gap junctions in the rat ZF compared with those in the ZR.

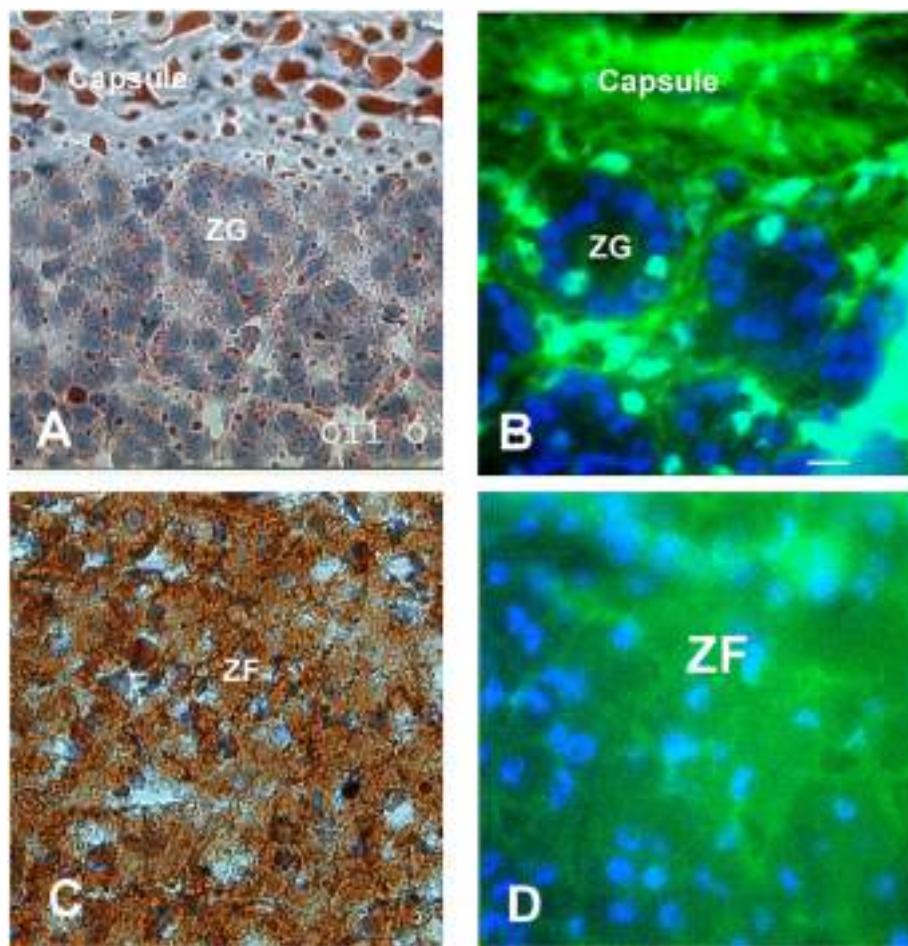
Consistent with the immunocytochemical quantitative analysis, when the expression of Cx43-encoded mRNA was analyzed with Northern Blot techniques in samples prepared from the capsule of the adrenal gland, which contain adherent zona glomerulosa cells, there was little gap junction RNA detected. In contrast, samples prepared from decapsulated glands, which contained mainly the ZF/ZR cells, had an abundance of gap junction RNA. It is thought that the detection of small amounts of Cx43-encoded mRNA in the zona glomerulosa with Northern Blot and almost no Cx43 gap junction proteins detected with immunocytochemical analysis may reflect possible contamination of the zona glomerulosa-enriched samples with cells from the ZF. In addition, some of the mRNA Cx43 detected with Northern Blot in the zona glomerulosa-enriched samples is thought to come from the presence of fibroblastic cells from the connective tissue capsule and trabeculae that project into the substance of the cortex in the sample (**Figure 3D**). In fact, these trabeculae within the substance of the gland were revealed with immunocytochemistry. To increase the accuracy for comparing the distribution of gap junctions with adrenal cortical zones, Oil Red O staining, which detects lipid droplets in the cytoplasm of cells in the ZF and ZR (56), has been used. The Oil Red O stain is much less abundant in zona glomerulosa cells that have few, if any, lipid droplets (56).



**FIGURE 3 | Gap junction (Cx43) localization and dye communication in the intact adrenal gland.** Immunohistochemical localization of Cx43 gap junction proteins revealed extensive staining in the zona fasciculata (ZF) and zona reticularis (ZR), while there was limited staining in the zona glomerulosa (ZG) [left panels: **(A,C)**]. Correspondingly, lucifer yellow dye communication between cells was more abundant in the inner zones of the adrenal cortex (ACTH responsive areas) than in the outer zone [right panels: **(B,D)**]. Capsule (arrowheads), connective tissue trabecule (arrow), and Cx43 (white puncta). Bars: **(A,B)** 50  $\mu$ m and **(C,D)** 30  $\mu$ m [modified from Ref. (30)].

The pattern of Oil Red O staining distinguished the cells of the zona glomerulosa from cells of ZF and ZR and substantiated that the cells which lack Cx43 gap junction staining are indeed cells of the zona glomerulosa (**Figure 4**) (30).

In parallel to the differential localization of gap junction plaques is the pattern of dye communication in the intact adrenal cortex. Specifically, lucifer yellow dye moved extensively in the inner cortex while dye communication was limited to the capsular projections of connective tissue between the cells of the zona glomerulosa (**Figures 3B,D and 4B,D**) (5, 30). Dye communication was not observed between cells of the zona glomerulosa. This lack of dye movement would be consistent with the observed absence of Cx43 protein in immunofluorescence studies and would further support the suggestion that other connexin family members do not assemble into functional gap junctions in this zone. In the inner two cortical zones, where gap junctions were plentiful, dye was transferred (30). It should be noted that while Cx43 was found in the inner two zones, it is possible that connexin types, other than Cx43, may be located



**FIGURE 4 | Communication in the intact adrenal cortex.** Oil Red O staining was used to distinguish the lipid-rich zona fasciculata and zona reticularis from the zona glomerulosa (**A,C**). Lucifer yellow dye was transferred between the fibroblasts of the connective tissue capsule and trabeculae; however, communication was absent between cells of the zona glomerulosa (**B**). The cells of the inner two cortical zones, particularly those in the zona fasciculata, exhibited extensive dye communication (**D**). Note the Lucifer yellow and Oil Red O staining are diffuse and seen throughout the cytoplasm, which somewhat obscures the cell boundaries and nuclei. Bars: (**A–D**) 30  $\mu$ m [modified from Ref. (30)].

in these inner two zones since not all of the 21 known connexin family members have been evaluated. In addition, there is a possibility that other, yet to be discovered, connexin type could be detected in the future. It is speculated that while cells of the zona glomerulosa, for reasons yet to be determined, are less dependent on cell-cell communication, the communication of a regulatory molecule between cells of the inner two layers modulates their functions.

## ROLE OF GAP JUNCTIONS IN ADRENAL CORTICAL FUNCTION

### Steroidogenesis

Gap junctions, for years, have been suggested to play a key role in a number of physiological phenomena (3, 57, 58). However, in early years, this proposed role was solely based on the observations that dye and current could move between cells that had gap junctions and not on experimental evidence that demonstrates

actual physiological responses. Adrenal cortical cells were one of the first endocrine cell types in which a direct relationship was established between gap junctions and physiological responses (59). Furthermore, the findings in the adrenal cortex have been used to lend support to the speculations that gap junctions influence physiological events in other cell types (60, 61).

The first set of experiments, which demonstrated functional gap junction-mediated communication, were studies in which adrenal cells were placed into culture with ovarian granulosa cells. It is well documented that cells of the inner adrenal cortical zones possess specific receptors for ACTH and, when bound to its receptor, ACTH elicits a number of responses (62, 63), including an activation of cAMP-dependent protein kinase A (PKA) (59, 64), an increase in steroid synthesis (7, 65, 66), and a decrease in proliferation of cells in culture (2, 8, 67). In the adrenal/granulosa coculture populations, a cytochemical method was used to specifically localize free catalytic subunits (C) of PKA at subcellular, light microscopic levels of resolution

(59, 68). In these studies, heterotypic, adrenal/granulosa, cell pairs were demonstrated to form gap junctions and ACTH treatment activated PKA in the adrenal cell populations (59). Moreover, ACTH stimulation not only, as expected, activated PKA in the adrenal cells but also with time, within the granulosa cells that were in contact with adrenal cells. The ovarian granulosa cells lacked ACTH receptors but had receptors for follicle-stimulating hormone (FSH), which activates PKA (69). Treatment of the coculture cell population with FSH activated PKA in the granulosa cells and in the adrenal cells that formed cell contacts with granulosa cells (**Figure 5**). Importantly, adrenal cell secretion of steroid hormones was increased (59). The finding that hormone treatment stimulated bidirectional exchange of a signal that initiated PKA activation in adrenal/granulosa coculture cell populations provided compelling evidence that the hormone-induced intercellular communication, which activated PKA, could serve a biological role.

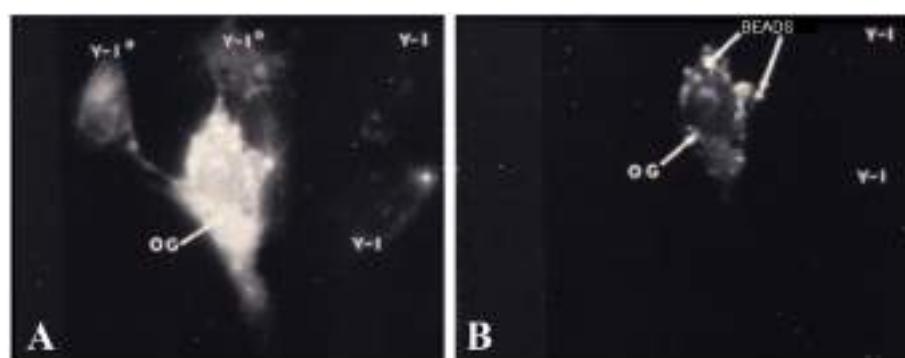
In further support of the biological role of gap junctions in adrenal cortical function, inhibition of gap-junctional communication with 18-alpha glycyrrhetic acid (18-alpha GA) treatment decreased the steroidogenic responsiveness of bovine adrenal cells in culture to ACTH (4). Specifically, it was demonstrated that 18-alpha GA treatment decreased the steroidogenic response to a submaximal dose of ACTH but did not decrease steroidogenesis if cells were treated with saturating doses of ACTH (4). A similar decrease in ACTH-stimulated steroidogenesis was demonstrated in bovine and human cell lines and in rat primary cell populations treated with 18-alpha GA (8). The effects of treatment with this glycyrrhetic acid derivative were also substantiated in experiments in which Cx43 antisense transfection techniques were used to inhibit gap junction-mediated communication (8). Cell populations expressing Cx43 antisense grew faster, had decreased capacity for cell-cell communication, and had a diminished steroidogenic responsiveness to ACTH treatment compared with null transfected control populations (8). This decrease in ACTH-mediated steroidogenesis following gap junction inhibition by either chemical treatment or Cx43 antisense expression is consistent with the concept that

gap junction-mediated communication is a necessary factor in hormonal response. It has been suggested that gap junction communication of cAMP signaling, and subsequent PKA activation in the recipient cell, may increase the efficiency of hormone response by facilitating amplification of ACTH signaling throughout the population. This would be particularly useful at submaximal stimulation doses of the hormone, as suggested by Munari-Silem, when only a small population of the cells may be responding to ACTH (4).

In addition to 18-alpha, 18-beta glycyrrhetic acid (18-beta GA) has also been used in a number of studies to inhibit gap junction communication (70–72). Huang and colleagues found that treatment with 18-beta GA increased the basal levels of steroidogenesis secreted in rat primary adrenal cell cultures (73). These findings are interesting given that the increased steroidogenesis appears to be PKA-independent and would suggest a direct effect of the 18-beta GA on the production of steroidogenic enzymes in addition to their effect on inhibition of gap junction-mediated cell communication. It should be noted that neither cell communication nor the effect of ACTH stimulation was measured in this study. Further investigations are needed to determine if the concentrations of 18-beta GA used in this study eliminated cell-cell communication, and more importantly, if the ACTH-stimulated steroidogenesis at submaximal and saturating treatment doses were altered. It should also be noted that both 18-alpha and 18-beta GA have formulas very similar to that of cortisol and may be mistaken, with some assay methods, for an adrenal steroid (74).

## Proliferation

In the intact adrenal gland, it was noted that the cells of the zona glomerulosa, which had few if any gap junctions, divided rapidly. In contrast, cells of the inner zones, where gap junctions were more abundant, proliferated more slowly (6, 40). Based on this inverse relationship between the rate of proliferation in the adrenal zones and the presence of gap junctions, it was suggested that gap junctions play a role in regulating cell proliferation in the adrenal cortex.



**FIGURE 5 | Cocultured adrenal/granulosa cell pairs.** Adrenal cell clusters viewed with optics specific for fluorescein isothiocyanate (**A**) or rhodamine isothiocyanate (**B**) were treated with follicle-stimulating hormone (FSH) for 30 min. Abundant protein kinase dissociation was initially observed in granulosa cells (OG) and could be seen, after 5–15 min, in two adrenal cells (Y-1\*) that are in contact with the granulosa cell, while two nearby adrenal cells (Y-1) that are not in contact with the granulosa cell failed to dissociate PKA (**A**). Granulosa cells were identified by pre-labeling them with rhodamine-coated beads (**B**) [modified from Ref. (59)].

In the zona glomerulosa, it has been suggested that the lack of gap junctions and thus capacity for direct communication of growth regulatory molecules would result in more cell proliferation in this zone. Along these same lines of evidence, the slower proliferation rate of cells in the inner zones, where gap junctions are more abundant (5, 40), would be consistent with their greater capacity to communicate growth inhibitory signals. Many factors are known to regulate growth, and gap junction-mediated communication may play a key role by facilitating the movement of these factors between the cells.

In support of the hypothesis that gap junctions contribute to the control of adrenal cell proliferation, possibly by allowing passage of molecules between cells, are the findings that cell proliferation of bovine adrenal cortical cell (SBAC) populations was significantly increased if gap junction protein expression was suppressed by Cx43 gap junction cDNA antisense transfection techniques (8). The average number and size of gap junction plaques decreased while the rate of population growth increased in these studies (8). Conversely, when adrenal cells were treated with ACTH, which increased gap junction protein expression, there was a corresponding decrease in cell population growth (8). Both the ACTH-stimulated increase in gap junction protein expression and decrease in cell population growth were mimicked by treatment with the second messenger, dibutyryl cyclic adenosine monophosphate [DbcAMP (1 mM)]. It is, thus, thought that the ACTH-induced alterations in Cx43 gap junction protein expression and proliferation are modulated by a PKA mechanism. Further, the findings of an inverse relationship between gap junctions and proliferation would suggest that, in the adrenal cortex, along with the multiple mechanisms known to be involved in controlling adrenal gland structure and function, ACTH-stimulated intercellular communication *via* gap junctions may represent an important factor in adrenal gland behavior. However, not only is gap junction-mediated cell communication thought to regulate ACTH-induced changes in steroidogenesis and proliferation but also there is compelling evidence that ACTH serves to regulate gap junction protein expression and stability at the cell surface.

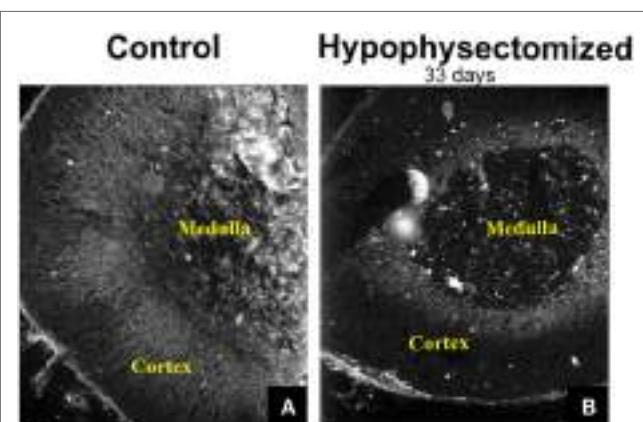
## REGULATION OF GAP JUNCTION EXPRESSION

In a number of studies of adrenal cells in culture, ACTH has been demonstrated to increase gap junction protein expression and to increase the size and number of surface gap junction plaques (4, 7, 8). These effects on gap junctions can be mimicked by treatments that increase cAMP levels and activate PKA (59, 64). Specifically, an increase in gap junction plaque size and number at the cell surface as well as the decrease in gap junction plaque disassembly (internalization to form annular gap junctions) was reported following DbcAMP treatment. These results are consistent with the theory that the changes in gap junctions following ACTH treatment were dependent on cAMP and the activation of protein kinase. Conversely, elevation of cAMP and PKA activation has been demonstrated to decrease the number of annular gap junctions in adrenal cortical cells (75). This suggests that PKA activation may decrease gap junction plaque internalization,

which would further contribute to the observed increase in gap junction plaques at the cell surface following treatments that elevate cAMP levels in adrenal cell populations.

In addition to the findings made in adrenal cell cultures, the relationship between gap junction protein expression, occurrence, distribution, and ACTH levels in the body have been evaluated in studies in which the tropic state of the adrenal gland was altered by surgical removal of the pituitary (46). The removal of the pituitary, termed hypophysectomy, eliminates the source of ACTH since the pituitary secretes this as well as a variety of other hormones that are either produced by cells of the anterior pituitary (growth hormone, gonadotrophins, prolactin, and thyroid stimulating hormone) or by cells of the hypothalamus and then stored in the pituitary (antidiuretic hormone and oxytocin) (1, 76). The elimination of ACTH by hypophysectomy led to a profound atrophy of the cortex, which was more marked in the inner zones (ZF and ZR) than in the zona glomerulosa (46). While increasing ACTH levels increased gap junctions, eliminating ACTH by perturbing the pituitary–adrenal gland axis by hypophysectomy in mice led to diminished Cx43 gap junction expression mainly in the ZF (Figure 6) (31). If these hypophysectomized animals were treated with ACTH, Cx43 gap junction plaque size was increased (46). The increase in gap junction protein expression occurred in the ACTH-dependent zones (ZF and ZR) with no change in the ACTH-independent zone (zona glomerulosa). Thus Cx43 gap junction protein expression can be regulated *in vivo* as well as in adrenal cell populations maintained in culture, in further support that ACTH can modulate gap junctions.

The molecular mechanism for how ACTH may modulate gap junctions has not been demonstrated in the adrenal gland. It is well documented, however, that ACTH treatment results in PKA activation (64); reviewed by Ruggiero and Lalli (77), and in a number of other cell types, activation of PKA has been shown to increase gap junction plaque assembly by facilitating the phosphorylation of gap junction protein (78). Specifically, PKA activation results in the phosphorylation of serines (S364, S365,



**FIGURE 6 | Immunohistochemical demonstration of gap junction (Cx43) distribution in the adrenal gland.** Gap junction proteins were abundant in the inner cortex of the mouse adrenal (A). Hypophysectomy led to diminished Cx43 gap junction expression mainly in the zona fasciculata at 33 days post-surgery (B) [modified from Ref. (31)].

S369, and S373) on the C-terminal tail of Cx43 (78). Treatment with ACTH, based on this information, would be expected to phosphorylate one or more of these serines. There is a need, however, for increased information on the relationship between ACTH treatment and connexin phosphorylation if we are to fully understand the role of gap junction function in adrenal cell behavior.

To date, the largest increase in information on gap junction regulation and function comes from the knowledge of which Cx43 C-terminal tail amino acids are phosphorylated and dephosphorylated (22, 78–81). Phosphorylation/dephosphorylation events on the C-terminal tail of Cx43 are dependent on activation of PKA, as well as a number of other kinases including protein kinase C (PKC), tyrosine kinase, mitogen-activated protein kinase (MAPK), and casein kinase 1 (22, 82–84). These protein kinases, through triggering the phosphorylation of particular amino acids on the tail of Cx43, regulate gap junctions (assembly into plaques, stability at the cell surface, gap junction plaque disassembly, and cell-cell communication). Specifically, there are at least 11 serine and 2 tyrosine residues on the C-terminal tail of Cx43 (Cx43-C-terminus) that, when phosphorylated, result in either an increase in gap junction function (S325, S328, S330, S364/365, and S373) or the downregulation of gap junction activity (Y247, S255, S262, Y265, S279/282, and S368) (85–87). These observations have been made mainly from studies in clonal lines of murine fibroblasts (L929 cells), fibroblasts derived from Cx43 knockout and wild-type mice, rat liver epithelial cells (T51B), HeLa cells, and rat primary granulosa cells (88). It should be noted that ACTH-mediated activation of either S364, S365, S369, S373, or other possible amino acids of the C-terminal tail of Cx43 has not, to our knowledge, been examined. Furthermore, protein kinase-mediated phosphorylation of the C-terminus of Cx43 has been demonstrated to play a role in gap junction plaque internalization and annular gap junction vesicle formation (89, 90). Specifically, MAPK, casein kinase 1, and PKC activation increases the number of annular gap junction vesicles while decreasing gap junctions on the cell surface (69, 80, 89–92). In the case of PKC-mediated hyperphosphorylation of Cx43, it is thought that phosphorylation makes this connexin more vulnerable to proteolytic degradation, thus decreasing gap junction-mediated communication. The interplay between the various kinase pathways in Cx43 phosphorylation and cell-cell communication have not been elucidated in the adrenal cortex. Such studies, however, are needed if we are to fully understand the role of ACTH in regulating those endocrine cell responses that are dependent on the communication of molecules between cells. Certainly, in the adrenal gland, there is a need for cells to cooperate and get information from one another. Gap junctions provide this function, and when gap junctions are lost from a population, pathological conditions, including cancers, are thought to develop (42, 93, 94).

## LOSS OF GAP JUNCTIONS AND ADRENAL CORTEX CANCER

The loss of gap junction function has been implicated in tumor development (93–99). Most tumors associated with the adrenal

cortex develop in the ZF (100) where, under normal conditions, gap junctions tend to be large and abundant (6, 40, 42). The most common types of adrenal gland tumors are adrenal adenomas, which are non-cancerous tumors of the cortex (101, 102). Adrenocortical carcinoma, cancer of the adrenal cortex, is rarely observed; however, patients with certain inherited genetic disorders are at a higher risk of developing this cancer (102).

In the adrenal cortex, a decrease in gap junction expression has been correlated with the stage of tumor differentiation/progression (42). Specifically, in studies to characterize gap junctions during tumorigenesis, the number of gap junctions observed per cell was quantitated and compared in normal human cells of the adrenal gland ZF, as well as cells from adenoma and carcinoma adrenal gland tissues. Cells from the ZF of normal adrenal glands displayed a much higher number of gap junctions per cell ( $13.78 \pm 1.93$  SEM) than when compared with benign adrenocortical adenomas ( $4.6 \pm 1.17$  SEM;  $p \leq 0.05$ ). The number of gap junctions demonstrated between cells of malignant adrenocortical tumors ( $1.42 \pm 0.58$  SEM;  $p \leq 0.05$ ) were significantly lower than both the normal and benign cells (42). These observations are consistent with the hypothesis that a capacity for increase in proliferation coupled with the loss of terminal differentiation as the tumor progresses from non-malignant to malignant reflects the loss of gap junction-mediated communication of growth regulatory molecules between cells. Further, it has been suggested that the increased capacity for tumor metastasis may result from the loss of the cell–cell adhesion that is provided not only by adhesion junctions but also by gap junctions (103–105). Interestingly, exogenous Cx43 can decrease cell proliferation and contribute to reversion of the transformed phenotype (106, 107). Both observations are suggestive of a relationship between gap junctions, differentiation, and proliferation. There is a hope that the induction of gap junctions in malignant cells will provide a novel therapeutic strategy for treating adrenal cancer and, in addition, pharmacological methods and manipulations designed to increase gap junctions may someday serve as a therapeutic method.

While some investigators have suggested the loss of gap junctions in adrenal tumor cell populations, others have demonstrated that metastatic tumors are correlated with an increase in the expression of connexins dependent on the tissue type (108–111). In few cases, tumor cells are capable of communication with other tumor cells through gap junctions; however, they do not communicate with normal cells (112). The difference in the detection of changes in some studies compared with others may reflect the complex nature of cancers in addition to the adrenal zones of origin of the various tumors. Further investigations of the relationship between gap junction expression and tumor progression are needed. This is specifically true when studying the adrenal gland, given the number of adrenal incidentalomas that are commonly detected and the need to differentiate benign from malignant detections. The reported lack of gap junctions in the carcinoma cell population, if found to be widespread in different tumor populations, could be used as an additional method of determining the capacity for metastasis and as an indicator of the rate at which the tumor may proliferate. Furthermore, if cancer cells of the adrenal are capable of communicating with

one another but not with normal cells as suggested by Yamasaki in other tissues (112), it would be possible to design protocols that use gap junction-mediated communication to selectively kill the cancer cells while leaving the normal healthy cells intact, the bystander effect (113–115).

## CROSS TALK BETWEEN CELLS OF THE CORTEX AND MEDULLA

It is becoming clear that the cells of the medulla and the cortical cells are physically interwoven with one another (116) and, thus, the possibility of gap junction-mediated communication between chromaffin cells and adrenocortical cells may exist. In humans, as well as other mammals, for example cortical cells can be found within the medulla in contact with chromaffin cells (116–118). Furthermore, inner cortical cells of the ZR are in direct contact with the chromaffin cells of the medulla (1), and gap junctions between these cells could possibly provide a mechanism for direct communication of information. Certainly, there is a functional relationship between the cortical and medullary cells, since the adrenal androgens, particularly dehydroepiandrosterone (DHEA), which is produced in ZR following ACTH stimulation, can inhibit chromaffin cell proliferation and differentiation (119, 120). In addition, cells of the cortex may influence catecholamine release from chromaffin cells while the chromaffin cells may regulate adrenal cortical steroid hormone production (10, 116, 121). Gap junctions may provide a mechanism for some of the functional interdependency, especially, among those cells at the medullary–cortical interface area and among islands of cortical cells located within the medulla.

In the medulla, several different connexin types (Cx29, Cx36, Cx43, and Cx50) have been reported to be expressed, depending on the species (41, 122). While some investigators have not reported gap junction plaques in the medulla, others have demonstrated clusters of medullary cells that express Cx43 antigens (5). It is thought that gap junctions in the medulla play a role in the catecholamine (epinephrine and norepinephrine) secretory process and, thus, helps in mediating the body's response to stress (54, 123–125). In addition, stress causes a well-orchestrated, cascade of events that result in the release of a number of other hormones, including glucocorticoids from the adrenal cortex (126). Gap-junctional coupling may complement electrical coupling to enhance communication of signals needed for synchronized cellular hormone release in response to ACTH or low synaptic activity during stress (125).

## SUMMARY

Gap junctions are thought to be important in regulating cell population growth, cell morphology, differentiation, cell migration, wound healing, and cell function (2, 93, 108). By analyzing the frequency, distribution, and function of these junctions within intact adrenal glands as well as in adrenal cells in culture following ACTH stimulation, the role of connexins in these processes may be clarified. The specific spatial abundance of gap junction proteins in the adrenal cortical zones is intriguing with regard to its functional implications. It can be suggested that cells

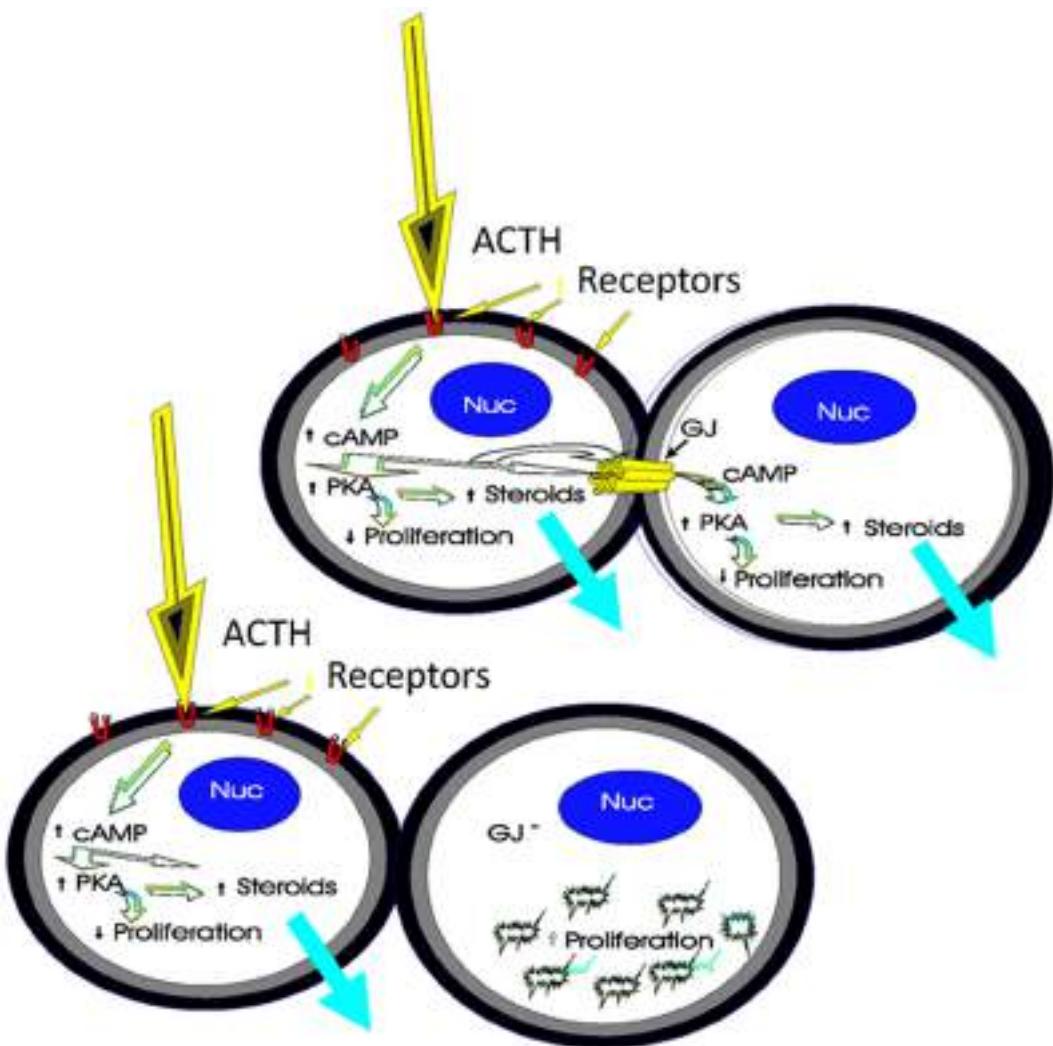
of the zona glomerulosa, which express fewer gap junctions than the two other cortical zones, may be less dependent on cell–cell communication for normal function than the cells of the ZF or ZR. Further, a relationship between proliferation rates, steroidogenesis, and gap junction presence or absence in the gland has been suggested (Figure 7). Studies to demonstrate the effects of gap junction overexpression and inhibition in the adrenal glands are needed, if we are to resolve some of the questions of how gap junctions function as adrenal cells undergo the morphological and proliferative changes associated with the establishment and maintenance of cortical zonation and hormone-mediated steroidogenesis.

Intact adrenal glands and adrenal cell cultures provide us with a valuable paradigm for studying gap junctions and the cellular mechanisms involved in hormone responses. Indeed, study of adrenocortical cells *in vivo* and *in vitro* is important not only for an understanding of adrenal gland function *per se* but also for providing insight into the foundation of cell communication and biological response in other tissues. But, beyond cell communication, it is becoming apparent that gap junctions play a role in providing adhesive forces needed to hold cells together (103–105).

In addition to gap junction-mediated communication and adhesion, the gap junction internalization process that results in increased annular gap junction vesicles within the cytoplasm and fewer surface gap junction plaques could be important to events in the adrenal gland. Annular gap junctions were reported in the adrenal gland as early as 1988 with transmission electron microscopy (49). It has been demonstrated that annular gap junctions undergo degradation (13, 51, 52). Relatively large numbers of annular gap junctions occur in the adrenal gland, and it could be postulated that gap junction turnover is high in this tissue. Rapid turnover could be needed for migration of the cells to maintain the cortical zones during cytogenesis, for example. Such suggestions are not without precedent. In the ovary, for example, gap junctions are thought to allow the movement of molecules to the egg from the follicular granulosa cells (60, 61). In the case of the ovary, phosphorylation of the C-terminal tail of connexin has been demonstrated to be regulated by the peptide hormone, luteinizing hormone (127). Large numbers of annular gap junctions are reported during ovulation, and it is thought that if gap junctions are not internalized from the surface of the granulosa cells that the egg will remain “trapped” within follicle and ovulation will not occur (128). Gap junctions in a similar way could potentially retard the migration of cells from one area to another (centripetal migration) by tethering the cells to one another. Inhibiting cell migration through the granulosa cell layer of the follicle, in the case of the ovary, or as cells migrate in the adrenal gland could keep cells connected to one another if gap junctions fail to internalize. Such possibilities, however, are yet to be demonstrated.

## REMAINING QUESTIONS

It is clear from early studies that there is a relationship between gap junctions and ACTH-mediated responses in the adrenal cortex. There are, however, very few recent articles available



**FIGURE 7 | Schematic of the role of gap junctions in steroidogenesis and cell proliferation.** The binding of ACTH to its receptors on coupled cortical cells stimulates cyclic adenosine monophosphate (cAMP) to activate protein kinase A (PKA), thereby increasing steroidogenesis and decreasing proliferation. Gap junction-mediated movement of cAMP between cells in the population would amplify hormonal responses in contacting cells, thus increasing ACTH-mediated steroidogenesis. An increase in the number of gap junctions, increases ACTH-mediated steroidogenesis while decreasing proliferation. However, if gap junctions are decreased as a result of molecular manipulations or chemical treatments, for example, ACTH stimulation in one cell does not affect steroidogenesis in the second cell, and there would be a loss in coordinated cell function between cells [modified from Ref. (130)].

on gap junctions in the adrenal cortex. Yet, there are important questions that remain to be answered. For example, in human conditions with diminished circulating ACTH levels, are there changes in the expression or distribution of adrenal cortical gap junctions, as seen following hypophysectomy in rodents (30)? There is dramatic remodeling and relocation of gap junction plaques in the diabetic heart (129). Would the remodeling of gap junctions in the adrenal occur in this and in other endocrine related diseases? Adrenal cortical steroidogenesis is influenced mainly by the activation of PKA (59, 64). In tissues, other than the adrenal, PKA has been shown to phosphorylate the C-terminal tail of Cx43 gap junction proteins (22, 78–81). Such PKA-mediated connexin phosphorylations have been demonstrated to be critical in regulating gap junction assembly and stability

(22, 78–81). In the adrenal cortex following ACTH treatment, it is presumed that gap junction protein phosphorylation occurs. However, which Cx43 C-terminal tail amino acids are phosphorylated is unknown. Further, the interplay between PKA and the other protein kinases (PKC, tyrosine kinase, MAPK, and casein kinase 1) in the regulation of gap junctions and adrenal cortical functions needs to be investigated. It has become clear that connexins can have multiple regulatory functions, which depend upon their phosphorylated state (22, 82, 84). Unfortunately, the role of protein kinase-mediated connexin phosphorylation in the adrenal cortex, although described in other tissues, has not been investigated, to our knowledge, in the adrenal cortex. However, one would predict that as cells alter their function that the need for gap junction-mediated cell communication, and thus

gap junction expression and phosphorylation, may also change. Moreover, the molecules moving between cells may change. One molecule that has been demonstrated to transfer between gap junctions of adrenal cells to regulate steroidogenesis is cAMP (59, 68). But surely, more than this, one molecule is moving between the cells of the adrenal. The abundance of gap junctions in the inner cortex would suggest a high demand for communication of molecules in these zones. The following question has yet to be answered: how movement of molecules between cells serves to regulate the cascade of events involved in proliferation but more importantly in differentiation of the cells such that transcription factors needed for the production of cortisol in one zone of the adrenal but for androgens in another zone.

The study of adrenocortical cells *in vivo* and *in vitro* is important not only for an understanding of adrenal gland function *per se* but also for providing insight into the foundation of cell communication and biological response in other tissues. Further study will

elucidate how gap junctions and cell–cell communication as well as gap junction-mediated adhesion is related to adrenal gland development cell differentiation and steroidogenic function.

## AUTHOR CONTRIBUTIONS

CB wrote sections of the manuscript, prepared figures, and edited the manuscript. SM wrote sections of the manuscript, prepared figures, and edited the manuscript.

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# Adrenal Gland Microenvironment and Its Involvement in the Regulation of Stress-Induced Hormone Secretion during Sepsis

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Survival of all living organisms depends on maintenance of a steady state of homeostasis, which process relies on its ability to react and adapt to various physical and emotional threats. The defense against stress is executed by the hypothalamic–pituitary–adrenal axis and the sympathetic–adrenal medullary system. Adrenal gland is a major effector organ of stress system. During stress, adrenal gland rapidly responds with increased secretion of glucocorticoids (GCs) and catecholamines into circulation, which hormones, in turn, affect metabolism, to provide acutely energy, vasculature to increase blood pressure, and the immune system to prevent it from extensive activation. Sepsis resulting from microbial infections is a sustained and extreme example of stress situation. In many critical ill patients, levels of both corticotropin-releasing hormone and adrenocorticotropin, the two major regulators of adrenal hormone production, are suppressed. Levels of GCs, however, remain normal or are elevated in these patients, suggesting a shift from central to local intra-adrenal regulation of adrenal stress response. Among many mechanisms potentially involved in this process, reduced GC metabolism and activation of intra-adrenal cellular systems composed of adrenocortical and adrenomedullary cells, endothelial cells, and resident and recruited immune cells play a key role. Hence, dysregulated function of any of these cells and cellular compartments can ultimately affect adrenal stress response. The purpose of this mini review is to highlight recent insights into our understanding of the adrenal gland microenvironment and its role in coordination of stress-induced hormone secretion.

**Keywords:** stress system, hypothalamic–pituitary–adrenal axis, immune–adrenal crosstalk, ACTH, glucocorticoids

## INTRODUCTION

In order to survive all, living organisms must maintain a steady state of homeostasis. This unique capacity to react and adapt to various physical and emotional threats in higher vertebrates is mediated by a coordinated action of the nervous, endocrine, and immune systems, known as stress system (1). Activation of the stress system, which is composed of the hypothalamic–pituitary–adrenal (HPA) axis and the sympathetic–adrenal medullary system, leads to increased synthesis and release of glucocorticoids (GCs) and catecholamines (CAs) from the adrenal cortex and medulla, respectively (2–4). The main function of these hormones is maintenance and restoration of basal and stress-related

body homeostasis. This protective action of adrenal hormones is mostly accomplished by their metabolic, cardioprotective, and anti-inflammatory actions. In particular, both GCs and CAs are known to acutely enhance plasma glucose levels and promote an increased cardiac output and high blood pressure, while protecting against excessive inflammation (5–8).

Secretion of adrenal hormones during stress-free conditions is regulated centrally and is characterized by ultradian rhythms (9). An early morning release of adrenocorticotrophic hormone (ACTH) into circulation is enhanced by increased concentrations of corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) in the hypophyseal portal system of anterior pituitary (10, 11). Consequently, elevated plasma levels of ACTH stimulate adrenocortical cells to produce and release GC hormones. In turn, as a part of negative feedback, GCs act directly on the pituitary gland to reduce ACTH secretion and on hypothalamic neurons to reduce CRH release (11, 12). Systemic rise in CA levels is in turn initiated by activation of the sympathetic nervous system and splenic nerves innervating the adrenal medulla (13, 14).

One of the extreme examples of sustained and severe physical stress is sepsis syndrome. It is characterized by abnormal host response to infection, resulting in systemic inflammation that frequently culminates in a life-threatening dysfunction of multiple organs (15, 16). Severe sepsis remains the leading cause of mortality worldwide, and its incidence is increasing (17, 18). An intact function of the stress system, and in particular the activation of GC and CA production, is critical to survive this adverse condition (19, 20). During sepsis, this homeostatic function of the stress system is frequently impaired with mechanisms remaining largely unknown (21, 22). Consequently, many critically ill patients demonstrate suppressed ACTH and CRH levels while having normal or elevated cortisol levels (22). Hence, a key involvement of pituitary-independent factors was proposed (2). Among these, reduced GC metabolism and activation of local adrenal microenvironment seem to play crucial role (23, 24) (**Figure 1**).

As regulation of the stress system and importance of impaired metabolism of GCs during sepsis were recently presented and discussed in many review articles (25–27), the main purpose of this mini review is to highlight recent insights into our understanding of the role local adrenal gland microenvironment in the regulation of stress-induced hormone secretion during sepsis.

## ADRENAL GLAND MICROENVIRONMENT

Within adrenal gland, two embryonically different tissues coexist: mesodermally derived, steroid-producing cortex and ectodermally derived, CA-producing medulla. Within these two environmental niches, interplay between various cells takes place including adrenocortical and chromaffin cells, neuronal cells, immune cells, endothelial cells, and glia cells (28). The adrenal gland is a source of many bioactive substances including steroid hormones, CAs, cytokines, neurotransmitters, and neuropeptides (**Table 1**). These substances are known to interact with various cell types within adrenal gland itself, thereby influencing its function during stress conditions and disease (24, 28).

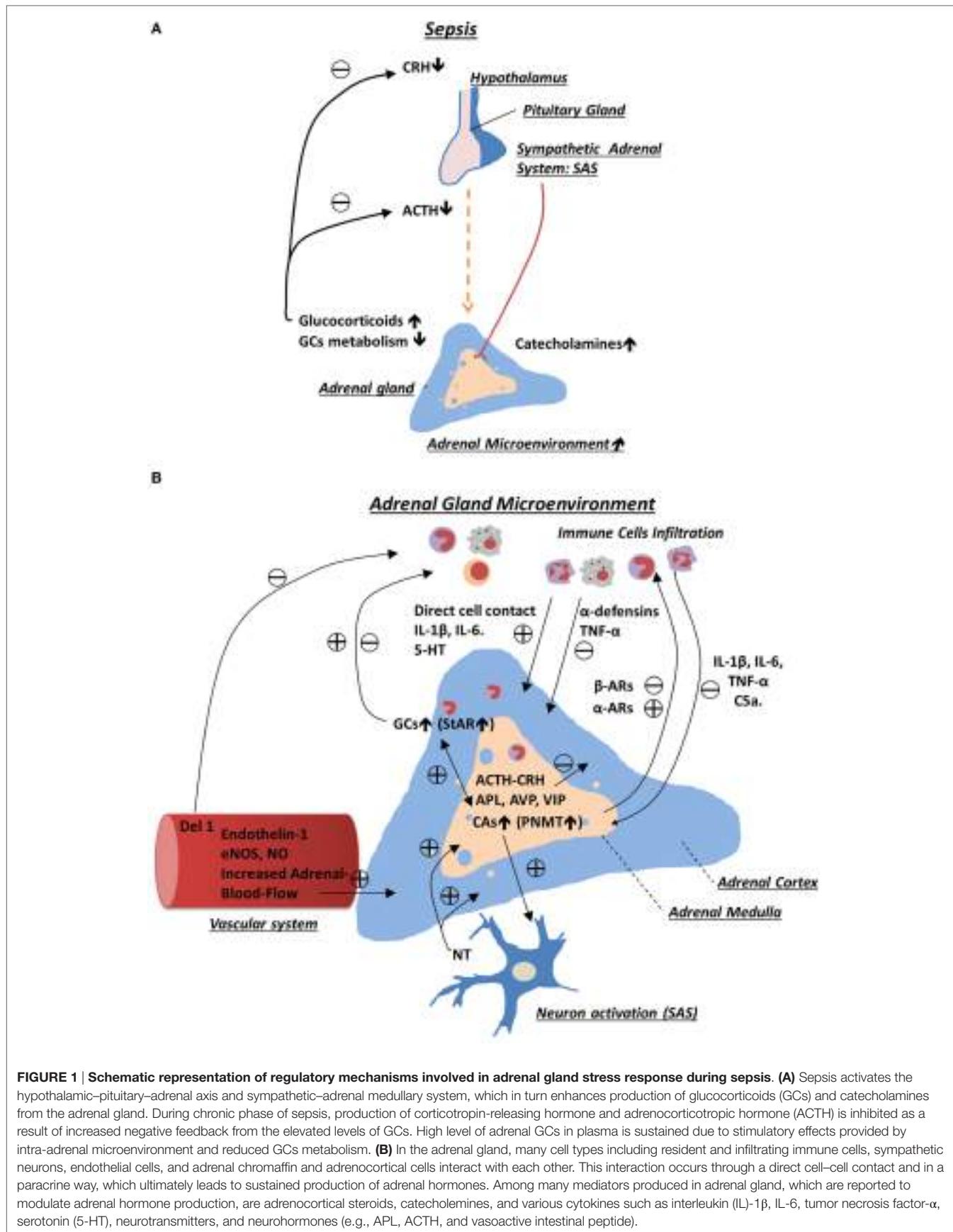
## Adrenal Cortex and Medulla Interactions during Sepsis

Ample evidence exists suggesting a bidirectional interaction between adrenal cortex and the medulla (28). Despite a classical view on the adrenal gland anatomy, demonstrating a clear separation of the cortex and medulla, it has been well documented that these two endocrine tissues are morphologically intermingled (29, 30). This close localization, in turn, enables direct cell-cell and paracrine interactions between adrenocortical and chromaffin cells involving their secretory products.

Consequently, the adrenal cortex-derived GCs were found to enhance synthesis of CAs both *in vitro* (31) and *in vivo* (32–34). GCs were found to execute these effects by upregulating expression of phenylethanolamine N-methyltransferase (PNMT), which gene encodes for an enzyme that catalyzes the synthesis of epinephrine from norepinephrine. Furthermore, studies using various knockout mice, which were deficient in steroidogenic factor-1, glucocorticoid receptor, or corticotropin-releasing hormone type 1 receptor, clearly demonstrated that lack of one of these key steroidogenic regulators impair PNMT gene expression and thus basal and stress-induced epinephrine production (35–38). Similarly, an intact function of adrenal medulla is important to maintain adrenocortical function. It has been shown that coculture of bovine adrenocortical cells with chromaffin cells enhanced a basal GC secretion by 10-fold (39). Furthermore, CAs were found to enhance production of various steroids including cortisol, aldosterone, and androstenedione, through mechanism involving upregulation of steroidogenic acute regulatory protein (28, 40, 41).

During sepsis, an enhanced production of epinephrine and norepinephrine (42–44) and increased secretory capacity of adrenal chromaffin cells were reported (45). Unlike expression of tyrosine hydroxylase, which enzyme is involved in first step of CA biosynthesis, upregulation of PNMT gene expression in adrenal medulla was found to be regulated independently of splanchnic nerve stimulation (28). Instead, an extremely high concentration of GCs was reported to be involved in regulation of PNMT expression during sepsis (46). Consequently, stress-induced expression of PNMT is strongly attenuated in CRH-deficient mice (47).

In addition, adrenal chromaffin cells release many active neuropeptides and transmitters, including neuropeptide Y (NPY), vasoactive intestinal peptide (VIP), or substance P, with known stimulatory action on GCs production (48, 49). For example, VIP, which levels increase during experimental endotoxemia in adrenal medulla, was found to stimulate DHEA, testosterone, androstenedione, cortisol, and aldosterone secretion in adrenocortical cells (48–52). Among other mediators found in adrenal medulla, which may influence GC production during sepsis, are angiotensin, AVP, CRH, ACTH, and apelin (53–55). Although direct stimulatory effects of angiotensin 2, CRH, and ACTH on adrenal hormone production are well described, the degree to which each of these peptides contributes to hormone production during sepsis conditions has not been fully elucidated yet (2, 56). Another interesting peptide found recently to be expressed in the adrenal medulla is apelin. Apelin is a neurohormone, which acts through its receptor APJ expressed in various organs including



**FIGURE 1 | Schematic representation of regulatory mechanisms involved in adrenal gland stress response during sepsis. (A)** Sepsis activates the hypothalamic–pituitary–adrenal axis and sympathetic–adrenal medullary system, which in turn enhances production of glucocorticoids (GCs) and catecholamines from the adrenal gland. During chronic phase of sepsis, production of corticotropin-releasing hormone and adrenocorticotrophic hormone (ACTH) is inhibited as a result of increased negative feedback from the elevated levels of GCs. High level of adrenal GCs in plasma is sustained due to stimulatory effects provided by intra-adrenal microenvironment and reduced GCs metabolism. **(B)** In the adrenal gland, many cell types including resident and infiltrating immune cells, sympathetic neurons, endothelial cells, and adrenal chromaffin and adrenocortical cells interact with each other. This interaction occurs through a direct cell-cell contact and in a paracrine way, which ultimately leads to sustained production of adrenal hormones. Among many mediators produced in adrenal gland, which are reported to modulate adrenal hormone production, are adrenocortical steroids, catecholamines, and various cytokines such as interleukin (IL)-1β, IL-6, tumor necrosis factor-α, serotonin (5-HT), neurotransmitters, and neuropeptides (e.g., APL, ACTH, and vasoactive intestinal peptide).

**TABLE 1 |** Intra-adrenal interaction involved in a regulation of adrenal stress response.

Cell interactions	Mediators	Action
<b>Immune–adrenal crosstalk</b> (macrophages, neutrophils, mast cells, lymphocytes)	Glucocorticoids (GCs)	Stimulatory effects on naïve and inhibitory actions on activated immune cells
	Cytokines	Stimulatory [interleukin (IL)-1 $\beta$ , IL6] or inhibitory action [tumor necrosis factor (TNF)- $\alpha$ ] on adrenal steroidogenesis
	Catecholamine (CA)	Anti-inflammatory effects through activation of $\beta$ -adrenergic receptors (ARs) (increase IL-10, decrease TNF- $\alpha$ in macrophage, and decrease NO and ROS in neutrophils) Pro-inflammatory effects through activation of $\alpha$ -ARs
	Direct contact	Stimulatory effects on adrenal DHEA synthesis
	$\alpha$ -Defensins	Inhibitory effects on steroidogenesis through decreased sensitivity of adrenal cells to adrenocorticotrophic hormone (ACTH)
	Neutrophil extracellular traps	Destruction of adrenal cells and promotion of hemorrhages
	GCs	Stimulatory action on CA synthesis (through PNMT upregulation)
	CAAs	Stimulatory action on adrenal steroidogenesis
	Neurotransmitters (NT) (substance P, vasoactive intestinal peptide, neuropeptide Y, etc.)	Stimulatory and inhibitory action on adrenal steroidogenesis
	Local corticotropin-releasing hormone–ACTH system	Stimulatory action on adrenal steroidogenesis
<b>Adrenocortical–adrenomedullary interactions</b> (adrenocortical cells and chromaffin cells)	Apelinergic system	Stimulatory action on adrenal steroidogenesis
	Endothelin-1	Direct stimulatory effect on GC production
	eNOS and NO	Possible stimulatory effect on steroidogenesis
	Developmental endothelial locus 1	Regulation of immune cells recruitment into adrenal gland
<b>Vasculature</b> (endothelial cells)	Adrenal blood flow	Stimulatory effect on steroidogenesis
	NT (acetylcholine)	Increased steroidogenesis via enhanced sensitivity of adrenal cells to ACTH
		Increased production and secretion of CAAs
<b>Innervation</b> (sympathetic neurons)		

hypothalamic neurons, anterior pituitary, and adrenal gland. It was recently found to modulate neuroendocrine response to stress through stimulation of secretion of both ACTH and corticosterone directly and *via* AVP- and CRH-dependent pathways (54, 55, 57).

Recently, a population of nestin-positive glia-like multipotent stem cells was identified in adrenal medulla of nestin-GFP transgenic mice (58). Interestingly, when subjected to chronic

stress, this population was able to give rise to new chromaffin cells, suggesting a direct involvement of these cells in the adrenal stress adaptation (58–60). It is therefore very interesting to investigate the fate of this cells and their potential interaction with adrenocortical cells during sepsis conditions.

In summary, an intact function of both adrenal cortex and adrenal medulla is of pivotal importance in the regulation of adrenal stress response. Consequently, any disorders or medications that can attenuate adrenal cortex function will also ultimately affect CAAs production by chromaffin cells and *vice versa* potentially impacting the outcome of many stress-related disorders (19).

## Immune–Adrenal Crosstalk

In the last 40 years, it has become evident that immune and endocrine systems interact with each other at the level of adrenal gland and that this interaction is crucially involved in the regulation of adrenal gland function during normal and stress conditions (61). This immune–endocrine crosstalk is mostly executed by bidirectional action of paracrine factors, such as steroid hormones, CAAs, and various vasoactive or proteolytic enzymes, as well as by direct cell–cell contacts and activation of toll-like receptors (TLRs) (28, 62).

## Immune–Adrenal Interaction at the Level of Adrenal Cortex

During non-stress conditions, many immune cells can be found in the innermost zone of adrenal cortex known as *zona reticularis*; these are particularly tissue macrophages (63), dendritic cells (64), mast cells (65), and lymphocytes (66). However, some of them, especially macrophages and mast cells, were also reported in other parts of the adrenal gland including subcapsular region and in the adrenal medulla (64). In non-stress conditions, adrenal-resident immune cells play important homeostatic functions by sensing pathogens, removing apoptotic cells, and promoting tissue remodeling through, e.g., secretion of growth factors (24, 67). Recently, a new population of adrenal macrophages was identified based on the high expression of a complement receptor immunoglobulin, CRIg, which molecule is known to mediate phagocytosis of pathogens and apoptotic cells (68). During systemic inflammation induced either by LPS injection or CLP, highly dynamic changes within adrenal immune cell populations can be observed. For instance, it has been shown that already within first 3 h after administration of LPS or induction of peritonitis in rodents, a rapid infiltration of neutrophils takes place, while the number of local dendritic cells and macrophages declines (69–71).

Using electron microscopy, close cell–cell localization between local immune cells and surrounding adrenocortical, chromaffin, or endothelial cells was demonstrated (64, 66). This in turn enables various bidirectional and direct interactions between each cell type. For example, a functional crosstalk between adrenocortical cells, expressing major histocompatibility complex class II molecules (MHC/HLA), and leukocytes was described. Indeed, a co-incubation of T cells with primary cultures of human adrenocortical cells promoted adrenal androgen and cortisol secretion (66). Besides direct

interaction, various immune cells were found to regulate adrenal hormone production in a paracrine way through secretion of cytokines, such as interleukins (IL) 1 and 6 (72), and pro-opiomelanocortin (POMC)-derived peptides, e.g., ACTH and biogenic amines (28, 73).

The ample experimental evidence exists demonstrating that cytokines are critically involved in ACTH-independent activation of GC production during sepsis (72). For example, injection of IL-1 $\beta$  enhanced GC production in hypophysectomized rats (74). Furthermore, an impaired synthesis of GCs after LPS injection was observed in mice receiving sera against tumor necrosis factor (TNF)- $\alpha$  and IL-6 and, to a lesser extent, IL-1 $\beta$ - or IL-6-deficient mice (75, 76). However, it has been demonstrated that cytokine levels found normally in plasma are far too low to induce hormone production from adrenocortical cells. Therefore, the main source of cytokines must come from the adrenal gland itself (28). Sustained production of cytokines in highly anti-inflammatory environment of the adrenal gland during systemic inflammation was found to be enabled by increased expression of migration inhibitory factor (77). During sepsis, various cell types present within adrenal gland microenvironment may be a potential source of pro-inflammatory cytokines. For example, adrenocortical cells were found to express several TLRs and secrete several cytokines in response to bacterial ligands (78, 79). However, in a recent study, inactivation of myeloid but not adrenocortical TLR signaling resulted in significant reduction of intra-adrenal cytokine levels and activation of the HPA axis after LPS administration (80). The latter observation suggests that immune cells are the key sources of cytokines in the adrenal gland during sepsis. Due to high expression of both cytokine and GC receptors in adrenocortical and immune cells, respectively, cytokines and adrenal hormones are known to regulate production in each other. For example, IL-1 $\beta$  or IL-6 was found to increase adrenal hormone production, whereas TNF stimulation demonstrated rather opposite effect (81–83). In turn, adrenal steroid hormones are known to influence immune cells function. In particular, it has been demonstrated that in naïve immune cells, GCs can activate several inflammatory-related genes, e.g., TLRs, yet in cells treated with LPS, they inhibit inflammation (6). In a recent study, a chronic exposure to GCs was found to cause a shift in the innate–adaptive balance of the immune response, particularly, influencing the chemokine–chemokine receptor networks (84). Besides cytokines, other biologically active substances, such as serotonin (5-HT) or histamine, which are stored by mast cells show stimulatory effects on adrenal steroidogenesis (85, 86).

During prolonged sepsis, the immune–adrenal interaction may also result in the suppression of adrenal hormone production. Despite their important role in host defenses against bacterial infections limiting bacterial spread in circulation (87), a prolonged exposure of adrenal cells to neutrophil-derived antimicrobial agents, such as ROS and proteolytic enzymes secreted during formation of neutrophil extracellular traps, results in tissue damage (88). In addition, neutrophils were found to secrete corticostatins, e.g.,  $\alpha$ -defensins, which substances are known to interfere with ACTH-mediated increase in adrenocortical hormone production (89). In a recent study using developmental endothelial locus 1 (Del-1)-deficient mice (90), an association

between higher amount of infiltrating neutrophils and impaired adrenal corticosterone production after systemic LPS administration was found (91).

### Immune–Adrenal Interaction at the Level of Adrenal Medulla

Activation of the sympathetic nerve system during sepsis results in enhanced production and secretion of CAs from the adrenal chromaffin cells (44) through a mechanism involving an increased release of Ca<sup>2+</sup> from endothelial reticulum (45). An early increase in endogenous CA level plays an important protective role as its absence due to either pharmacological or surgical intervention resulted in induction of hypotension and increased mortality of rats during experimental endotoxemia (20, 43). One of the mechanisms involved in the protective role of CAs, besides well-known vasopressor function, is the anti-inflammatory action on various immune cells. The immunomodulatory effects of CAs are mediated *via* their direct interactions with  $\beta$ -adrenergic receptors (ARs) expressed by a variety of immune cells. In particular, it has been shown that CAs promote IL-10 secretion while decreasing production of pro-inflammatory TNF cytokine in LPS-treated macrophages (92). Furthermore, incubation of neutrophils with CAs decreased NO production and ROS generation (93). However, these immune-suppressive effects of CAs should be carefully interpreted as they may correspond predominantly to the leukocyte populations or be restricted to  $\beta$ -AR activation. In fact, recently both epinephrine and norepinephrine were shown to increase IL-6 production in endothelial cell line: HMEC-1 and in human skin microvascular endothelial cells (94). It has been also found that activation of  $\alpha$ -ARs in macrophages by CA may potentiate inflammation by increasing TNF- $\alpha$  production (95). Recently, phagocytes were shown to be able not only to secrete CAs in response to LPS stimulation but also to possess all major CA-generating and -degrading enzymes required for their production and inactivation (96). This discovery adds additional complexity to already complex immune–chromaffin cell interaction. During sepsis, the adrenal medulla shows high degree of immune infiltration (69). This suggests that overactivation of immune cells could potentially impair production and secretion of adrenomedullary hormones or induce structural damage. Indeed, during experimental sepsis induced by CLP, systemic inflammation resulted in a strong apoptosis in the adrenal chromaffin cells, which process required activation of receptors for complement C5a (97). The latter observation suggests that during progression of sepsis, an increased activation of immune system promoting apoptosis of chromaffin cells may impact stress-induced CA production.

### Adrenal Vasculature

Vascular system plays a key role in the proper functioning of many vital organs during sepsis. Besides enabling the secretion of steroid hormones and CAs into circulation, adrenal vasculature was found to control leukocyte infiltration through expression of adhesion molecules and Del-1 protein (91).

Adrenal cortex is characterized by high density of endothelial cells that stay in close vicinity with steroid-producing cells. Consequently, both adrenal hormones and endothelial cell-derived products were shown to influence function of adrenal

vasculature and steroid-producing cells, respectively. For example, human adrenocortical cells increase production of aldosterone and cortisol once incubated with endothelial cell-conditioned media. Although the exact composition of the latter media was not determined, this stimulatory effect was found to require cAMP, but not PKA, pathway (98). Endothelial cells are known to produce several factors that, e.g., nitric oxide (NO) or endothelin-1, could potentially influence adrenal hormone production. In particular, in rat and human adrenocortical cells, it has been found that endothelin-1 can directly promote GC synthesis and potentiate angiotensin II and ACTH-induced aldosterone production (99). Besides endothelin-1, nitric oxide (NO) was found to exert stimulatory effect on adrenal steroidogenesis by mediating the acute response to ACTH (28). In fact, blocking of NO production either by using NO synthase inhibitor or NO scavengers resulted in decreased ACTH-mediated corticosterone release (100). However, in other studies using perfused adrenal glands administration of L-arginine, the substrate for nitric oxide synthesis did not change corticosterone response to ACTH, suggesting an indirect modulatory action of NO (101).

During sepsis, in many critically ill patients, an increased size of adrenal gland resulting from the hypervascularization and increased blood flow and adrenal hyperplasia were found to positively correlate with survival rate (102). Although an intact activity of splanchnic nerve is crucial in regulation of adrenal blood flow, other factors such as VIP, NPY, or neuropeptides were found also to be involved (103, 104). Furthermore, a positive correlation between an increased blood flow and enhanced adrenal steroidogenesis was demonstrated (105). Indeed, an enhanced adrenal blood flow was found to participate in angiotensin II- and ACTH-induced aldosterone and cortisol production. This stimulatory action on

adrenal vascular response to angiotensin II and ACTH was found to be mediated by vasoactive substances released from granules of mast cells (106). However, more recently, additional involvement of the cytochrome 450 in controlling the adrenal blood flow after AII and ACTH was demonstrated in rats (24, 107).

## SUMMARY

Sepsis and septic shock strongly impacts body homeostasis, which if not sufficiently counteracted by activated stress system results in increased mortality of critically ill patients (56, 108, 109). During chronic phase of sepsis, elevated adrenal hormone secretion was reported to be mediated by pituitary-independent mechanisms including activation of intra-adrenal microenvironment (22, 23). An increasing amount of experimental studies support the key involvement of several cell types and cellular systems present within adrenal gland microenvironment in the sustained production of adrenal hormones during sepsis. Consequently, any impairment in function of these systems can ultimately affect adrenal stress response (2, 24).

## AUTHOR CONTRIBUTIONS

WK, MS, and SB have all made substantial, direct, and intellectual contribution to the work and approved it for publication.

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# Role of ACTH in the Interactive/Paracrine Regulation of Adrenal Steroid Secretion in Physiological and Pathophysiological Conditions

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In the normal human adrenal gland, steroid secretion is regulated by a complex network of autocrine/paracrine interactions involving bioactive signals released by endothelial cells, nerve terminals, chromaffin cells, immunocompetent cells, and adrenocortical cells themselves. ACTH can be locally produced by medullary chromaffin cells and is, therefore, a major mediator of the corticomedullary functional interplay. Plasma ACTH also triggers the release of angiogenic and vasoactive agents from adrenocortical cells and adrenal mast cells and, thus, indirectly regulates steroid production through modulation of the adrenal blood flow. Adrenocortical neoplasms associated with steroid hypersecretion exhibit molecular and cellular defects that tend to reinforce the influence of paracrine regulatory loops on corticosteroidogenesis. Especially, ACTH has been found to be abnormally synthesized in bilateral macronodular adrenal hyperplasia responsible for hypercortisolism. In these tissues, ACTH is detected in a subpopulation of adrenocortical cells that express gonadal markers. This observation suggests that ectopic production of ACTH may result from impaired embryogenesis leading to abnormal maturation of the adrenogonadal primordium. Globally, the current literature indicates that ACTH is a major player in the autocrine/paracrine processes occurring in the adrenal gland in both physiological and pathological conditions.

**Keywords:** ACTH, aldosterone, cortisol, Cushing's syndrome, aldosterone-producing adenoma, hyperplasia, adrenocortical cells, VEGF

## INTRODUCTION

The adrenal cortex is a heterogeneous tissue that not only contains steroidogenic cells but also hosts various cell types that are able to locally release a wide variety of bioactive signals. This histological organization results in a complex interactive network that participates in the regulation of both basal and ACTH-induced corticosteroidogenesis. The intracortical sources of regulatory factors include chromaffin cells arranged in cords or islets, nerve fibers originating from extraadrenal neurones or cell bodies located in the adrenal medulla, cells of the immune system, including lymphocytes,

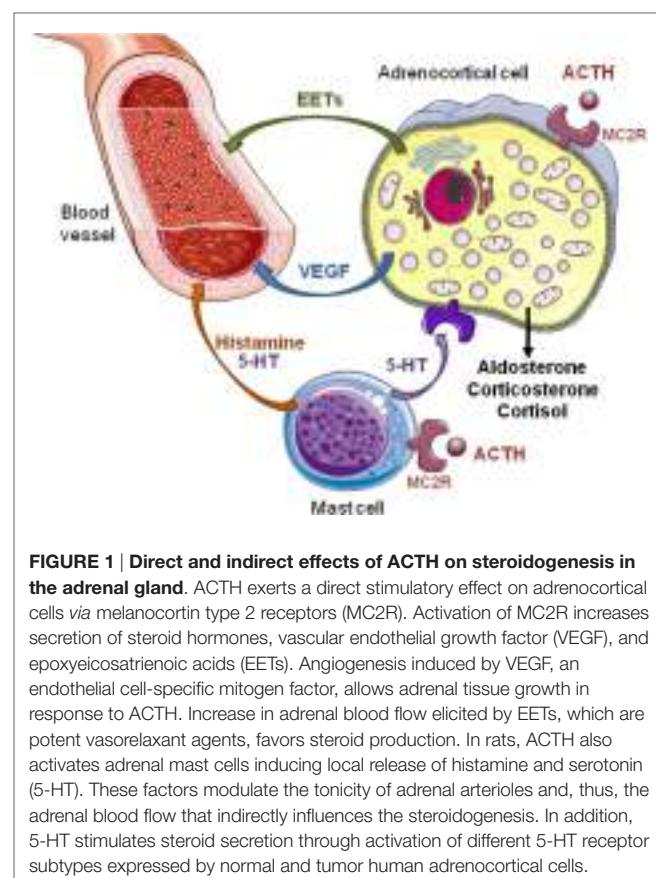
macrophages/monocytes and mast cells, endothelial cells, and adipocytes. These autocrine/paracrine mechanisms have been extensively reviewed during the past years (1–5). However, their physiological role, especially their exact contribution to the regulation of corticosteroid synthesis remains a matter of debate although some data indicate that intraadrenal regulatory signals may mediate parts of the biological effects of ACTH on the adrenal cortex. Interestingly, adrenocortical neoplasms associated with steroid hypersecretion exhibit molecular and cellular defects that tend to reinforce the potency of paracrine factors to activate corticosteroidogenesis. For instance, abnormal expression of ACTH has been reported in adrenocortical cells in both tumors and hyperplasias responsible for hypercortisolism (6–11).

In the present review article, we have updated the current knowledge on the role of ACTH in the cell-to-cell communication processes occurring in the adrenal cortex in both physiological and pathological conditions. We will also discuss the potential interest of the intraadrenal ACTH regulatory loop for the clinical management of patients with primary adrenal excess of corticosteroids.

## EFFECT OF ACTH ON THE ADRENAL VASCULATURE

The adrenal cortex is a richly vascularized organ. This extensive vasculature is essential for delivery of tropic hormone and steroid hormones precursors to the gland and secretion of mature hormones into the blood flow. Furthermore, the establishment of such dense vascular network ensures that every adrenocortical cell is in contact with at least one endothelial cell (12, 13). This remarkable histological organization allows paracrine regulation of adrenocortical cells by endothelial cells through release of endothelins, adrenomedullin, nitric oxide, and prostacyclin (14). For instance, Rossi et al. have shown that endothelin-1 released by endothelial cells is an important regulator of aldosterone secretion, and may then indirectly influence arterial blood pressure (15, 16). The release of endothelin by adrenocortical sinusoids is thought to mediate the modulation of adrenal steroidogenesis by the adrenal blood (17). Interestingly, ACTH appears able to both act on development and maintenance of the adrenal vasculature and regulate the adrenal blood flow (18, 19), influencing thus steroid production through an indirect effect in addition to its intrinsic steroidogenic action on adrenocortical cells (**Figure 1**).

The modulation of adrenal angiogenesis by ACTH may involve several bioactive signals. Thrombospondins (TSPs) represent a wide family of extracellular proteins consisting of five members, TSP1–5, which can bind multiple cell surface molecules, including heparin sulfate proteoglycans, low-density lipoprotein receptor-related protein, integrins, CD36, and CD47 (20). Owing to the great diversity of their binding partners, TSPs are involved in various biological processes, such as cell adhesion, spreading and migration, and angiogenesis (20, 21). In this respect, TSPs are known to inhibit angiogenesis by preventing migration of capillary endothelial cells. Interestingly, adrenocortical cells release high amounts of TSP2 in response to ACTH (22), suggesting that TSP2 may mediate some of the biological actions of



**FIGURE 1 | Direct and indirect effects of ACTH on steroidogenesis in the adrenal gland.** ACTH exerts a direct stimulatory effect on adrenocortical cells via melanocortin type 2 receptors (MC2R). Activation of MC2R increases secretion of steroid hormones, vascular endothelial growth factor (VEGF), and epoxyeicosatrienoic acids (EETs). Angiogenesis induced by VEGF, an endothelial cell-specific mitogen factor, allows adrenal tissue growth in response to ACTH. Increase in adrenal blood flow elicited by EETs, which are potent vasorelaxant agents, favors steroid production. In rats, ACTH also activates adrenal mast cells inducing local release of histamine and serotonin (5-HT). These factors modulate the tonicity of adrenal arterioles and, thus, the adrenal blood flow that indirectly influences the steroidogenesis. In addition, 5-HT stimulates steroid secretion through activation of different 5-HT receptor subtypes expressed by normal and tumor human adrenocortical cells.

ACTH in the adrenal cortex, especially centripetal adrenocortical cell migration, which is a fundamental process in the dynamic organization and remodeling of the adrenal cortex (23). However, the physiological role of TSP2, which is primarily expressed in zona glomerulosa and zona fasciculata, remains unclear since TSP2-null mice exhibit no alteration in corticosteroid secretion or adrenal development (24). In addition, syndromes of ACTH excess, including Cushing's disease and 21-hydroxylase deficiency, are associated with adrenocortical hyperplasia, a process that underlies active angiogenesis. Conversely, several observations indicate that vascular endothelial growth factor (VEGF) plays a pivotal role in the trophic effects of ACTH on the adrenal vasculature (25). VEGF is a widely expressed cytokine that acts as an endothelial cell-specific mitogen and angiogenic factor. In the bovine adrenal gland, VEGF is expressed in zona glomerulosa and zona fasciculata cells and its release is stimulated by ACTH (26). Upregulation of VEGF by ACTH has also been reported in human adrenal (27). The effect of ACTH on adrenal VEGF production involves transcription-independent mechanisms, including stabilization of VEGF mRNA by the HuR protein (28). Conversely, ACTH suppression by dexamethasone in mice results in progressive decrease of VEGF expression in adrenocortical cells and regression of the vascular network (29). Interestingly, ACTH also stimulates VEGF expression in human fetal adrenocortical cells, suggesting that VEGF is an important mediator of the trophic action of ACTH during the adrenal development (30).

Consistently, an increase in VEGF expression in adrenocortical cells has been noticed in the regenerating adrenal cortex in rats (31, 32). It is noteworthy that, in parallel to its effect on adrenal vasculature development *via* local synthesis of VEGF, ACTH also favors adrenal tissue growth through an antiapoptotic action on adrenocortical cells (29) and stimulation of synthesis of growth factors (30). Finally, VEGF may be involved in the capacity of ACTH to induce endothelial fenestration, a phenomenon that favors cell-to-cell interactions (26, 33).

The mechanism by which ACTH modulates the adrenal blood flow is obviously not univocal. Adrenocortical cells themselves are able to release vasorelaxant agents in response to ACTH. These compounds include metabolites of arachidonic acid, such as epoxyeicosatrienoic acids (EETs) (34). In addition, in the rat adrenal gland, capsular mast cells modulate the tonicity of adrenal arterioles and, thus, the adrenal blood flow through local release of histamine and serotonin (5-hydroxytryptamine; 5-HT) (17, 35). Interestingly, rat adrenal mast cells are sensitive to the action of ACTH, suggesting that they may represent an important intermediate in the effect of corticotropin on the adrenal blood flow (35). In humans, the presence of mast cells has been reported in both the subcapsular region of the normal adrenal gland (36) and various types of adrenocortical tumors, including deoxycorticosterone-secreting tumors, aldosterone-producing adenomas (APAs), and adrenocortical carcinomas (37–39). However, there is currently no data reported in the literature indicating that human adrenal mast cells are target cells for ACTH.

## INDIRECT EFFECTS OF ACTH ON CORTICOSTEROIDOGENESIS THROUGH ADRENOCORTICAL CELL SECRETORY PRODUCTS

Adrenocortical cells are important sources of bioactive compounds that are able to modulate steroidogenesis through autocrine/paracrine actions. In man, it is doubtful that corticosteroid may affect their own secretion in physiological conditions. Conversely, the adrenal cortex is known to express the diverse components of the renin–angiotensin system, leading to local synthesis of angiotensin II and potential autocrine/paracrine stimulation of aldosterone secretion (40). Interestingly, renin production, which primarily occurs in zona glomerulosa cells, is stimulated by ACTH and reduced by hypophysectomy or dexamethasone administration (41, 42). This observation may explain why plasma renin levels are usually not suppressed in patients with overt ACTH-dependent hypercortisolism (43, 44). In fact, it is conceivable that, in this condition, ACTH-induced adrenal renin secretion may compensate inhibition of renal renin synthesis by hypervolemia and hypertension secondary to cortisol excess.

Adrenocortical cells also synthesize and release various cytokines, such as interleukin-1 (IL-1), IL-3, IL-6, and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) (1). These signals may influence the steroidogenic and mitogenic activities of adrenocortical cells *via* autocrine/paracrine processes (5). Indeed, ACTH has been shown to modulate cytokine production by adrenocortical cells, either positively (IL-6) or negatively (TNF- $\alpha$ ) (45, 46). Intraadrenal

cytokines may, thus, represent important effectors of ACTH capable of potentiating or attenuating its action on adrenocortical cells. However, there is currently no evidence that the impact of ACTH on adrenal cytokine synthesis may be involved in the pathogenesis of ACTH-dependent adrenal hyperplasia and/or hypercortisolism.

Several types of adrenocortical neoplasms are associated with illicit neuroendocrine differentiation of adrenocortical cells (47). This is especially true for APAs that have been shown to express synaptophysin, neuronal cell adhesion molecule, neuron specific enolase, and SV2 (48, 49). In addition, we have observed that APA cells may also abnormally synthesize 5-HT that is able to stimulate aldosterone secretion through activation of the overexpressed serotonergic type 4 (5-HT4) receptor (50, 51). Because APA cells also express high amounts of the ACTH receptor, i.e., the melanocortin receptor type 2 (MC2R) (51–53), it is conceivable that locally produced 5-HT may act as an amplifier of the stimulatory effect of ACTH on aldosterone secretion by APA tissues.

Inhibins and activin are dimeric peptides belonging to the TGF- $\beta$  family. Inhibins are formed by combination of the  $\alpha$ -subunit encoded by INHA and A or B isoform of the  $\beta$ -subunit, encoded by INHBA and INHBB, respectively. Alternatively, activin is a homodimer composed of two  $\beta$ -subunits. The action of activin is mediated by its specific receptors type I and II, and the intracellular proteins SMAD. Inhibins counteract the biological effects of activin by antagonizing activin type II receptor and formation of an inactive complex with the TGF $\beta$  type III receptor  $\beta$ -glycan. Adrenocortical cells are able to express both  $\alpha$  and  $\beta$  subunits (54–56). In particular, the  $\alpha$ -inhibin is expressed in the zona reticularis under the positive control of ACTH, whereas  $\beta$ -subunits are mainly present in the outer cortex. Both activin receptors and the inhibin co-receptor  $\beta$ -glycan are also detected in the adrenal cortex (55, 56). It has been demonstrated that ACTH stimulates secretion of inhibin A and B, without modifying production of activin A (55). These data indicate that ACTH also controls corticosteroidogenesis through modulation of the intraadrenal activins/inhibins ratio.

## INDIRECT EFFECTS OF ACTH ON CORTICOSTEROIDOGENESIS THROUGH NON-STEROIDOGENIC ADRENAL CELLS

The adrenal gland is surrounded by adipose tissue and its cortical region contains adipocytes either isolated or arranged in small islets (57). Like cells of the immune system, adipocytes release a wide panel of cytokines, suggesting that they could influence the adrenocortical function through a cell-to-cell communication process. For instance, adipocytes have been shown to activate aldosterone release by secreting soluble bioactive factors, which have not been yet characterized (57–59). Conversely, leptin exerts an inhibitory action on ACTH-induced corticosteroid secretion in human adrenocortical cells without affecting their viability and proliferation (60–63). It is possible that peri- and intraadrenal adipocytes may be controlled by ACTH and, thus, constitute a relay in the action of the hormone on the adrenal cortex. In support of this hypothesis, it has been reported that

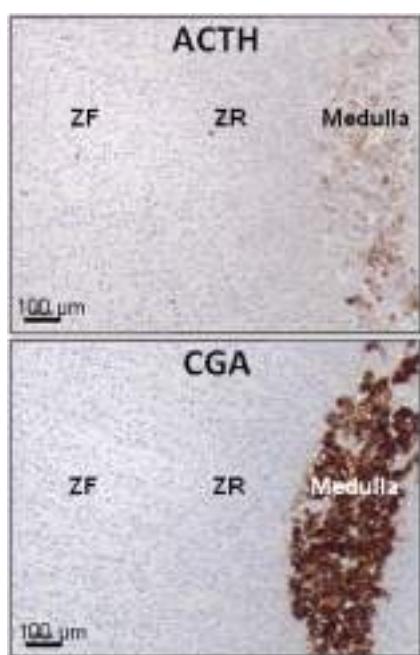
murine adipocyte cell lines and immortalized adipocytes express the MC2R, and ACTH regulate adipocyte functions in these models (64–66). In addition, patients with congenital adrenal hyperplasia due to 21-hydroxylase deficiency, a condition which is associated with chronically high plasma ACTH levels, can present with adrenal myelolipoma (67–69), suggesting a role of ACTH in the development of lipomatous tissue inclusions in the adrenal glands. However, in contrast to the observations made in murine cells, human mature adipocytes only express low levels of the MC2R and ACTH does not influence lipolysis in the mature human adipose tissue (70).

## PARACRINE CONTROL OF ADRENAL STEROIDOGENESIS BY INTRAADRENAL ACTH

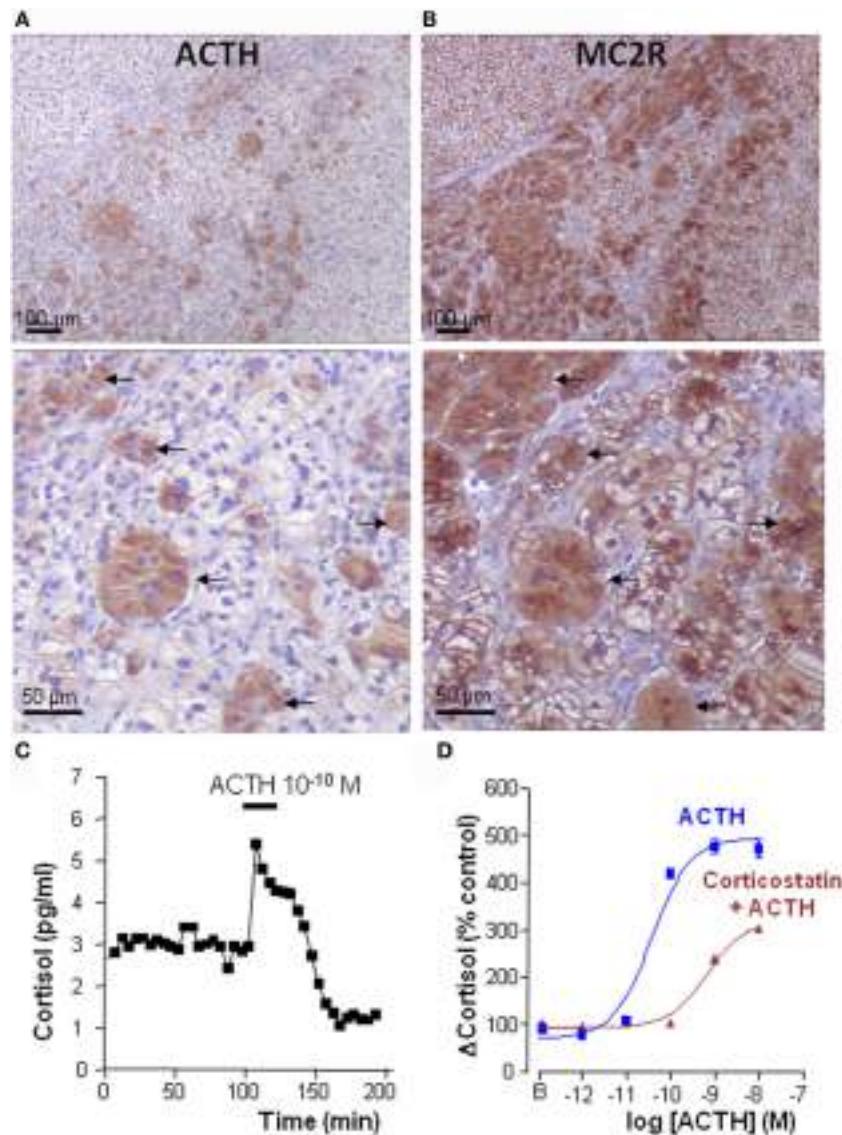
In the normal adrenal gland, chromaffin cells release detectable amounts of ACTH that is a major mediator in the corticomedullary functional interaction (71, 72) (**Figure 2**). This secretory process can be activated by corticotropin-releasing hormone (CRH) that is expressed in the adrenal medullary tissue (11). As chromaffin cells are also regulated by splanchnic nerves and proinflammatory cytokines, it seems possible that they may be important intermediates in the activation of the adrenal cortex during stress and inflammation. However, the physiological role of the paracrine control of corticosteroidogenesis by adrenomedullary ACTH remains unclear (3).

Conversely, clinicopathological studies have shown that paracrine interactions involving ACTH produced by chromaffin cells may play a role in the pathogenesis of hypercortisolism. Pheochromocytoma can occasionally produce ACTH resulting in ACTH-dependent Cushing's syndrome (73–75). Hypercortisolism seems to be mainly the consequence of the endocrine corticotrophic action of ACTH whose plasma levels are typically elevated in this situation (76). Nevertheless, this mechanism may not be exclusive. In fact, hyperplasia of the adrenal cortex adjacent to pheochromocytoma has also been observed, indicating that ACTH originating from the pheochromocytoma tissue can stimulate adrenocortical cells in a paracrine manner (11, 77–80). Such a histological pattern is close to what is observed in corticomедullary mixed tumors that are composed of intermingled adrenocortical and pheochromocytoma tissues (81–84). These rare tumors are sometimes associated with hypercortisolism, suggesting that pheochromocytes release paracrine signals capable of activating glucocorticoid synthesis (81, 84). ACTH could be one of them although it is not excluded that catecholamines may exert a stimulatory action on cortisol production through illicit expression of adrenergic receptors in tumor adrenocortical cells (85, 86).

Alternatively, the occurrence of ectopic production of ACTH in the adrenal cortex has already been reported. Very rarely, ACTH-positive cells in the adrenocortical tissue can reveal adrenal micro-metastases of an ACTH-secreting cancer. In the published cases, plasma ACTH levels were strongly elevated, as a result of ACTH secretion by the primary tumor (87–89). More surprisingly, a subpopulation of adrenocortical cells has been shown to produce detectable amounts of ACTH in various types of adrenal neoplasms. A first case of adrenocortical cortisol-secreting adenoma associated with production of ACTH by tumor cells, has been described in 2001 by Hiroi et al. (6). Illicit synthesis of ACTH was considered to result from abnormal pituitary differentiation of the tissue as witnessed by co-expression of 17-hydroxylase and pituitary homeobox factor-1 mRNAs by adrenocortical cells (6). At the ultrastructural level, tumor cells exhibited characteristics of both steroidogenic cells and neuroendocrine cells, and the tumor was, thus, referred to as an adrenocortical–pituitary hybrid adenoma. Bilateral macronodular adrenal hyperplasia (BMAH), a rare cause of primary adrenal hypercortisolism, has also been found to contain ACTH-producing cells. This observation was first reported by Pereira et al. who also noticed that ACTH-positive cells were labeled by antibodies to chromogranin A (CGA), suggesting that these cells may correspond to intracortical chromaffin cells (90). Subsequently, several teams reported expression of proopiomelanocortin (POMC) and ACTH in groups of adrenocortical cells in isolated BMAH cases (8, 10, 91) (**Figure 3**). In one case, it could be shown that ACTH-positive cells also expressed 17-hydroxylase but were negative for pituitary corticotroph markers (8). The role of ACTH in the pathogenesis of BMAH has been more extensively investigated in a large series of 30 cases (9). Adrenal hyperplasia samples expressed POMC mRNA at variable levels. Proconvertase 1, a protease implicated in the maturation of POMC into ACTH, was also visualized in clusters of adrenal cells, indicating that ACTH could be generated from POMC in the BMAH tissues. In agreement with this



**FIGURE 2 |** Presence of ACTH in the normal human adrenal. ACTH immunoreactivity is exclusively located in some chromaffin cells (high panel), identified as chromogranin A (CGA) immunoreactive cells (low panel), in the medulla. ACTH was detected by using antibodies against the N-terminal region of the peptide. ZF, zona fasciculata; ZR, zona reticularis. Illustration of the data published in Ref. (9).



**FIGURE 3 |** Intraadrenal ACTH regulation of cortisol secretion in bilateral macronodular adrenal hyperplasia (BMAH). **(A,B)** Consecutive sections of a BMAH tissue labeled with ACTH and MC2R antibodies. **(A)** Heterogeneous distribution of ACTH-producing cell clusters in BMAH. **(B)** The ACTH receptor MC2R is highly expressed in ACTH-producing cells (arrows) and in their vicinity. **(C)** ACTH sensitivity of BMAH tissues. Application of exogenous ACTH stimulated cortisol secretion by perfused BMAH explants. **(D)** Corticostatin, a MC2R antagonist, inhibited *in vitro* the cortisol response of cultured BMAH cells to ACTH. Illustration of the data published in Ref. (9).

finding, ACTH immunoreactivity was detected, as previously noticed, in some adrenocortical cells either isolated or arranged in small groups disseminated in the tissues. The presence of corticotropin can also be seen in some chromaffin cells of the adrenal medulla. Surprisingly, adrenocortical ACTH-positive cells also displayed characteristics of steroidogenic cells, such as the presence of numerous lipid inclusions and several markers of steroidogenic differentiation, including steroidogenic factor 1 (SF1), 17-hydroxylase, and the HDL-cholesterol receptor scavenger receptor B1 (SRB1). It could be, thus, concluded that they constitute a subcategory of adrenocortical steroidogenic cells characterized by an unusual capacity to synthesize ACTH.

BMAH specimens were found to express very low levels of T-pit [a transduction factor which controls pituitary corticotrophs differentiation (92)], confirming that ectopic synthesis of ACTH in adrenocortical cells does not result from illicit corticotrophic-like differentiation of the latter (9) but may rather be considered as an additional feature of neuroendocrine differentiation of the hyperplastic tissues (47, 93, 94). Interestingly, ACTH-containing cells were also positive for gonadal markers like the gonadal marker insulin-like 3 (INSL3), exhibiting thus a pseudo-gonadal phenotype (8, 9, 95, 96). This observation is concordant with expression of POMC and synthesis of ACTH previously reported in testicular Leydig cells and ovarian granulosa cells (97, 98).

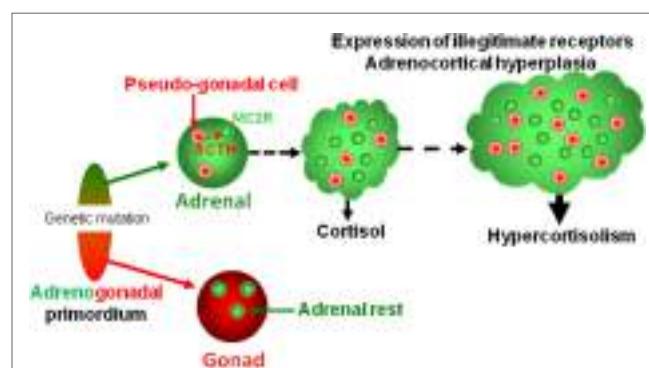
Because the adrenal glands and gonads both originate from a same tissue precursor, the adrenogonadal primordium, it is likely that the occurrence of these pseudo-gonadal cells in BMAH tissues may be the consequence of altered embryogenesis, explaining the bilaterality of the lesions.

Perfusion studies revealed that ACTH is released by BMAH tissues *in vitro* in a pulsatile mode (9), in agreement with older clinical studies showing a clear pulsatility of cortisol secretion in patients with BMAH (99). The ectopic secretion of ACTH by the hyperplastic adrenal glands has also been detected *in vivo* in two patients through adrenal vein catheterization (9). Taken collectively, these data suggested that, in BMAH tissues, intraadrenal ACTH may exert an autocrine/paracrine action to stimulate cortisol secretion, supplying therefore circulating ACTH that is suppressed by cortisol excess. This hypothesis was supported by statistical analyses showing positive correlations between ACTH and cortisol levels in BMAH culture medium. In addition, basal plasma cortisol concentrations measured *in vivo* were positively correlated with both the levels of POMC mRNA and the ACTH histological score in the tissues. Most importantly, MC2R antagonists, such as corticostatin and ACTH (7–38), were found to inhibit *in vitro* spontaneous and ACTH-evoked cortisol secretion by BMAH explants (9) (**Figure 3**). Clinical studies with MC2R antagonists are now mandatory to confirm that cortisol production is actually dependent on intraadrenal ACTH in patients with BMAH.

Bilateral macronodular adrenal hyperplasia tissues also constitute an interesting model for the study of the regulation of MC2R expression in the adrenal cortex. MC2R mRNA is globally underexpressed in BMAH samples versus normal adrenals (100). In fact, we could observe that, at variance with the normal adrenal cortex that is diffusely labeled by anti-MC2R antibodies, BMAH explants exhibit heterogeneous distribution of the receptor that appears highly expressed in the vicinity of clusters of ACTH-producing cells and more weakly at distance (**Figure 3**). Indeed, as previously established in the normal adrenal gland (101–103), MC2R seems to be upregulated by ACTH in BMAH tissues. In fact, MC2R mRNA levels were positively correlated with POMC mRNA rates and MC2R-like immunoreactivity was principally visualized in the vicinity of ACTH-positive cells (9). Interestingly, ACTH-producing cells were also found to express the receptor, suggesting that intraadrenal ACTH possibly exerts autocrine actions in BMAH.

All these data indicate that intraadrenal ACTH plays a pivotal role in the pathogenesis of hypercortisolism associated with BMAH. Deciphering the mode of regulation of ACTH production by BMAHs is, thus, essential for the comprehension of the pathophysiology of the disease. At variance with pituitary ACTH, intraadrenal ACTH does not seem to be regulated by cortisol, as suggested by the lack of action of dexamethasone and the glucocorticoid receptor antagonist RU486 on ACTH release by BMAH explants (9). Conversely, we noticed that ligands of various membrane receptors that are known to be abnormally expressed by BMAH cells, i.e., 5-HT, LH/hCG, and glucose-dependent insulinotropic peptide (GIP), are able to activate ACTH production from BMAH tissues *in vitro* (9). This surprising finding indicated that activation of illicit

membrane receptors may stimulate cortisol production *via* two mechanisms, including a direct effect on corticosteroidogenesis, as previously shown in BMAH cell culture (93, 104), and an indirect action mediated by ACTH secretion (9). Consistently, MC2R antagonists were found to partially inhibit *in vitro* the cortisol response evoked by GIP in perfused BMAH samples. Taken together, these results suggest that intraadrenal ACTH may be regarded as a common intermediate and amplifier of the action of several illicit membrane receptors in BMAH tissues. Targeting the MC2R with specific antagonists may, thus, represent an efficient strategy for the treatment of BMAH-associated hypercortisolism. The pathophysiology links between intraadrenal ACTH and abnormally expressed receptors may be more complex and could form a complete auto-amplification loop in the hyperplastic tissues. In fact, although the presence of the LH and GIP receptors in adrenocortical cells may be simply considered as features of pseudo-gonadal differentiation of BMAH tissues, overexpression of some membrane receptors, such as 5-HT receptors, may result from local production from ACTH. This hypothesis is supported by intriguing observations recently performed in another type of adrenal hyperplasia associated with hypercortisolism, namely primary pigmented adrenocortical disease (PPNAD). The disease is caused in most patients by germline inactivating mutations that affect the PRKAR1A gene (105), resulting in constitutive activation of protein kinase A (PKA) in adrenocortical cells (106). Since MC2R are positively coupled to the cAMP/PKA pathway, it can be considered that PRKAR1A mutations partly mimic the action of ACTH on adrenal steroidogenic cells. Interestingly, PPNAD have been found to overexpress several types of 5-HT receptors, including the



**FIGURE 4 | Putative pathophysiological mechanism responsible for bilateral macronodular adrenal hyperplasia.** Both gonads and adrenals originate from the adrenogonadal primordium. It can be speculated that the causative genetic mutations may alter differentiation and/or separation of the adrenogonadal primordium leading to the presence of pseudo-gonadal cells in the adrenals. Secretion of ACTH by intraadrenal pseudo-gonadal cells may progressively stimulate, *via* activation of MC2R, both cortisol secretion and growth of the adrenocortical tissue, leading to bilateral adrenal hyperplasia associated with hypercortisolism. In parallel, sustained activation of the PKA pathway consecutive to activation of MC2R by intraadrenal ACTH may activate expression of some illegitimate receptors. Activation of steroidogenesis by ligands of illegitimate receptors further reinforces cortisol hypersecretion through both an intrinsic stimulatory action and an indirect effect *via* local release of ACTH.

5-HT<sub>4</sub> receptor (107). It seems, thus, conceivable that abnormal expression of 5-HT receptors in BMAH tissues may result from exposure of adrenocortical cells to locally produced ACTH whose release is subsequently increased by the 5-HT signaling pathway.

There is now no doubt that BMAH is a genetically determined condition, *ARMC5* being a major susceptibility gene of the disease (108). It is now well established that the development of macronodular adrenal hyperplasia requires inactivation of the two *ARMC5* alleles, respectively, by the first germline mutation and a secondary somatic genetic event. Surprisingly, inactivation of *ARMC5* expression in the human adrenocortical cell line H295R, which reproduces the molecular defects observed in adrenocortical cells of patients with BMAH, results in a decrease in expression of steroidogenic enzymes (108, 109). It seems, thus, that a second line event is necessary for the emergence of hypercortisolism. We hypothesize that *ARMC5* mutations may alter differentiation and/or separation of the adrenogonadal primordium leading to the presence of pseudo-gonadal cells in the adrenal areas. Progressive expression of POMC and ACTH by these cells may then result in cortisol hypersecretion. Because illicit expression of ACTH has also been observed in non-*ARMC5*-mutated adrenal hyperplasias, it seems that abnormal differentiation of adrenocortical cells is a frequent histological feature in BMAH tissues whatever the causative firstline genetic defect (**Figure 4**).

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## CONCLUSION

In addition to its well-known action on adrenocortical cells to promote steroidogenesis through activation of the MC2R, ACTH exerts multiple effects through the paracrine communication processes that occur in the adrenal gland in both physiological and pathophysiological conditions. ACTH can also be abnormally produced in adrenal neoplasms in which the hormone acts, thus, as an autocrine/paracrine factor to activate steroid secretion. This new pathophysiological concept opens novel avenues for the development of original pharmacological treatments of primary adrenal syndromes of steroid excess.

## AUTHOR CONTRIBUTIONS

HL, MT, and EL: wrote the paper. CD, EL: acquisition, analysis, and interpretation of data. EL: designed the figures. JB: revised the paper and gave intellectual contribution.

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# Effects of Chronic ACTH Excess on Human Adrenal Cortex

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Chronic ACTH excess leads to chronic cortisol excess, without escape phenomenon, resulting in Cushing's syndrome. Excess adrenal androgens also occur: in females, they will overcompensate the gonadotrophic loss, inducing high testosterone; in males, they will not compensate it, inducing low testosterone. Chronic ACTH excess leads to chronic adrenal mineralocorticoid excess and low aldosterone levels: after an acute rise, aldosterone plasma levels resume low values after a few days when ACTH is prolonged. Two other mineralocorticoids in man, cortisol and 11 deoxycorticosterone (DOC), at the zona fasciculata, will not escape the long-term effect of chronic ACTH excess and their secretion rates will remain elevated in parallel. Over all, the concomitant rise in cortisol and 11 DOC will more than compensate the loss of aldosterone, and eventually create a state of chronic mineralocorticoid excess, best evidenced by the accompanying suppression of the renin plasma levels, a further contribution to the suppression of aldosterone secretion. Prolonged *in vivo* stimulation with ACTH leads to an increase in total adrenal protein and RNA synthesis. Cell proliferation is indicated by an increase in total DNA the resulting adrenocortical hyperplasia participates in the amplified response of the chronically stimulated gland, and the weight of each gland can be greatly increased. The growth-stimulatory effect of ACTH *in vivo* most likely proceeds through the activation of a local and complex network of autocrine growth factors and their own receptors; a number of compounds, including non-ACTH proopiomelanocortin peptides such as  $\gamma$ 3-MSH, have been shown to exert some adrenocortical growth effect.

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## INTRODUCTION

The pituitary–adrenal axis is a central actor in Endocrinology. Through it, the fine tuning of corticosteroids secretion is maintained, from fetal to adult life, under basal and stressful conditions, with immediate and/or long-term consequences. Altered ACTH secretion induces catastrophic clinical situations: adrenal insufficiency on the one hand, Cushing's syndrome on the other hand. Both are debilitating conditions that severely alter the quality of life, create multiple complications, and, ultimately may lead to premature death. Besides ACTH-dependent Cushing's syndrome, there are many situations where ACTH is chronically oversecreted. This review will examine the effects of chronic ACTH excess on adrenal cortex in man, and concentrate on steroid secretion and adrenal cortex growth.

Rare and particular situations will also be addressed.

## THE VARIOUS SITUATIONS IN MAN WITH CHRONIC ACTH EXCESS

The many situations that are associated with chronic ACTH excess in man are presented in **Table 1**. Depending on the «quality» of the original adrenal cortex they can be artificially distinguished in three different groups.

### There Are Three Situations Where the Adrenal Cortex Is Originally, Functionally, and Anatomically Normal

- Healthy volunteers administered with exogenous ACTH or its synthetic analog Cortrosyn (ACTH<sub>1-24</sub>).
- Patients with ACTH-dependent Cushing's syndrome, either Cushing's disease or the ectopic ACTH secretion syndrome. Excess ACTH is chronically produced by a pituitary or a non-pituitary tumor and acts on a basically normal adrenal cortex.
- Patients with the syndrome of general resistance to glucocorticoids.

These three situations allow measuring the effects of chronic ACTH excess on both corticosteroid secretions and adrenal cortex growth.

### There Are Two Situations Where the Adrenal Cortex Has, Congenital or Acquired, Intrinsic Steroidogenic Defects

- The various types of congenital adrenal hyperplasias (CAHs) associated with altered cortisol synthesis.
- Treatment of ACTH-dependent Cushing's syndrome with anticortisol drugs that inhibit steroidogenesis (metyrapone, ketoconazole, etomidate, aminoglutethimide, LCI699).

**TABLE 1 | Conditions with chronic ACTH excess in man.**

#### Normal adrenal cortex<sup>a</sup>

ACTH/Cortrosyn administration to normal volunteers  
ACTH-dependent Cushing's syndrome

Cushing's disease; ectopic ACTH secretion syndrome  
General resistance to glucocorticoids

#### Abnormal adrenal cortex: disorders of steroid synthesis<sup>b</sup>

Congenital adrenal hyperplasia  
21 Hydroxylase-, 11 hydroxylase-, 17 hydroxylase deficiencies  
Inhibitors of adrenal steroidogenesis  
Metyrapone, ketoconazole, etomidate, aminoglutethimide, LCI699

#### Abnormal adrenal cortex: «missing» adrenal glands

Acquired destruction  
Addison's disease  
Bilateral adrenalectomy  
Adrenolytic drugs (*o,p'*DDD)  
Congenital developmental defects

<sup>a</sup>Model to study both the functional and anatomical effects of chronic ACTH excess.

<sup>b</sup>Model to study the anatomical effects of chronic ACTH excess.

In both situations, chronic ACTH excess is an adapted response to chronic cortisol deprivation. They preclude studying the effects of chronic ACTH excess on corticosteroid secretions. Still the effects on adrenocortical growth can be evaluated in a pertinent fashion.

### In the Last Situations, the Adrenal Cortex Is Simply—And Anatomically—Missing or Compromised

- Bilateral adrenalectomy, usually for ACTH-dependent Cushing's syndrome.
- Adrenolytic treatments (*o,p'*DDD) of ACTH-dependent Cushing's syndrome.
- Acquired adrenal destructions (infections, hemorrhages, autoimmune, bilateral metastases...).
- Congenital developmental defects (genetic syndromes).

Evidently, none of these situations help to study the «adrenal» effects of chronic ACTH excess. They would be, however, adapted to study the non-adrenal effects of chronic ACTH—and proopiomelanocortin (POMC)-related peptides excess. Indeed, by opposition with the sole situation where ACTH itself—or its synthetic analog Cortrosyn—are exogenously administered to healthy volunteers, all other situations with chronic excess of endogenous ACTH are accompanied by parallel excess of other non-ACTH POMC-derived peptides.

## CHRONIC ACTH EXCESS IN MAN AND CORTICOSTEROID SECRETION

### Cortisol

#### Chronic ACTH Excess Leads to Chronic Cortisol Excess

Experiments in healthy volunteers, more than half a century ago, have shown the effects of repeated ACTH administrations (1, 2).

- When the same dose of ACTH is exogenously administered daily in man, a stepwise increase in daily cortisol secretion is observed over the days [see Figure 8 in Ref. (1)].
- This «amplifying» phenomenon has now a molecular explanation: adrenocortical cells exposed to ACTH *in vitro* acquire an increased number of ACTH receptors (MC2R) and an increased rate of protein Gs expression (3–6).
- Through the cAMP pathway the binding of ACTH and the transducing apparatus are both amplified, explaining the higher sensitivity and the greater response potential of chronically stimulated cells.
- No escape phenomenon is observed, although the cortisol oversecretion tends to plateau after several days.
- Over a wide range, the cortisol response is proportional to the dose of administered ACTH.

In patients with ACTH-dependent Cushing's syndrome, chronic excess of endogenous ACTH also leads to chronic

cortisol excess. Interestingly in many patients with Cushing's disease, cortisol excess is associated with «normal» ACTH plasma values compared with those seen in the morning in normal subjects (7, 8). These values are considered «abnormally» normal or «inappropriate» in face of the hypercortisolism that should normally totally suppress ACTH secretion. Furthermore, these levels remain constant over the day, with no circadian variation, and ACTH acts on hyperresponsive adrenocortical cells; indeed, acute ACTH stimulation of the hyperplastic adrenals in Cushing's disease patients triggers a much higher and more lasting response than would be observed in normal subjects given the same dose [see Figure 2 in Ref. (2)].

Chronic ACTH excess in case of non-pituitary tumors (the ectopic ACTH secretion syndrome) has the exact same consequences on cortisol secretion. Because ACTH plasma levels are often higher in these patients, they also have—in general—higher cortisol oversecretion (8).

In the syndrome of general resistance to cortisol, the glucocorticoid receptor type 2 is mutated with a loss of function. It is an autosomal dominant familial disease (9–11). All cells and tissues have lost their normal sensitivity to cortisol. At the hypothalamic–pituitary level, it is felt as an apparent lack of cortisol, which—naturally—induces an adapted response with chronic increase in ACTH secretion (12). This natural human situation offers a privileged model to observe the response of a perfectly normal adrenal cortex to chronic ACTH excess: unsurprisingly chronic cortisol excess is observed, but without the clinical features of Cushing's syndrome. Together with cortisol, other ACTH-dependent corticosteroids, androgens dehydroepiandrosterone (DHEA), and mineralocorticoids deoxycorticosterone (DOC) are oversecreted by the zona fasciculata (see further).

A state of chronic and acquired general resistance to cortisol can be artificially created in Cushing's disease patients chronically treated with RU486 (Mifepristone). The drug is an antagonist to the glucocorticoid receptor type 2, the acute administration of which triggers an immediate pituitary ACTH retort in normal subjects (13, 14). As expected, under long-term RU486 administration some clinical peripheral features of the Cushing's syndrome are ameliorated such as hyperglycemia (15); as expected also, in the patients with Cushing's disease, the hypothalamic–pituitary–adrenal axis is stimulated (by the “apparent” cortisol deprivation) and ACTH is acutely and/or chronically increased further with plasma levels raising up *ca.* three times above their baseline initial values (16, 17). In the SEISMIC study, several patients developed high blood pressure, edema, and hypokalemia suggestive of a state of chronic hypermineralocorticism (17). All ACTH-dependent corticosteroids presumably increase in parallel: cortisol, the clinical impact of which remains blunted at the glucocorticoid receptor by the drug; but also adrenal androgens and DOC, the actions of which are not opposed at their respective androgen and mineralocorticoid receptors (for both DOC and cortisol) and which may thus induce serious clinical features: possibly hyperandrogenism in women; hypertension and hypokalemia in both sexes (18).

## Adrenocortical Androgens

### Chronic ACTH Excess Leads to Chronic Adrenal Androgens Excess with Contrasted Impacts in Females and Males

Adrenal androgens are produced by the zona fasciculata/reticularis, and—as cortisol—are under the same—and dominant if not exclusive—control of ACTH and the cAMP pathway. Acute administration to healthy volunteers induces abrupt increase in circulating adrenal androgens (19). The situation has been particularly studied in Cushing's disease patients. The specific adrenal androgen DHEA sulfate (DHEAS) is chronically increased both in males and females (20). Thus DHEA, DHEAS, and Δ-4-androstenedione are elevated in Cushing's disease patients (21–23). Yet, the impact of chronic ACTH excess upon the overall androgenic status is contrasted between females and males:

- In both females and males, chronic excess of androgens, and cortisol, both lead to suppressed gonadotroph function directly at the hypothalamic–pituitary levels.
- In females, because the pituitary gonadotrophic function normally accounts for only half of the total circulating androgens, the excess adrenal androgens will eventually overcompensate the gonadotrophic loss and induce an overall excess of circulating androgens: their peripheral transformation to testosterone and dihydrotestosterone may lead to a moderate state of androgen excess in females with its clinical impact: hirsutism, infertility [see Figure 3 in Ref. (22)].
- In males, because the pituitary gonadotrophic/gonadal function normally accounts for the vast majority of circulating androgens (*ca.* 90%), the excess of adrenal androgens will not compensate that which has been lost due to the cortisol-induced suppression of the gonadotrophic/gonadal function. The overall circulating androgens will eventually be abnormally low (testosterone), with a clinical impact: decreased sexual activity, infertility (24).

In a way similar to that observed with cortisol, chronic increase in adrenal androgens will be observed in other situations with chronic ACTH excess such as the ectopic ACTH secretion syndrome, and the general resistance to glucocorticoids. In the latter situation, as evoked earlier, excess androgens in females may be the dominant symptom that should alert on the diagnosis in the absence of «Cushing's» features; it may provoke precocious puberty in children (12).

In patients with Cushing's disease treated with the antiglucocorticoid RU486, the inescapable rise in ACTH secretion will—theoretically—increase further the state of hyperandrogenism in females and this should be a major drawback for the use of this compound .... Surprisingly, there is no report on the clinical or hormonal androgen status of women in the SEISMIC study (17).

Dissociation between cortisol and adrenal androgens is observed, however, in the particular situation of patients resuming normal corticotroph function after long-term ACTH excess. After successful pituitary surgery in Cushing's disease patients, DHEAS remains suppressed for months or years after plasma cortisol has normalized (21).

## Mineralocorticoids

### Chronic ACTH Excess Leads to Chronic Adrenal Mineralocorticoid Excess and Low Aldosterone Levels

It has long been known that acute ACTH administration to man leads to immediate aldosterone secretion; yet this effect is only transient and is not maintained when ACTH is prolonged over periods of time, and aldosterone plasma levels resume low values after a few days (25, 26).

Yet this “escape” phenomenon on aldosterone will not prevent the establishment of a state of chronic mineralocorticoid excess, for two reasons:

- This “escape” will *only concern aldosterone*, at the zona glomerulosa. Its exact mechanism is not entirely understood: it is suggested that increased concentrations of cortisol in the adrenal cortex directly inactivates the last steps of aldosterone synthesis in the ZG (27); there is also evidence that initial ZG cells undergo a differentiation process toward cortisol producing cells (28).
- Two other mineralocorticoids in man, cortisol and 11 DOC, at the zona fasciculata, will not escape the long-term effect of chronic ACTH excess and their secretion rates will remain elevated in parallel (29).

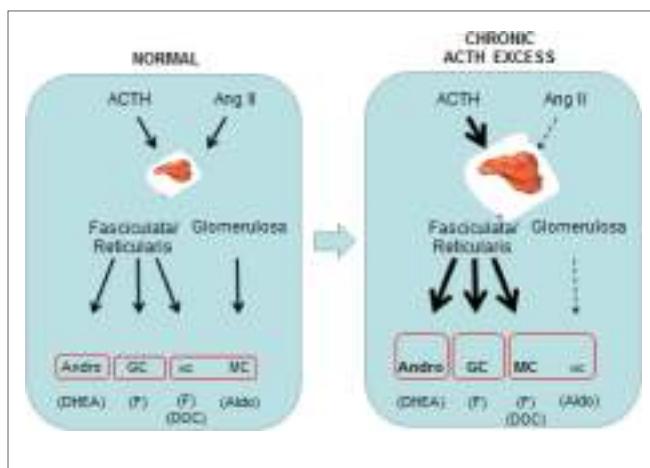
Over all, the concomitant rise in cortisol and DOC, even though each of these molecules is intrinsically less potent than aldosterone at the mineralocorticoid receptor, will more than compensate the loss of aldosterone, and eventually create a state of chronic mineralocorticoid excess, best evidenced by the accompanying suppression of the renin plasma levels, a further—if not exclusive—contribution to the suppression of aldosterone secretion (30). This general mineralocorticoid effect is in correlation with the level of ACTH excess: its clinical consequences (high blood pressure, hypokalemic alkalosis) are more frequent in patients with the ectopic ACTH secretion syndrome than in those with Cushing’s disease (31).

As expected, patients with the syndrome of general resistance to glucocorticoids have parallel increases in cortisol and DOC, and hypokalemic hypertension may be another—sometime predominant—clinical presentation in these patients, as reported in the first published cases (9).

These complications may also occur in patients with Cushing’s disease treated by RU486 (Mifepristone). The drug induces a further increase in cortisol and probably DOC (this latter steroid rarely if ever measured in these patients!), which both can interact, without any opposition, at the mineralocorticoid receptor (18), and see **Figure 1**.

## CHRONIC ACTH EXCESS IN MAN AND ADRENAL CORTEX GROWTH

The central role of ACTH on the adrenal cortex trophicity has long been known. Hypophysectomy results in adrenal cortex atrophy that is restored by the sole administration of ACTH (32). Thus, *in vivo*, ACTH is the predominant if not the

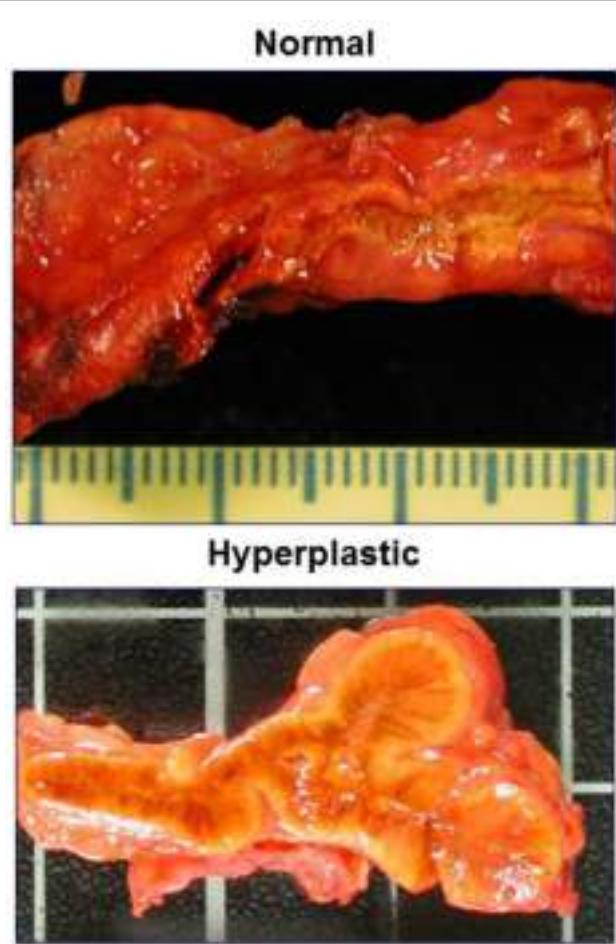


**FIGURE 1 |** Schematic overview of the comparative effects of normal and prolonged ACTH excess in man on adrenal cortical steroids at the zona glomerulosa and the zona fasciculata/reticularis.

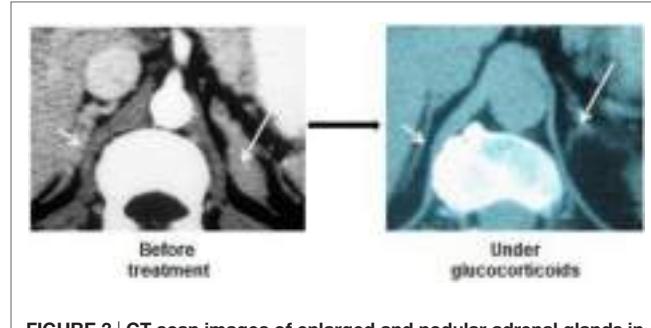
exclusive trophic factor for the adrenals. More recently, various animal models, which eliminate ACTH or its receptor (MC2R) in transgenic mice, have confirmed the central role of ACTH to maintain normal adrenal cortex growth (33–35). Prolonged *in vivo* stimulation with chronic ACTH administration or oversecretion eventually leads to an increase in total adrenal protein and RNA synthesis. Cell proliferation is indicated by an increase in total DNA (32) the resulting adrenocortical hyperplasia participates in the amplified response of the chronically stimulated gland, and the weight of each gland can be greatly increased.

The exact mechanism whereby ACTH promotes adrenocortical growth still is complex and remains partially understood, since *in vitro* studies show a paradoxical negative effect of ACTH on adrenocortical cell proliferation (36). The growth-stimulatory effect of ACTH *in vivo* most likely proceeds through the activation of a local and complex network of autocrine growth factors and their own receptors; a number substances, including non-ACTH POMC peptides such as  $\gamma$ 3-MSH, have been shown to exert some adrenocortical growth effect (28).

In ACTH-dependent Cushing’s syndrome, chronic ACTH excess leads to bilateral adrenal hyperplasia: both adrenals are enlarged, their weight is increased in comparison with normal glands, and the histological appearance shows diffuse widening of the fasciculata/reticularis zona (**Figure 2**). This hyperplasia is typically homogeneous and rather symmetrical. In some cases however, it may be asymmetrical, and/or one or the two glands may bear nodular zones or authentic nodules embedded within the diffuse hyperplasia. Today, CT-Scan allows to see the adrenal hyperplasia *in vivo* in patients with ACTH-dependent Cushing’s syndrome, and to observe its fate after the suppression of ACTH, that is after successful pituitary surgery: the two enlarged glands and the nodules progressively shrink and can even become atrophic until normal ACTH secretion is spontaneously restored, which may take months or years (37, 38). In parallel to these anatomical changes, baseline cortisol and its response to the acute stimulation by ACTH is suppressed, and progressively resume



**FIGURE 2 | Comparing the macroscopic aspect of normal adrenals and those of a Cushing's disease patient.**



**FIGURE 3 | CT-scan images of enlarged and nodular adrenal glands in a patient with 21 hydroxylase deficiency.** Regression under ACTH suppression by large doses of glucocorticoids.

normal testicular tissue, are loaded with the MC2R (40). The close phenotype shared by TART and fetal adrenals, including classical markers of adrenal steroidogenesis, highly favors the hypothesis that TART develops from an original adrenal cortical cell type.

- In Cushing's disease patients, particularly those treated by "total" bilateral adrenalectomy, a large increase in ACTH secretion can be triggered. It may play a role in the growth of adrenal rests, locally (adrenal extrusions that have escaped the surgeon tools!), but also at distance, in the testis, and many other places (41, 42).

## RARE AND PARTICULAR SITUATIONS

### Mimicking Chronic ACTH Excess

There are several pathological conditions where pituitary ACTH is actually suppressed and cortisol is oversecreted in response to molecular phenomenon which occur directly at the adrenal level, and, somehow, mimick chronic ACTH excess:

- ACTH may be produced locally by tumoral adrenal cells, and its autocrine action may participate in cortisol oversecretions in cases of bilateral macronodular adrenal hyperplasia (43).
- In the absence of ACTH, its signaling pathway may be nevertheless overactivated and generate excess of corticosteroid secretion: adrenocortical tumors which express illegitimate G-protein coupled receptors (44, 45), activated mutated MC2R (46), mutated PRKAR1A in the Carney complex (47, 48), and mutated PKACa in adrenocortical adenomas (49).
- It has also been suggested auto antibodies acting at the ACTH receptor might be responsible for excess cortisol secretion in some cases of Wulffraat (50). Yet little confirmation has been obtained since the original paper.

It is interesting to observe that all these situations eventually result in the overactivation of the cAMP signaling pathway, and always occur in benign tumors. They concur with the idea that chronic ACTH excess, or chronic activation of its signaling pathway at the same time may have some growth effect but also a differentiation action.

over months or years. As already mentioned, cortisol response is restored more rapidly than androgen response.

A rather similar presentation is observed in patients with CAH: both glands are enlarged, and become smaller under glucocorticoid administration (**Figure 3**).

Some studies seem to demonstrate that chronic ACTH excess also favors the appearance of adrenocortical nodules, and it has been suggested that some of them ultimately become autonomous (i.e., ACTH-independent). Yet, it is common observation that suppression of chronic ACTH excess, in Cushing's or CAH patients, reduces both the hyperplastic and nodular parts of the enlarged glands (**Figure 3**).

Interestingly, the growth promoting effect of chronic ACTH excess can be exerted also at distance, on adrenal rests:

- Testicular adrenal rests may develop as local tumors which impinge the normal spermatogenesis, and may become cause of infertility in poorly controlled male patients with CAH (39). There is some evidence that lowering ACTH plasma levels may shrink these tumors which, in contrast with the

## When “Normal” ACTH Is Too Much!

In the syndrome of apparent mineralocorticoid excess, the loss of function of 11 hydroxysteroid dehydrogenase type II, particularly at the kidney, locally enhances the mineralocorticoid action of cortisol (51).

In the glucocorticoid-remediable aldosteronism, gene rearrangement induces the “ectopic” expression of the Aldo synthase gene within the ZF cells and thus provokes the oversecretion of aldosterone under the action of ACTH (52).

In these two situations, the HPA axis and cortisol secretion are normal. Yet, it drives a state of “apparent” or “real” mineralocorticoid excess, and the treatment option is indeed to suppress ACTH secretion.

## Non-ACTH POMC Peptides

As mentioned earlier, ACTH is part of a larger polypeptide precursor, POMC the enzymatic processing of which liberates ACTH itself and a number of other “non-ACTH” POMC-derived peptides. Among these peptides, the lipotropins (beta- and gamma-lipotropins) exert a definitive action on the melanocytes in man, and are responsible, together with ACTH, for skin hyperpigmentation that is observed in all situations of excess endogenous ACTH secretion. Beta-endorphin is an opioid peptide, which is a processing product of POMC. It circulates in blood, in parallel with the ACTH that is secreted by normal or tumoral pituitary or non-pituitary corticotroph cells. Yet, even at extremely high plasma values, circulating beta-endorphin has no known actions in man, and does not exert any analgesic action

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- Experimental evidence suggest that non-ACTH N-terminal POMC peptides may exert a growth effect on the adrenal cortex (54, 55). The small peptide N-POMC<sub>1–28</sub>, which bears two intra-molecular disulfide bridges, is responsible for this action which has also been described in the human adreno cortical tumors cells NCI-H295 (56). Yet the receptor for the N-POMC<sub>1–28</sub> peptide remains elusive (55).

## Chronic Stress and Pseudo-Cushing

Pseudo-Cushing corresponds to these situations in man where an authentic hypercortisolism is biologically present (increased urinary cortisol, abnormal response to dexamethasone suppression test) and may be confused with “endogenous” Cushing’s disease. Yet, in Pseudo-Cushing there is no pituitary adenoma; the ACTH excess is thought to be “functional,” driven by the overactivity of the hypothalamus and oversecretion of corticotrophin-releasing hormone under chronic stress, severe depression, intense physical activity, and anorexia nervosa (57).

## AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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# The Potential of ACTH in the Genesis of Primary Aldosteronism

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Aldosterone is a homeostatic hormone, rising in volume depletion, sodium deficiency, and potassium loading, in response to angiotensin II and elevation of plasma potassium. Pathophysiologically, in primary aldosteronism (PA) aldosterone levels are inappropriate for the patient's sodium and potassium status, and thus outside the normal feedback loop. ACTH is equivalent with AII and  $[K^+]$  in elevating aldosterone: its effects differ from those of the other secretagogues in four ways. First, it is not sustained; second, it raises aldosterone and cortisol secretion with equal potency; third, it is outside the normal feedback loops, reflecting the epithelial action of aldosterone; and finally its possible role in driving inappropriate aldosterone secretion (aka PA) is not widely recognized. Thirty years ago, it was shown that on a fixed sodium intake of 175 meq/day 36 of 100 unselected hypertensives, in whom PA has been excluded on contemporary criteria, had 24 h urinary aldosterone levels above the upper limit of normotensive controls. More recently, the dexamethasone enhanced fludrocortisone suppression test (FDST) showed 29% of unselected hypertensives to have plasma aldosterone concentrations above the upper limit of normotensive controls. In subjects negative for PA on the FDST, 27% were extremely hyper-responsive to ultra-low dose ACTH infusion; the remaining 73% showed minimal aldosterone elevation, as did normotensive controls: all three groups had negligible cortisol responses. On treadmill testing, no differences were found between groups in (minimally altered) ACTH and cortisol levels: hyper-responders to ultra-low ACTH, however, showed a major elevation in PAC. The implications of these studies, when validated, are substantial for PA, in that approximately half of hypertensive patients appear to show inappropriate aldosterone levels for their sodium status. The physiological role(s) of ACTH as an acute aldosterone secretagogue, and the mechanisms whereby its continuous secretion is curtailed, remain to be established.

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## BACKGROUND

For many years, most studies on aldosterone have focused on its action on renal salt and water transport, undeniably its major homeostatic role. More recently, following the RALES trial of the effects of spironolactone in progressive cardiac failure (1), a range of extraepithelial effects, primarily cardiovascular, has been shown in animal studies (2–4). The doses of aldosterone (or DOCA) used, and the extent of salt loading, make extrapolation to physiology difficult, and to human pathophysiology doubly difficult. That said, PA clearly has a higher cardiovascular risk profile than age-, sex-, and blood pressure-matched essential hypertension (EH) (5, 6): whether this is a primary effect of

aldosterone on the heart and/or blood vessels, or secondary to the increased total body sodium, is yet to be established (7).

The secretory response to what are commonly regarded as its principal regulators (angiotensin II, plasma potassium concentration) is prolonged as long as the stimulus persists. In contrast, ACTH elevates aldosterone acutely but transiently. At least two features of this response are unclear. The first is the organ(s) and processes targeted by the acute elevation; the second the mechanism(s) whereby this acute response is curtailed. Given that the circadian rhythm of aldosterone secretion clearly precedes that of cortisol, both steroids may have roles as "Zeitgebers," effecting diurnal changes in their respective physiologic target tissues. In addition to its circadian variation, however, ACTH is elevated by stress, physical, or psychological. Whereas stress responses to elevated cortisol have been widely studied, those in response to elevated aldosterone appear to have escaped attention. It may be that they form part of the classical "fight or flight" response, by acutely (non-genomically) activating mineralocorticoid receptors (MR) in the vasculature, a physiologic aldosterone target tissue expressing the specificity-conferring enzyme 11 $\beta$ -hydroxysteroid dehydrogenase type 2 (11 $\beta$ -HSD2) (8). A similar rapid vasoconstrictor response follows the acute aldosterone elevation upon orthostasis.

What curtails the elevation of aldosterone secretion despite continued high ACTH levels is currently unclear. One attractive possibility is that it reflects a genomic effect of maintained high levels of glucocorticoid secretion. Heterozygous glucocorticoid receptor (GR) ( $\pm$ ) null mice show persistently elevated levels of both corticosterone and aldosterone levels, consistent with elevated levels of ACTH and impaired glucocorticoid negative feedback on aldosterone secretion (9). To date, there appear to be no reports on levels of aldosterone in patients with hypercortisolism due to GR abnormalities leading to increased ACTH levels. There are, of course, additional/other mechanisms whereby the acute elevation of aldosterone in response to ACTH might be curtailed.

Finally, it is accepted that under normal circumstances, ACTH acts with equivalent potency as a secretagogue for cortisol and aldosterone. Recently, as will be discussed in detail below, there is evidence that aldosterone secretion appears supersensitive to very low dose ACTH at levels which do not elevate cortisol secretion (10).

## PRIMARY ALDOSTERONISM

The first description of the role of ACTH came over 50 years ago, with the description by Sutherland et al. of a patient with "hypertension, increased aldosterone secretion and low plasma renin activity relieved by dexamethasone" (11). This constellation, variously termed glucocorticoid-suppressive hyperaldosteronism (GSH) or glucocorticoid-remediable aldosteronism (GRA) is now termed familial hyperaldosteronism Type-1, and its genesis demonstrated in an early paper from the Lifton laboratory (12). This heritable condition reflects a crossover event during ancestral meiosis, wherein a chimeric gene with the 5' end of CYP11B1 (11 $\beta$  hydroxylase) and the 3' end of CYP11B2 (aldosterone synthase) are fused. This results in a sequence, which is expressed

throughout the zona fasciculata, producing high levels of aldosterone in response to normal levels of ACTH, rather than the normally much lower levels from aldosterone producing cell clusters (13) in the zona glomerulosa. Suppression of ACTH secretion by dexamethasone lowers aldosterone levels and replaces (more or less) the secretion of endogenous glucocorticoid.

Arguably, the first of the studies directly addressing the potential role of ACTH in PA was by Kem et al. (14). Seven PA patients [three ideopathic adrenal hyperplasia (IAH) and four aldosterone producing adenoma (APA)] were studied, with the conclusion that "these data indicate that ACTH frequently is the dominant stimulus of the episodic secretion of aldosterone in patients with either adrenal adenomas or hyperplasia." Couple this with a report 4 years later by Helber et al. (15) entitled "Evidence for a subgroup of essential hypertensives with non-suppressible excretion of aldosterone during sodium loading." The effect of sodium loading (175  $\mu$ g/day for 6 days) on 24 h urinary aldosterone (UA) was determined in three groups – 56 healthy controls, 100 patients with EH, and 16 patients with PA (12 APA, 4 IAH). In healthy controls, the upper limit of UA was <6  $\mu$ g/day; all the PA patients were unsuppressed (IAH 8–20, APA 23–64  $\mu$ g/day); 64 of the EH patients suppressed UA to levels in the normal range; but 36 did not, with values above 6 and up to 16  $\mu$ g/day. Non-suppressing EH patients showed a mean UA value of  $10 \pm 3$  (SD)  $\mu$ g/day; those who suppressed a mean of  $2.7 \pm 1.4$  (SD). Plasma [K<sup>+</sup>] in the 36 non-suppressors was  $3.81 \pm 0.44$  meq/L and in the 64 EH suppressors was  $4.26 \pm 0.37$  meq/L ( $p < 0.001$ ). Finally, in response to 4 weeks on 200 mg/day spironolactone, blood pressure fell by 22 mmHg in non-suppressors, 21 mmHg in the PA patients, and 9 mmHg in the 64 EH patients who suppressed. The authors conclude, "We believe, therefore, that our patient groups with inadequate high aldosterone suppression during Na<sup>+</sup> loading may reflect qualitatively a similar pathogenetic defect in mineralocorticoid secretion as "hyperplasia patients" with hypermineralocorticoidism."

These data, based on 24 h urinary rather than spot plasma aldosterone, remained unremarked for more than three decades. By the use of variously stringent cut-offs in both the screening test for aldosterone [the aldosterone to renin ratio (ARR)] followed by confirmatory testing, PA is currently gazetted to affect 5–13% of referred hypertensives. Add the 16 confirmed PA to the 36 non-suppressors found by Helber et al. in patients referred to the University of Cologne, and the figure stands at 45%, which would currently be regarded as totally out of court, given the understandable focus on the most florid expression of PA, sometimes dubbed "low hanging fruit."

Where this at face value improbable figure starts to make sense is the incorporation of ACTH status into confirmatory/exclusion testing. Currently, any one of half a dozen procedures are used around the world, of which the fludrocortisone-saline suppression test (FST) is sometimes regarded as the "gold standard" – 4 days of fludrocortisone, sodium, potassium supplementation, and often (but not always) hospitalization. In a series of papers from the Piaditis group in Athens (10, 16–19), the potentially confounding effect of ACTH on values obtained in the FST has been addressed in two ways. First is the inclusion of 2 mg dexamethasone at midnight on the last day of testing

[fludrocortisone suppression test (FDST)], with initial results that were arresting, in both a group of control normotensives with normal adrenals on imaging, and a second group of essentially unselected hypertensives (16).

On the FDST, the 97.5% upper limit of normal for plasma aldosterone concentration (PAC) was found to be 74 pmol/L, and for ARR 32 pmol/mL, in the 72 normotensive controls. Among the 180 hypertensives, 56 (31%) showed upright PAC values above 74 pmol/L, combined with an ARR >32 pmol/mU on day 5, evidence for a much higher percentage of unselected hypertensives having inappropriate aldosterone secretion, i.e., PA. In prior studies from the same group, the classical saline infusion test (SIT) was compared with a post-dexamethasone SIT; the latter showed a similar (24%) prevalence of PA, more than double the 11% found on the commonly used SIT (17). More recently, much larger groups of controls ( $n = 100$ ) and hypertensives (327) have been studied, with results essentially identical to the initial study, and a prevalence of PA of 29% (18). A recent overview by Piaditis and colleagues (19) compares these latter figures for prevalence with those from other centers absent dexamethasone loading, which range from 1 to 18%.

There are a number of factors that may conspire toward explaining the difference with and without dexamethasone. The first is the reliance on plasma cortisol as a surrogate for ACTH: just as their circadian secretion appears different, the responses to stress (see below) may differ. Second is the single make-or-break value for PAC, close to the nadir of circadian levels, on which both screening (ARR, PAC above some arbitrary cut-off) depend. It is just possible that Helber et al. got it right, and that UA integrates secretion over 24 h, which may be more pathophysiologically relevant. Finally, it may be that in the historically nephrocentric universe of stimuli to aldosterone secretion angiotensin II reigns supreme, plasma  $[K^+]$  admitted almost as an afterthought, and a continuous role of ACTH almost entirely neglected. We may have to extend the sentence quoted earlier from Kem et al. "These data indicate that ACTH frequently is the dominant stimulus of the episodic secretion of aldosterone in patients with either adrenal adenomas or hyperplasia" (14) from the pathophysiological to the realm of physiology, in control normotensives [to halve the 97.5% cut-off in the FST (6 meq/L) to 3 meq/L in the FDST], and (incidentally) in only ~70% of essential hypertensives.

There would also appear to be another dimension in which ACTH may be a probable actor in inappropriate hypersecretion of aldosterone. The data also come from the Athens group, and address patients with EH who previously tested negative for PA by the FDST. In this study (10), 113 of such hypertensives and 61 normotensive controls underwent an ultra-low dose ACTH-stimulation test, followed by a treadmill test. On the basis of the cut-off values for aldosterone (47 ng/dL) and ARR (2.7 ng/dL/ $\mu$ U/mL) at 15- or 30-min post ACTH (97.5% percentile value in controls), 30/113 (27%) of hypertensives recorded values for both PAC and ARR above the cut-off (HYPER group), and 83/113 (73%) did not (EH: ESHT group). Mean values for aldosterone post-ACTH were identical in the control and ESHT groups, and approximately threefold higher at both 15 and 30 min in the HYPER group; cortisol levels post-ACTH were the same in all three groups.

The authors then extended their studies from testing the adrenal responses to ultra-low ACTH to real life (more or less), with all participants undergoing a treadmill stress test to 80% of age-adjusted maximal cardiac pulse, 24 h after the ultra-low ACTH test in each project. When the results of both tests were aggregated and analysed, the 30 patients in the HYPER group showed a twofold to threefold greater elevation over baseline compared with controls or ESHT: no group showed an elevation in plasma cortisol at peak or recovery.

These studies are prismatic, but not perfect. In addition to issues canvassed in the accompanying editorial (20), other questions might be raised. Variances for all groups in terms of response to ultra-low ACTH are extraordinarily low, even when expressed as they are at SEM; this is much less the case for aldosterone responses to treadmill testing, but those for the other two groups remain tight. In contrast, the variances in terms of cortisol response are enormous. The FDST test was done on days 4–7, after ultra-low ACTH on day 2 and the treadmill on day 3. While this may have been convenient, it presumably excluded an unspecified number of subjects who had undergone the two previous tests. There would thus appear to be a corpus of data on such subjects, in addition to the 113 hypertensives who "failed" the FDST. From this perspective, these patients may have been of more interest than the adventitious KCNJ5 data.

These questions said and done, the data from these studies (10, 16–19) taken together argue powerfully that we need to reconsider our currently accepted values for the prevalence of inappropriate aldosterone secretion (aka PA). The current figures of 1–18% of hypertension – on the basis of strict or relaxed cut-offs, referred or general practice patients – are testimony to diagnostic frailty rather than variation in patient populations: what the first three of the Athens studies have shown is a potential confounder in unrecognized roles for ACTH in testing. The implication of the three studies (16–18) is that ~30% of unselected hypertensives have autonomous aldosterone secretion independent of the classic secretagogues, a much higher figure than is currently acknowledged.

The ultra-low ACTH/treadmill studies open a quite distinct scenario, that of heightened zona glomerulosa sensitivity to everyday stresses, accounting for a similar fraction of hypertensive subjects. Just as the driver of PA is unclear in the majority of PA diagnosed post-FDST, so it is for the genesis of the adrenal sensitivity. What needs to be done is to validate the generalizability of past findings, not by slavishly repeating the present protocols, given the difficulty in persuading control subjects to undergo imaging and FDST, but by doing a comparison of the seated saline suppression test (SSST) with and without 2 mg dexamethasone at midnight on the day before (FSSST). In preliminary studies, Michael Stowasser's laboratory has shown excellent comparability between the SSST and the FST (21). Subjects negative on the FSSST might then be enrolled in an ultra-low dose ACTH study, including if possible a 24 h urine to measure integrated aldosterone secretion, in addition to values obtained during the test.

If, as I anticipate, this series of findings from Athens can be generalized, we need to recognize that PA affects not ~10%

of hypertensives but over half. Currently, major interest and considerable resources are devoted to seeking, for example, additional somatic mutations possibly involved in oversecretion of aldosterone from APA: it may be much less fun grinding through clinical studies, but the outcome for hypertensive patients is likely to be far greater. If over half of hypertensive patients have PA, and fewer than 1% are even screened let alone diagnosed and treated in any jurisdiction, the case for inclusion of low dose MR antagonist in first line antihypertensive therapy would appear to be incontrovertible.

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# Role of ACTH and Other Hormones in the Regulation of Aldosterone Production in Primary Aldosteronism

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The major physiological regulators of aldosterone production from the adrenal zona glomerulosa are potassium and angiotensin II; other acute regulators include adrenocorticotrophic hormone (ACTH) and serotonin. Their interactions with G-protein coupled hormone receptors activate cAMP/PKA pathway thereby regulating intracellular calcium flux and CYP11B2 transcription, which is the specific steroidogenic enzyme of aldosterone synthesis. In primary aldosteronism (PA), the increased production of aldosterone and resultant relative hypervolemia inhibits the renin and angiotensin system; aldosterone secretion is mostly independent from the suppressed renin–angiotensin system, but is not autonomous, as it is regulated by a diversity of other ligands of various eutopic or ectopic receptors, in addition to activation of calcium flux resulting from mutations of various ion channels. Among the abnormalities in various hormone receptors, an overexpression of the melanocortin type 2 receptor (MC2R) could be responsible for aldosterone hypersecretion in aldosteronomas. An exaggerated increase in plasma aldosterone concentration (PAC) is found in patients with PA secondary either to unilateral aldosteronomas or bilateral adrenal hyperplasia (BAH) following acute ACTH administration compared to normal individuals. A diurnal increase in PAC in early morning and its suppression by dexamethasone confirms the increased role of endogenous ACTH as an important aldosterone secretagogue in PA. Screening using a combination of dexamethasone and fludrocortisone test reveals a higher prevalence of PA in hypertensive populations compared to the aldosterone to renin ratio. The variable level of MC2R overexpression in each aldosteronomas or in the adjacent zona glomerulosa hyperplasia may explain the inconsistent results of adrenal vein sampling between basal levels and post ACTH

**Abbreviations:**  $\beta$ -AR,  $\beta$ -adrenergic receptors; 5-HT<sub>4</sub>R, serotonin receptor; AC, adenylate cyclase; ACTH, adrenocorticotropin hormone; APA, aldosterone-producing adenoma; APA, aldosterone-producing adenoma; APCCs, aldosterone-producing cell clusters; AT-1 receptor, angiotensin II type 1 receptor; ATP, adenosine triphosphate; AVS, adrenal venous sampling; BAH, bilateral adrenal hyperplasia; cAMP, cyclic adenosine monophosphate; CREB, cAMP response element binding protein; CYP11B1, cytochrome P450 family 11 subfamily B member 1 encodes 11-beta hydroxylase; CYP11B2, cytochrome P450 family 11 subfamily B member 2 encodes aldosterone synthetase; ET, endothelin-1; FH, familial hyperaldosteronism; FST, fludrocortisone suppression test; GIP, glucose-dependent insulinotropic peptide; GPCR, G-protein-coupled receptor; GRA, glucocorticoid-remediable aldosteronism; Gs- $\alpha$ , G stimulatory  $\alpha$  subunit; LH-HCG, luteinizing hormone/human chorionic gonadotropin; MC2R, melanocortin type 2 receptor; mRNA, messenger ribonucleic acid; PA, primary aldosteronism; PAC, plasma aldosterone; PKA, protein kinase; RAS, rennin–angiotensin system; RT-PCR, reverse transcriptase-polymerase chain reaction; StAR, steroidogenic acute regulatory protein; TRH, thyrotropin releasing hormone; UA, unilateral adrenalectomy; V1-AVPR, vasopressin receptor type 1; ZF, zona fasciculata; ZG, zona glomerulosa.

administration in the determination of source of aldosterone excess. In the rare cases of glucocorticoid remediable aldosteronism, a chimeric CYP11B2 becomes regulated by ACTH activating its chimeric CYP11B1 promoter of aldosterone synthase in bilateral adrenal fasciculate-like hyperplasia. This review will focus on the role of ACTH on excess aldosterone secretion in PA with particular focus on the aberrant expression of MC2R in comparison with other aberrant ligands and their GPCRs in this frequent pathology.

**Keywords:** ACTH, aldosterone regulation, melanocortin type 2 receptor, aberrant G-protein coupled receptors, primary aldosteronism

## INTRODUCTION

Primary aldosteronism (PA) was first described in patients with unilateral aldosterone-producing adenomas (1). It is characterized by increased aldosterone secretion causing salt retention, increased urinary potassium excretion, relative hypervolemia, suppressed plasma renin activity (PRA), and hypertension. PA is the most common curable form of secondary hypertension as it affects 4.3% of the general hypertensive population, 9.5% of patients referred to hypertension clinics (2), and up to 20% of those with resistant hypertension (3). PA is most often secondary to bilateral adrenal hyperplasia (BAH; 50–70% of cases) or to an aldosterone-producing adenoma (APA; 30–50% of cases) (4). The classical concept that a unique unilateral aldosteronoma is the causative lesion responsible for a high proportion of this surgically curable form of PA was recently challenged by the identification of zona glomerulosa (ZG) hyperplasia and nodulation adjacent to aldosteronomas when resected adrenals are examined carefully (5–7).

In order to prevent cardiovascular, metabolic and renal morbidities, early diagnosis and management of PA are mandatory (8–10). Unilateral adrenalectomy (UA) provides superior benefit compared to medical therapy in lateralized PA in terms of cardiovascular outcomes (11, 12), quality of life (12), and all-cause mortality (13); however, in BAH, pharmacological blockade of aldosterone excess using mineralocorticoid receptor antagonists, such as spironolactone or eplerenone, is the recommended treatment (4). Therefore, subtyping of PA is required to direct patients to surgical vs. medical therapy (4). To date, adrenal vein sampling (AVS) is the gold standard to differentiate lateralized from bilateral sources of PA (4) because adrenal imaging provides poor specificity in detecting lateralized cases (14) except in patients <35 years old (15).

In PA excess, plasma aldosterone concentration (PAC), despite suppressed renin activity, is not really autonomous, as frequently stated. It could be autonomous if it was solely or constitutively regulated by somatic and germline mutations of various ion channels genes regulating intracellular ionic homeostasis and cell membrane potential as reviewed elsewhere (16). In fact, several autocrine/paracrine hormones and regulatory mechanisms (17) activate variable levels of aberrant eutopic or ectopic receptors (18), which regulate aldosterone secretion either in unilateral adenomas or in BAH. In this review, we will focus on the role of one of these hormones, the adrenocorticotrophic hormone (ACTH), in stimulating aldosterone secretion in normal and

pathologic conditions and briefly mention others which play similar roles in PA.

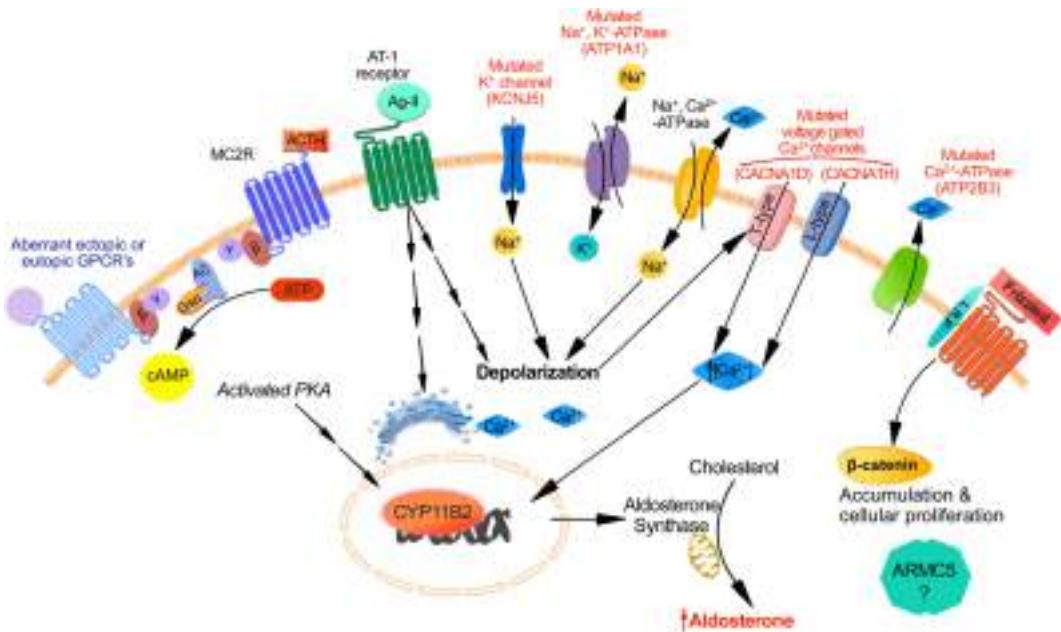
## NORMAL PHYSIOLOGY OF THE RENIN-ANGIOTENSIN SYSTEM

Renin is an enzyme produced primarily by the juxtaglomerular apparatus of the kidney and its release is the rate-limiting step in the regulation of the RAS (19, 20); it is controlled by four factors: (1) the macula densa comprises chemoreceptors for monitoring the sodium and chloride loads present in the distal tubule, (2) juxtaglomerular cells acting as pressure transducers that sense stretch of the afferent arteriolar wall and thus renal perfusion pressure, (3) the sympathetic nervous system (SNS), which increases the release of renin, particularly in response to upright posture, in addition to (4) inhibiting factors, including K<sup>+</sup>, Ca<sup>++</sup>, angiotensin II, and atrial natriuretic peptides (19).

The action of renin on angiotensinogen, synthesized in the liver, generates angiotensin I (19). Angiotensin-converting enzyme (ACE), localized in cell membranes particularly of the lung, cleaves angiotensin I into angiotensin II, which is the main biologically active angiotensin (19). Angiotensin II functions through the AT-1 receptor (AT1R) to maintain normal extracellular volume and blood pressure by increasing aldosterone secretion from the ZG via increased transcription of CYP11B2 (aldosterone synthase) (Figure 1) as well as constricting vascular smooth muscle, releasing norepinephrine, and epinephrine from the adrenal medulla, enhancing the activity of the SNS and finally promoting the release of vasopressin (19).

Zona glomerulosa cells are organized in rosette structures that spontaneously generate periodic depolarizing changes in membrane potential that are modulated in frequency by angiotensin II and extracellular K<sup>+</sup> (21, 22); Angiotensin II induces cell membrane depolarization most probably due to a Gi-mediated shift in the voltage dependence of channel activation toward more negative potentials thereby increasing intracellular Ca<sup>2+</sup> signal, which stimulates hormone-sensitive lipase and steroidogenic acute regulatory protein (StAR). Another mechanism by which angiotensin II binding to AT1R stimulates aldosterone secretion implicates activating the phospholipase C/inositol 1,4,5-trisphosphate pathway, releasing Ca<sup>2+</sup> stores from the endoplasmic reticulum, and activation of T-type voltage-gated Ca<sup>2+</sup> channels (23) (Figure 1).

Dopamine, atrial natriuretic peptide, and heparin inhibit aldosterone secretion. The secretion of aldosterone is restricted



**FIGURE 1 | Mechanisms responsible for aldosterone synthesis in zona glomerulosa cells under normal physiological conditions and excess production in primary aldosteronism.** The strongly negative resting membrane potential of zona glomerulosa (ZG) cells under resting physiological conditions is maintained by the concentration gradient of K<sup>+</sup> between the intracellular and extracellular space, which is generated by the activity of the Na<sup>+</sup>, K<sup>+</sup>-ATPase. Angiotensin II and increased K<sup>+</sup> lead to cell membrane depolarization, which opens voltage-dependent Ca<sup>2+</sup> channels. Furthermore, Angiotensin II acts through the Angiotensin II type 1 receptor (AT1R) inducing Ca<sup>2+</sup> release from the endoplasmic reticulum. Consequently, the increase in intracellular Ca<sup>2+</sup> concentration activates the calcium signaling pathway, which triggers activation of CYP11B2 transcription. The role for ACTH in the regulation of aldosterone secretion whether in normal physiology or in PA is in part determined by the level of expression of ACTH receptors (MC2R) in ZG cells. MC2R which is a GPCR coupled to the stimulatory Gs $\alpha$  subunit may induce an increase of intracellular cAMP concentration which activates protein kinase A thereby increasing CREB phosphorylation and CYP11B2 transcription. Aberrant expression of other GPCR may also be responsible for aldosterone excess despite a suppressed renin angiotensin system: eupotic GPCR include those for serotonin (5-HT<sub>4</sub>R); ectopic GPCR include those for glucose-dependent insulinotropic peptide (GIPR), luteinizing hormone/human chorionic gonadotropin (LH-hCG R),  $\beta$ -adrenergic receptors ( $\beta$ -AR), vasopressin (V1-AVPR) glucagon (glucagon receptor), TRH (TRH R), and Endothelin-1 ET<sub>A</sub> and ET<sub>B</sub> receptors. Other mechanisms implicated in PA involve somatic and germline mutations in ion channels genes regulating intracellular ionic homeostasis and cell membrane potentials: increase intracellular Na<sup>+</sup> concentrations and cell membrane depolarization result from KCNJ5 gain-of-function mutations affecting GIRK4 and ATP1A1 mutations of the Na<sup>+</sup>, K<sup>+</sup>-ATPase. Direct increase of intracellular Ca<sup>2+</sup> concentrations could also result from mutations in ATP2B3 encoding for the plasma membrane Ca<sup>2+</sup>-ATPase, mutations in CACNA1D affecting the Cav1.3 subunit of the L-type voltage-gated calcium channel or CACNA1H affecting the Cav3.2 subunit of the voltage-gated calcium channel. Finally dysregulation in cellular proliferation/apoptosis accelerating adenoma formation could be due either to activation of the Wnt/ $\beta$ -catenin pathway or to gene mutations such as ARMC5 although the mechanism of the latter mutation is not fully elucidated.

to the ZG because of zone-specific expression of aldosterone synthase (CYP11B2), which is regulated by the activation of calcium signaling (24).

## PATHOPHYSIOLOGY OF PRIMARY ALDOSTERONISM

The binding of free aldosterone to the mineralocorticoid receptor in the cytosol of epithelial cells (24), principally in the kidney, controls potassium homeostasis and maintains normal intravascular volume by increasing intestinal and renal Na<sup>+</sup> and Cl<sup>-</sup> absorption and reabsorption, respectively. Increased production of aldosterone in PA results in sodium retention, hypertension, and can also result in hypokalemia (20). In addition to the two most common subtypes of PA (BAH in 50–70% of the cases and APA in 30–50%), less frequent causes include primary (unilateral) adrenal hyperplasia (5%), aldosterone-producing adrenocortical carcinoma (<1%), familial hyperaldosteronism (1%), and ectopic aldosterone-producing adenoma or carcinoma

(<0.1%). The mechanisms implicated in the pathophysiology of PA are not fully elucidated. Somatic and germline mutations in ion channels genes regulating intracellular ionic homeostasis and cell membrane potential were described in sporadic APA and type-III familial PA (25–28) (Figure 1). Somatic mutations in the potassium channel gene KCNJ5 are found in almost 30–40% of aldosteronomas and alter channel selectivity allowing enhanced Na<sup>+</sup> conductance. Na<sup>+</sup> influx results in cell depolarization, the activation of voltage-gated Ca<sup>2+</sup> channels, aldosterone production, and cell proliferation (25, 29). Somatic and germline mutations in CACNA1D gene encoding a voltage-gated calcium channel result in channel activation and less depolarized potentials causing increased Ca<sup>2+</sup> influx, aldosterone production and cell proliferation in affected ZG cells (27, 30). Mutations in ATP1A1 gene (encoding the Na<sup>+</sup>/K<sup>+</sup> ATPase  $\alpha$  subunit) and ATP2B3 gene (encoding the plasma membrane Ca<sup>2+</sup> ATPase) were identified in 5.2 and 1.6%, respectively of patients in a series of APA (26). Mutations in CACNA1H gene, which encodes a voltage-gated calcium channel (Cav3.2) were discovered in children with PA; they

result in impaired channel inactivation and activation at more hyperpolarized potentials, producing increased intracellular  $\text{Ca}^{2+}$  and aldosterone excess (31). Different mutations in the genes described above are found in different aldosterone-producing nodules from the same adrenal, suggesting that somatic mutations are independent events (32, 33).

No mutations of any of the above ion channel genes were found in BAH or in ZG hyperplasia adjacent to the dominant aldosteronomas (26, 29, 32, 33); these findings suggest that nodule formation and excess aldosterone production are two dissociated events, implying a two-hit hypothesis for APA formation (16, 34). The first hit causing a unilateral aldosteroma or a dominant nodule adjacent to ZG hyperplasia may result from a somatic mutation in one of the genes described above, at least in approximately 60% of cases. Possible causes of the second hit that results in dysregulation in cellular proliferation/apoptosis accelerating adenoma formation could be due either to activation of the Wnt/ $\beta$ -catenin pathway (35, 36), PKA pathway, or to gene mutations such as *ARMC5* (37) (Figure 1). However, the pathophysiology of progression from normal adrenal to APA and the causes of diffuse bilateral hyperplasias, either as BAH or in mild form adjacent to the dominant aldosteroma, are still unknown. Aldosterone-producing cell clusters (APCCs), which have increased expression of CYP11B2, are nests of cells below the adrenal capsule. They protrude into cortisol-producing cells that are usually negative for CYP11B2 expression. Nishimoto et al. found that APCCs are common in normal adrenals, and they harbor a different mutational spectrum compared to APA suggesting that APCCs could be a precursor for APA (38). In addition, several hormones activating variable levels of eutopic or ectopic aberrant receptors (18) (Figure 1) as well as autocrine and paracrine regulatory mechanisms (17) can increase aldosterone secretion in PA (either APA or BAH) independently from the suppressed RAS (see later section).

## ROLE OF ACTH IN ALDOSTERONE PRODUCTION IN NORMAL PHYSIOLOGY

Adrenocorticotropic hormone can stimulate aldosterone secretion acutely and transiently under normal conditions, but to a lesser extent than angiotensin II and potassium. ACTH is a 39-amino-acid peptide, which results from the cleavage of its proopiomelanocortin (POMC) precursor by prohormone convertases PC1/3 and may be further cleaved by PC2 to generate  $\alpha$ -melanocyte-stimulating hormone ( $\alpha$ -MSH) (39, 40). It is mainly produced in the anterior pituitary corticotropes, but is also produced in brain, adrenal medulla, skin, and placenta (41–43). ACTH can induce aldosterone production at lower doses than the ones needed for cortisol and DHEA production (44). Furthermore, ACTH stimulates aldosterone production acutely and sometimes chronically.

### Acute Effects of ACTH

The initial binding of ACTH to its specific melanocortin type 2 receptor (MC2R) stimulates both cortisol and aldosterone secretion. MC2R (45) is a seven transmembrane domain receptor that belongs to the family of melanocortin receptors (MCRs) (45).

Five MCRs constitute a distinct family of G-protein coupled hormone receptors (GPCR); MC2R is the smallest MCR and GPCR (45, 46). MC2R is expressed in zona fasciculata (ZF) and ZG cells (47). The binding of ACTH to its MC2R induces the dissociation of Gs- $\alpha$  subunit and activation of adenylyl cyclase (AC) that generates cAMP from ATP (48) (Figure 1). cAMP molecules bind to specific domains of the regulatory subunits of protein kinase A (PKA) thereby dissociating the tetramer and releasing the catalytic subunit (PRKACA) from its inactivating regulatory subunits. Activated PRKACA phosphorylates and activates steroidogenic acute regulatory protein (StAR) as well as cAMP response element binding protein (CREB), thereby increasing StAR expression. On the other hand, activation of the PKA pathway induces a slow but sustained calcium influx through the L-type calcium channels. The subsequent increase in intracellular calcium activates calcium/calmodulin-dependent protein kinase and steroidogenesis (49, 50).

### Chronic Effects of ACTH

In contrast to *in vivo* studies that suggest that ACTH is a short-term stimulator of aldosterone production, *in vitro* studies showed that ACTH can act as a major stimulus of aldosterone secretion. Continuous intravenous infusion of ACTH leads to a transient stimulation of aldosterone secretion, whereas its pulsatile administration leads to a sustained stimulation of aldosterone up to 72 h (51). Moreover, chronic exposure to ACTH (2 days or more) leads to transformation of ZG cells into ZF-like cells with elongated mitochondria with lamellar and tubular cristae becoming round with ovoid cristae; at the functional level, the synthesis of angiotensin II receptors, steroidogenic enzymes, and their products is altered (52–56).

## ROLE OF ACTH IN EXCESS ALDOSTERONE SECRETION IN PA

### Diurnal Rhythmicity of Aldosterone

In recumbent normal subjects on a regular diet, the circadian rhythm of PAC is regulated by the activity of plasma renin independently of ACTH (57). In contrast, patients with PA have a circadian rhythm of PAC mediated by changes in ACTH rather than by the suppressed plasma renin–angiotensin II levels (58). Several groups described that PAC falls following overnight sleep when ACTH levels are low despite upright posture or angiotensin II infusion. Similarly, they noted a marked increase in PAC shortly after ACTH administration (59–63), which was higher compared to normal controls or patients with essential hypertension (64, 65). Furthermore, abolition of diurnal rhythm by dexamethasone in PA demonstrates the impact of ACTH on adrenal steroidogenesis (66). Administration of dexamethasone 0.75–2.0 mg per day for 2 days decreased aldosterone levels by a mean of 49% in a group of 15 patients with aldosteromas; in 33%, the suppression was greater than 80% (67).

### ACTH Role in Familial Hyperaldosteronism

Familial hyperaldosteronism (FH) type-1, previously known as glucocorticoid-remediable aldosteronism (GRA), was first

described as a form of hyperaldosteronism relieved by dexamethasone (68). It is suspected in young PA patients whose relatives suffer from cerebrovascular accidents. It is an autosomal dominant disease whereby the promoter of the chimeric 11 $\beta$ -hydroxylase/aldosterone synthase gene belongs to the 5' end of CYP11 B1 (11 $\beta$  hydroxylase) and drives the expression of the 3' end of CYP11 B2 (aldosterone synthase) ectopically in ZF cells under the main regulation by ACTH (69); in these patients, dexamethasone usually decreases aldosterone secretion by more than 80% or to <4 ng/dL (67), but the diagnosis is now performed using genetic analysis. In contrast to FH type-1, FH type-2 is defined as PA in a patient with a first-degree relative (parent/sibling/offspring) with established PA but without FH type-1 gene rearrangement. Linkage analysis has mapped FH type-2 to chromosome 7p22 but no responsible gene has been identified yet (70). The prevalence of FH type-2 in PA is higher (1.2–6%) than FH type-1 (<1%). The FH type-3 and -4 are not regulated by ACTH stimulation, but they are caused by germline mutations in KCNJ5 (71) and CACNA1D/CACNA1H (30, 31) genes, respectively.

## ACTH Suppression or Stimulation Tests Can Reveal the Presence of PA

Based on the rationale that ACTH plays a more important role in PA than in normal subjects or those with essential hypertension, investigators in Athens compared the classical saline infusion test (SIT) to postdexamethasone SIT in 151 patients with single adrenal adenomas and detected almost double rate of aldosterone hypersecretion following dexamethasone administration (24 vs. 12%) (72). Similarly, they used a combined fludrocortisone-dexamethasone suppression test (FDST), which is a modification of the classic confirmatory fludrocortisone suppression test (FST) for the diagnosis of PA; it involves the administration of dexamethasone to hypertensives patients at midnight of the last day of the FST in order to eliminate the stimulatory effect of ACTH on aldosterone secretion. They demonstrated that the prevalence of PA rises from 5 to 13% with the usual diagnostic tests to 28.7–31% when using FDST; mineralocorticoid receptor blockade resulted in significant improvement in blood pressure in these patients (73–75). The same group administered an ultralow-dose (0.03  $\mu$ g) ACTH to 113 hypertensives without PA: the 30 patients (27%) who exhibited an aldosterone hyperresponse had significantly higher PAC, ARR, and PAC/ACTH ratio in the treadmill test; normalization of blood pressure by mineralocorticoid antagonists in these patients was also evident compared to the group of hypertensive not sensitive to ACTH/stress (76). Therefore, the benefit of mineralocorticoid blockade could extend even to hypertensive patients without confirmed PA who present an aldosterone hyperresponse to ACTH/stress, this category of hypertenives harboring a mild form of BAH. In contrast, another group examining the diagnostic accuracy of ACTH test in 158 hypertensive patients found that it was not very effective in differentiating between APA patients and non-PA patients (77).

## Use of ACTH to Identify the Source of Aldosterone Excess

Many efforts were conducted to find an easier and cheaper test than adrenal vein sampling (AVS), which is available only in tertiary care center with experienced angioradiologists to distinguish between lateralized and bilateral sources of aldosterone. Differential increase in PAC during upright posture was suggested to be a valuable tool to distinguish APA from BAH, but further studies showed that several APA and BAH had similar rise in PAC to upright posture (78). APA whether responsive or not to angiotensin II was found to be more sensitive to ACTH stimulation resulting in larger increase of PAC than in patients with BAH or essential hypertension (79–81). BAH patients also displayed increased response of PAC to ACTH administration than normal subjects or patients with essential hypertension (82). PAC increased more after ACTH bolus in the APA group compared with BAH group, which had an intermediate increase compared to normal controls (18, 66). A study in which patients received dexamethasone (1 mg) the evening before receiving i.v. injection of 50 IU of ACTH showed that the exaggerated PAC response was higher after 120 min in patients with APA than in BAH (83). It was suggested that this could be used for identifying the etiology subtype; however, significant overlap was present between APA, unilateral hyperplasia, and BAH cases and using an optimal cutoff value of the aldosterone >78 ng/dL for APA, provided a sensitivity of 76.8% and a specificity of 87.2% (83).

Kline et al. studied 65 patients with confirmed PA who were divided by histology into confirmed lateralized and non-lateralized; PAC in inferior vena cava (IVC) sampled during AVS before and after cosyntropin infusion was analyzed. Baseline and peak IVC aldosterone was higher in lateralized patients (APA) but incremental aldosterone rise was much greater in subjects with bilateral hyperplasia (84). This shows that ACTH can regulate APA as well as BAH, but that the effects are more pronounced in APA.

## Role of ACTH Stimulation during Adrenal Venous Sampling

The usefulness of ACTH stimulation in the conduct of AVS procedure is controversial and remains a matter of debate because of conflicting results. Some centers use cosyntropin infusion or bolus in order to minimize stress-induced or spontaneous fluctuations in aldosterone secretion when performing sequential non-simultaneous AVS, to maximize the gradient of cortisol from the adrenal vein to the inferior vena cava, and to maximize aldosterone secretion from an APA (85). In contrast, other groups found that ACTH-stimulation of aldosterone production from the contralateral gland or adjacent hyperplasia may reduce the gradient of aldosterone production resulting in incorrect lateralization (86, 87). The effect of both continuous ACTH infusion and bolus on the performance and interpretation of AVS in confirmed PA patients was investigated (88). Both methods lead to a significant increase in selectivity index for the right adrenal vein and ACTH bolus for the left adrenal vein. Lateralization index was not significantly affected after continuous ACTH infusion and

i.v. bolus. In 88 and 78% of the patients, the diagnosis obtained was the same before and after ACTH infusion and i.v. bolus, respectively (88). Recently, our group demonstrated that ACTH increased selectivity on both sides from 66.7% in basal samples to 91.8% poststimulation. A discordance of lateralization between basal and post-ACTH values was observed in 28% of cases, mostly lateralized cases basally that became bilateral post ACTH (87). The variation in the response to ACTH stimulation could be due to the variable expression of MC2R in APA (see later) (18). Careful examination of the levels of aldosterone in the adrenal vein contralateral to the dominant or lateralized APA and pathology confirmed the frequent presence of bilateral background hyperplasia and this could predict less favorable post-operative outcome with residual hypertension (35, 87).

## INCREASED BUT VARIABLE EXPRESSION OF MC2R IN PA

The explanation for the increased role of ACTH in the regulation of aldosterone in PA may be secondary to the overexpression and function of MC2R in this condition. The expression of MC2R mRNA was shown to be upregulated in human adrenocortical neoplasms specifically in functional adenomas in contrast to non-functioning adenomas and carcinomas (89). More specifically, a few studies have demonstrated increased ectopic expression of MC2R assessed by RT-PCR or transcriptome studies in resected aldosteronomas as compared to cortisol-secreting adenomas, non-functional adenomas, and adrenocortical carcinomas (90–92). A particularly pertinent informative study included 15 adrenal tumors (14 APA and 1 BAH); MC2R mRNA levels were increased by a mean of 3.9-fold in those tissues compared to normal adrenal (18). However great variability existed in the level of expression in each tumor as 4 had lower levels than normal (0.3-fold to 0.7-fold), while those with increased expression varied between 1.4- and 20.6-fold compared to normal. The data are limited to mRNA expression without available measurements at the protein levels (no good specific MC2R antibody), but correlated well with the *in vivo* increased response to ACTH administration. There is almost no data on MC2R expression in BAH as those patients are usually not operated, but in the only case with BAH studied by this group MC2R was 20-fold increased. These data appear to be compatible with the findings that the majority of patients stimulated with ACTH during AVS will have concordant results before and after ACTH as the majority overexpress MC2R; however, the 28% of discordant results we found (87) may be explained by cases where MC2R are relatively decreased in the dominant adenoma but is present in adjacent hyperplasia. This hypothesis remains to be validated in prospective studies.

## OTHER HORMONES AND ABERRANT RECEPTORS REGULATING ALDOSTERONE SECRETION IN PRIMARY ALDOSTERONISM

Adrenocorticotropin hormone is not the exclusive trigger of aldosterone secretion since several other hormones have a role in

the pathophysiology of PA in addition to the ion channels mutations. Serotonin plays a significant role in aldosterone synthesis in normal physiological and in PA. The administration of serotonin 5-HT<sub>4</sub> agonists such as metoclopramide, cisapride, and tegaserod resulted in higher stimulation of aldosterone in PA as compared to the physiological moderate increase in normal individuals (18, 93, 94). Whereas non-specific inhibitors of 5-HT such as cyproheptadine and ketanserin produced only minor and transient inhibition of aldosterone secretion in aldosteronomas (95, 96), specific 5-HT<sub>4</sub>R antagonists such as GR113808 were potent inhibitors of basal- and cisapride-induced aldosterone secretion (93). Chromaffin cells, endothelial cells, nerve terminals, and cells of the immune system are localized in the immediate vicinity of ZG cells and can secrete various factors to control aldosterone secretion (97). Local release of 5-HT by perivascular mast cells (MC) can activate 5-HT<sub>4</sub>R expressed in ZG cells and consequently stimulate aldosterone production (98). A role of MC in tumorigenesis was proposed (99, 100). The density of MC was found to be increased in APA tissues compared with normal adrenals (101). As the 5-HT<sub>4</sub>R have been found to be overexpressed in the majority of APA (but variable as MC2R) (17, 18, 102) and the ligand may be locally overexpressed also, a paracrine loop of regulation of aldosterone production appears to be present.

The compelling evidence supporting that various aberrant GPCR are frequently expressed in bilateral macronodular adrenal hyperplasia and Cushing's syndrome (103) led many researchers to investigate the presence of aberrant GPCR in PA. Adrenal production of aldosterone in APA and BAH was found to be under the influence of aberrant GPCR and their ligands, as demonstrated by *in vivo* and *in vitro* studies (104, 105). The expression of ectopic receptors, which are usually not expressed at significant levels in normal ZG cells include those for glucose-dependent insulinotropic peptide (GIPR) (106), luteinizing hormone/human chorionic gonadotropin (LH-hCG R) (18, 106–112), β-adrenergic receptors (β-AR) (113), vasopressin (V1-AVPR) (18, 106, 114, 115), glucagon (glucagon receptor), TRH (TRH R) (18, 112, 116) and Endothelin-1 ET<sub>A</sub> and ET<sub>B</sub> receptors (117). Using a microarray approach in 10 aldosteronomas compared with five normal adrenals and 13 cortisol-secreting adenomas, the six GPCRs with highest increase in expression included LHCGR, 5-HT<sub>4</sub>R, GnRHR, glutamate receptor metabotropic 3, endothelin receptor ET<sub>B</sub> receptors, and MC2R (92). **Table 1** summarizes the different types of aberrant ectopic or ectopic GPCR involved in aldosterone excess in PA. Co-expression of multiple aberrant GPCR was also reported; renin-independent stimulation of aldosterone secretion was observed *in vivo* following mixed meal, oral glucose, or administration of GIP, vasopressin, and tegaserod in a patient with unilateral source of PA (106). On the other hand, co-secretion of aldosterone and cortisol due to aberrant expression of GPCR was noted; in a patient with BMAH and β-AR-aberrant expression, isoproterenol stimulated both cortisol and aldosterone production (113).

Activating somatic *CTNNB1* (β-catenin) mutations have now been identified in tumors of three women with APAs, two who presented during pregnancy and one after menopause (118). All three had heterozygous activating mutations of *CTNNB1* and expressed aberrant LHCG and GNRH receptors at levels 100-fold

**TABLE 1 | Types of GPCR involved in aldosterone hypersecretion in patients with PA.**

Aberrant receptor	Phenotype	In vivo screening protocol	Targeted medical therapy
MC2R (eutopic) (90–92)	ACTH-dependent hyperaldosteronism	Cosyntropin	
GIP receptor (ectopic) (102)	Food-dependent hyperaldosteronism	Mixed meal Oral glucose	Octreotide, pasireotide GIPR antagonist
Vasopressin receptor (ectopic) (18, 102, 114, 115)	Upright posture-dependent hyperaldosteronism	Upright posture AVP/desmopressin	Specific AVP receptors antagonist
β-adrenergic receptor (ectopic) (113)	Upright posture Insulin-induced hypoglycemia Exercise/stress test hyperaldosteronism	Upright posture Isoproterenol ( $\beta$ 1-agonist)	$\beta$ -blockers
GnRH receptor, LH/hCG receptor (ectopic) (18, 102, 107–112)	Luteal phase of ovarian cycle/Pregnancy (transient) Postmenopausal (persistent)-dependent hyperaldosteronism	GnRH, hCG, Recombinant LH	Long-acting GnRH agonist (leuprolide acetate)
5-HT <sub>4</sub> receptor (eutopic) (18, 93, 94, 102)	Serotonin-dependent dependent	5-HT <sub>4</sub> receptor agonists (metoclopramide, cisapride, tegaserod)	5-HT <sub>4</sub> receptor antagonist (GR113808)
Glucagon receptor (ectopic) (18)	Hypoglycemia?	Intravenous glucagon	Octreotide
TRH receptor (ectopic) (18, 112, 116)	Hypothyroidism		
Endothelin-1 A and B receptors (ectopic) (117)			

higher than in other APAs. It was shown that the *CTNNB1* mutation led to activation of the WNT pathway; it was suggested that could be the cause of dedifferentiation of gonadal progenitor cells present in the adrenal tissues with increased expression of gonadal receptors. It is thought that the high levels of endogenous human chorionic gonadotropin (hCG) during pregnancy and of gonadotropin-releasing hormone (GnRH) and luteinizing hormone (LH) after menopause led to the identification of APAs in these patients (118).

It is currently unclear whether these aberrant regulatory secretory mechanisms by ACTH and other hormones and the overexpression of their GPCR in PA are secondary to unknown proliferative mechanisms or are primary and at least partially responsible for the abnormal proliferation, the initiation of diffuse BAH. However, they clearly play a role in aldosterone secretion which is not autonomous.

## CONCLUSION

Our understanding of the increased occurrence and complexity of molecular etiology and unique signature in each case of

PA has progressed greatly in recent years. The increased role of ACTH, of the variable expression of MC2R, and of other aberrant GPCR in PA should receive further attention in the future. The development of effective antagonists to MC2R and other aberrant GPCR could eventually offer interesting alternatives in patients with bilateral sources of excess aldosterone in combination with better antagonists of the mineralocorticoid receptor.

## AUTHOR CONTRIBUTIONS

NG, IB, and AL contributed to the conception and design of the manuscript as well as to drafting the review article and they all provided final approval of the version to be published.

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# The Role of *gsp* Mutations on the Development of Adrenocortical Tumors and Adrenal Hyperplasia

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Somatic GNAS point mutations, commonly known as *gsp* mutations, are involved in the pathogenesis of McCune–Albright syndrome (MAS) and have also been described in autonomous hormone-producing tumors, such as somatotropinoma, corticotrophoma, thyroid cancer, ovarian and testicular Leydig cell tumors, and primary macronodular adrenocortical hyperplasia (PMAH) (1–3). The involvement of *gsp* mutations in adrenal tumors was first described by Lyons et al. Since then, several studies have detected the presence of *gsp* mutations in adrenal tumors, but none of them could explain its presence along or the mechanism that leads to tumor formation and hormone hypersecretion. As a result, the molecular pathogenesis of the majority of sporadic adrenocortical tumors remains unclear (3). PMAH has also been reported with *gsp* somatic mutations in a few cases. Fragoso et al. identified two distinct *gsp* somatic mutations affecting arginine residues on codon 201 of GNAS in a few patients with PMAH who lacked any features or manifestations of MAS. Followed by this discovery, other studies have continued looking for *gsp* mutations based on strong prior evidence demonstrating that increased cAMP signaling is sufficient for cell proliferation and cortisol production (2, 4). With consideration for the previously reported findings, we conjecture that although somatic activating mutations in GNAS are a rare molecular event, these mutations could probably be sufficient to induce the development of macronodule hyperplasia and variable cortisol secretion. In this manuscript, we revised the presence of *gsp* mutations associated with adrenal cortical tumors and hyperplasia.

**Keywords:** *gsp*, GNAS, adrenal, tumors, hyperplasia, mutations

## INTRODUCTION

Heterotrimeric G proteins are the molecular switches that turn on intracellular signaling cascades in response to the activation of G protein-coupled receptors (GPCRs) by extracellular stimuli. Therefore, G proteins play a crucial role in defining the specificity and temporal characteristics of the cellular response. Heterotrimeric G proteins consist of three subunits,  $\alpha$ ,  $\beta$ , and  $\gamma$ , and their switching function depends on the ability of the G protein  $\alpha$ -subunit ( $G\alpha$ ) to cycle between an inactive GDP-bound conformation that is primed to interact with an activated receptor and an active GTP-bound conformation that can modulate the activity of downstream effector proteins. The  $\alpha$  subunit is a GTPase; therefore, when the receptor stimulates the G protein, this subunit releases GDP

and binds GTP. In this activated state, several  $\alpha$  subunit types act directly on effector molecules to modulate their activity. Some  $\alpha$  subunits show specificity for effectors; for example,  $\alpha S$  activates adenylate cyclases,  $\alpha I$  inhibits adenylate cyclases, and  $\alpha Q$  activates phospholipase C isoforms (5) (Figure 1).

The gene encoding the alpha subunit of stimulatory G proteins (GNAS, OMIM 139320) is located on chromosome 20q13.32 (6). Activating somatic mutations of stimulatory G protein called *gsp* mutations can result in the loss of intrinsic GTPase activity of the  $\alpha$  subunit with subsequent constitutive activation of adenylate cyclase (7, 8).

*gsp* mutations are involved in the pathogenesis of McCune-Albright syndrome (MAS), which is an endocrine disorder that is classically defined by the clinical triad of bone fibrous dysplasia, *café-au-lait* skin, and peripheral precocious puberty (1).

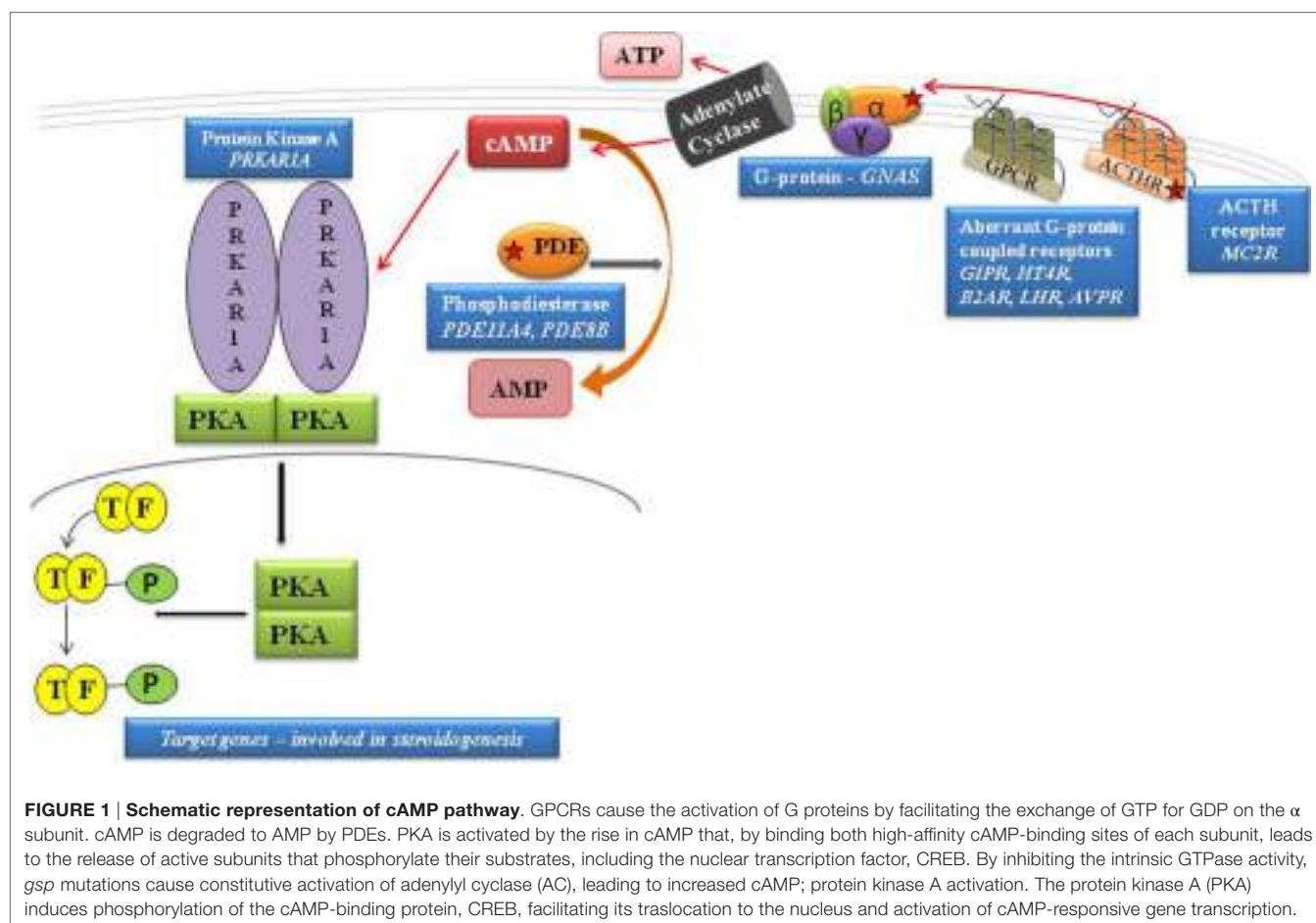
Patients with MAS present with a postzygotic *gsp* mutation in a mosaic distribution, resulting in varying degrees of tissue involvement that range from a single site to a widespread distribution. However, if these mutations were germline, they would be lethal. To date, this concept is supported by the absence of any cases resulting from vertical transmission and the discordance in disease among monozygotic twins (9). Adrenal hypercortisolism affects a minority of patients with MAS due to adrenal nodular hyperplasia and around 20 cases were described (8).

Naturally occurring mutations in codons 201 and 227, which alter the GTPase activity in the GNAS gene, have been described in autonomous hormone-producing tumors. Mutations involving substitution of either cysteine or histidine and, more rarely, serine for arginine at codon 201 or arginine for glutamine at codon 227 were first described in GH-producing pituitary tumors (10).

The *gsp* mutations have also been described in several tumors, such as somatotropinoma, thyroid tumor, ovarian and testicular Leydig cell tumors, and primary macronodular adrenocortical hyperplasia (PMAH), as well as in rare cases of corticotropinoma, cortisol, and aldosterone-secreting adrenocortical adenoma. All cases described were outside of the classical presentation of MAS (1, 2, 4, 7).

## cAMP/PKA Signaling in Adrenocortical Cells

The discovery of the role of cAMP (adenosine 3'5'-cyclic monophosphate) as an intracellular mediator introduced the concept of second messengers in signal transduction. cAMP is a nucleotide synthesized within cells using ATP, and it is under the action of a membrane-bound enzyme, adenylate cyclase. cAMP is continuously produced and inactivated by hydrolysis



**FIGURE 1 | Schematic representation of cAMP pathway.** GPCRs cause the activation of G proteins by facilitating the exchange of GTP for GDP on the  $\alpha$  subunit. cAMP is degraded to AMP by PDEs. PKA is activated by the rise in cAMP that, by binding both high-affinity cAMP-binding sites of each subunit, leads to the release of active subunits that phosphorylate their substrates, including the nuclear transcription factor, CREB. By inhibiting the intrinsic GTPase activity, *gsp* mutations cause constitutive activation of adenyl cyclase (AC), leading to increased cAMP; protein kinase A activation. The protein kinase A (PKA) induces phosphorylation of the cAMP-binding protein, CREB, facilitating its translocation to the nucleus and activation of cAMP-responsive gene transcription. [Adapted from Lacroix et al. (11)].

of 5'-AMP through a family of enzymes known as phosphodiesterase (12–14).

cAMP regulates many aspects of cell function, including enzymes involved in energy metabolism, cell division and differentiation, ion transport, ion channels, and contractile proteins. However, these effects are produced by a common mechanism, the activation of protein kinases by cAMP (Figure 2).

Proteins are phosphorylated by various protein kinases. The substrates of protein kinases and phosphatases include enzymes, neurotransmitter receptors, ion channels, and structural proteins, activating or inhibiting through phosphorylation, the target enzymes or ion channels. The increased cAMP production in response to the activation of  $\beta$ -adrenergic receptors affects several enzymes that are involved in glycogen metabolism, adipocytes, and muscle cells. The result consists of a coordinated response in which the energy stored as glycogen and fat becomes available as glucose, acting as a supply for muscle contraction (15).

cAMP, a second messenger, and its effector, protein kinase A (PKA), are key regulators of practically all cellular functions, such as cell growth and cell differentiation, and proliferation, and they mediate the effects of several hormones and neurotransmitters via GPCRs. This pathway is one of the major participants in the regulation of growth, proliferation, and hormonal secretion in adrenocortical cells (16, 17).

Once two cAMP molecules bind to each R subunit, the C subunits are released from the holoenzyme and can phosphorylate their targets, which are localized in the cytosol and in the nucleus. In adrenocortical cells, there is stimulation of glucocorticoid synthesis as well as transcriptional induction of steroidogenic enzymes and activation genes that are involved in cell replication (15, 18).

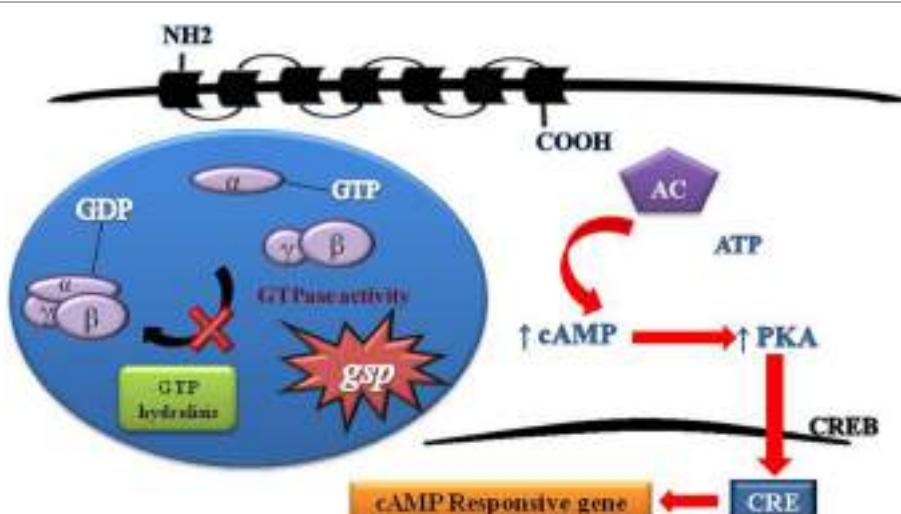
The majority of benign lesions of the adrenal cortex that lead to cortisol overproduction are linked to abnormalities of the cAMP pathway. Genetic defects in the cAMP/PKA that maintain pathway activation have been associated with adrenal disorders as follows:

- (1) Ectopic expression of G protein-coupled receptors (19, 20).
- (2) Somatic activating mutations in the gene coding for the  $\alpha_s$  protein (*GNAS*) (6, 10, 21).
- (3) Somatic activating mutations in the corticotropin receptor gene, *MC2R* (22, 23).
- (4) Germline and/or somatic inactivating mutations in the gene coding for the  $\beta$  subunit of PKA (*PRKAR1A*) (13, 14, 18).
- (5) Inactivating germline mutations in the genes coding two phosphodiesterases (*PDE8* and *PDE11A*) (24, 25).
- (6) Activating germline mutations in the gene coding for the  $\gamma$  subunit of PKA (*PRKACA*) (26).
- (7) Somatic mutations in *PRKACA*, which encodes the catalytic subunit of cyclic AMP-dependent protein kinase (27).
- (8) Germline and somatic inactivating mutations of *ARMC5* (28, 29).

Nevertheless, because these molecular events are not able to explain all cases of adrenocortical disorders, part of the molecular pathogenesis of the tumors causing Cushing's syndrome remains a challenge to scientific researchers.

## ***gsp* Mutations in Adrenal Cortical Tumors**

Somatic mutations of genes encoding components of the cAMP/PKA pathway (*GNAS*, *PRKAR1A*, *PDE8B*) and  $\beta$ -catenin (*CTNNB1*) have been reported in a small subset of adrenocortical tumors that produce cortisol (27, 30).



**FIGURE 2 | Schematic representation of G protein activation and signaling.** Heterotrimeric G proteins are composed of three distinct subunits alpha, beta, and gamma. Activity G protein depends on the alpha subunit. The alpha subunit contains high-affinity binding sites for guanine nucleotides (GDP) and has intrinsic GTPase activity. The GDP-bound form binds tightly to beta and gamma units in its inactive state. The GTP-bound form dissociates from beta and gamma units and serves as a regulator of effectors proteins. The receptor molecules cause the activation of G proteins by facilitating the exchange of GTP for GDP on the alpha subunit. The duration of subunit separation is timed by the rate of alpha subunit-mediated hydrolysis of GTP. The *gsp* mutations Arg201Cys and Gln227Arg at exons 8 and 9, respectively, of *GNAS* causing a constitutive signal of cAMP pathway.

The first report of the involvement of *gsp* mutations in adrenal tumors was by Lyons et al. In this study, the group tested the gene that encodes the alpha chain of Gi2, and the authors detected mutations that replaced arginine-179 with either cysteine or histidine in 3 of 11 tumors of the adrenal cortex and 3 of 10 endocrine tumors of the ovary (31).

Yoshimoto et al. systemically screened Gs alpha mutations in 197 human endocrine tumors. They included pituitary, thyroid, parathyroid, endocrine pancreas, and (cortex and medulla) adrenal tumors. They identified a unique, 29-year-old female patient with primary aldosteronism associated with a somatic *gsp* mutation in an aldosterone-producing adrenocortical adenoma. The authors commented that when the renin–aldosterone system is suppressed, aldosterone-producing adrenal adenomas become more sensitive to corticotropin stimulation. In this way, corticotropin is transmitted via Gs alpha-mediated cyclic AMP production. The study hypothesized that the *gsp* mutations may constitutively stimulate aldosterone synthesis in the glomerulosa zone, transmitting a constitutive signal via Gs-mediated cyclic AMP production, which would play an important role in the tumorigenesis of the aldosterone-secreting adenoma (3).

In 2000, Bugalho et al. described the presence of a mutation at codon 201 (CGT to TGT) in a patient with Cushing's syndrome due to a functioning adrenal adenoma (32). In 2004, Dall'Asta et al. also identified *gsp* mutations in one patient with ACTH-independent Cushing's syndrome. The presence of the *gsp* mutation seemed to alter the cortisol responses to agents via Gs protein-coupled receptors, whereas these responses are absent in other cases of adenoma-producing Cushing's syndrome without *gsp* mutations (33).

Moreover, Libé and Bertherat investigated the presence of genetic alterations on a series of 10 ACTH-independent Cushing's syndrome cases due to adrenocortical cortisol-secreting adenomas. The *gsp* mutation was identified in only one case, demonstrating that this abnormality is a rare cause of adrenocortical tumors. These findings suggest that different mechanisms are probably involved in adrenal tumorigenesis in primarily benign disorders (34).

A study conducted by Almeida et al. using the whole-genome expression profile (WGEP) of *PRKAR1A* and *GNAS*-mutant analysis revealed that not all cAMP activation is the same. Adrenal lesions harboring *PRKAR1A* or *GNAS* mutations share downstream activation of specific oncogenic signals (such as MAPK and cell cycle genes), but they differ substantially in their effects on others. These results support the hypothesis that several pathways can activate cAMP (13).

In 2013, Sidhu et al. described, for the first time, the presence of the p.R201C, a *GNAS* activating mutation in a malignant pediatric adrenocortical tumor. The malignant features described were as follows: areas of necrosis, microcytic degeneration, and both venous and capsular microinvasion. The tumor tissue also presented with abnormal allele-specific hypomethylation of the *KCNQ1OT1* gene involved in Beckwith–Wiedemann syndrome. Somatic mutations in these genes may constitutively activate the cAMP-protein kinase cascade, leading to cellular proliferation, which may then result in genomic instability and epigenetic alterations that give rise to ACTs and malignancy. This study suggested,

for the first time, that activation of the cAMP-PKA cascade alone may not be sufficient to cause malignant transformation in the adrenal cortex without resulting in secondary events (35).

Recently, somatic mutations of *PRKACA* (encoding the catalytic subunit of PKA) have been identified in more than one-third of the patients with Cushing's syndrome from sporadic adrenocortical adenomas; however, the molecular pathogenesis of the majority of sporadic adrenocortical tumors remains unclear (18, 27, 36).

In summary, apart from the known somatic mutations described in the literature, no other recurrent mutation by itself can explain the mechanism of tumor formation and hormone hypersecretion.

## ***gsp* Mutations in Primary Macronodular Adrenal Hyperplasia**

Over the last two decades, different studies have supported that multiple molecular mechanisms may be involved in the pathogenesis of PMAH (7, 12–14, 37). Several pathways were studied and analyzed for gene alterations, suggesting that there may be a heterogeneous group of diseases with a common presentation, ranging from subclinical hypercortisolism to overt Cushing's syndrome.

Bilateral adrenal hyperplasia may be part of MAS that is associated with hypercortisolism, especially in young children during the first years of age. The adrenal nodules of these patients carried the *gsp* mutation and adrenal cortical cells increased the levels of cAMP (6, 8).

In 2003, Fragoso et al. identified two distinct *gsp* somatic mutations affecting arginine residues in codon 201 of *GNAS* in a few patients with PMAH without any features or manifestations of MAS (2).

Subsequently, Hsiao et al. also reported the presence of *gsp* somatic mutation in one additional patient with PMAH (4). On the other hand, an additional study failed to observe *gsp* mutations in PMAH (37). Technical variations in the methodologies employed for the investigation of *gsp* mutations could explain the discrepancy in these published findings.

Almeida et al. described the analyzed the WGEP of primary pigmented nodular adrenocortical disease (PPNAD) patients associated with *gsp* mutations. The data indicated that cAMP activation in adrenal lesions harboring *gsp* mutations share the downstream activation of some oncogenic signals but differ significantly in their effects on others (13).

Considering the great variation of MAS, these cases might characterize late *gsp* somatic mutations considering that similar mutations have been described outside the context of MAS (e.g., acromegaly, thyroid cancer, and ovarian–testicular neoplasms) (7).

Based on strong previous evidence implying that increased cAMP signaling is sufficient for cell proliferation and cortisol production, we posit that although these somatic activating mutations in *GNAS* are a rare molecular event, they are probably sufficient to induce the development of macronodule hyperplasia and variable cortisol secretion (12, 38, 39).

Primary macronodular adrenocortical hyperplasia is a heterogeneous disorder that could be associated with genetic defects in

both germline and somatic levels. The presence of somatic *gsp* mutations was detected in rare cases with PMAH without MAS features. The role of *gsp* mutations in the development of this adrenal disorder remains partially unclear (2).

Recent studies have now indicated that PMAH is more frequently genetically determined than previously believed (28, 29). Germline mutations of *ARMC5* in ~50% of patients with apparently sporadic PMAH, and also in a large family with genetically transmitted PMAH. The *ARMC5* has no apparent link to the cAMP pathway, but its inactivation decreases the expression of both MC2R and various steroidogenic enzymes (19).

## Activation of Cyclic AMP Signaling in Lesions of the Adrenal Cortex due to Somatic GNAS Mutations

Aberrations in cAMP/PKA signaling are essential to the pathogenesis of benign cortisol-producing lesions of the adrenal cortex (40).

An important study by Almeida and coauthors analyzed the WGEF of adrenal lesions harboring somatic *GNAS* mutations in the normal adrenal pool and tissue with somatic *PRKAR1A* genes. They included three microdissected samples from adrenal lesions that were all caused by the same somatic *GNAS* mutation (p.R201H) as follows: a cortisol-producing adenoma from patients with PMAH and Cushing's syndrome and hyperplasia from patients with MAS and Cushing's syndrome. The results of this study showed that the MAPK and p53 signaling pathways were highly overexpressed in all lesions compared with normal tissues. *GNAS*-mutant tissues were significantly enriched for extracellular matrix receptor interaction and focal adhesion pathways compared with *PRKAR1A* mutants. In addition, *NFKB*, *NFKBIA*, and *TNFRSF1A* were overexpressed in *GNAS*-mutant adrenal tissue (13).

*NFKB* is a transcription activator nuclear factor kappa light-chain enhancer of activated B cells, a complex protein that controls DNA transcription, cytokine production, and cell survival. The nuclear factor kappa-beta controls the expression of various genes that are involved in cancer-related processes, including immune and inflammatory responses; cell adhesion, proliferation, differentiation, and apoptosis; and angiogenesis (13).

*TNFRSF1A* is a tumor necrosis factor receptor member of the superfamily 1A and the protein encoded by this gene is a member of the TNF-receptor superfamily. This protein is one of the major receptors for tumor necrosis factor-alpha. This receptor can activate NF-kappa-beta and mediate apoptosis, and it functions as a regulator of inflammation (13).

The study of adrenal lesions harboring *PRKAR1A* or *GNAS* mutations, conducted by Almeida et al. suggested that cAMP signaling inhibitors could be used as molecularly designed therapies for subclinical CS in the context of bilateral adrenal hyperplasia (13).

Despite the importance of the cAMP pathway, these molecular events are a rare cause of adrenocortical tumors and adrenal

hyperplasia, showing that several other genes should be involved in the cAMP pathway as long as other genes are involved in adrenal tumorigenesis and hyperplastic development.

## NEW INSIGHTS

This review aimed to provide a summary of the main studies on the role of cAMP and *gsp* mutations on the development of adrenocortical tumors and adrenal hyperplasia. Throughout this review, we showed that *gsp* mutations are a rare cause of adrenal disorders and involvement of *gsp* mutations in increasing the cAMP levels is not always detected.

The molecular pathogenesis of cortisol-producing adrenal adenomas and adrenal hyperplasia is not well understood.

Somatic mutations in the gene encoding beta-catenin (*CTNNB1*) have primarily been found in non-secreting adrenocortical adenomas, and there is some evidence that increased endocrine activity may be linked to aberrant cAMP signaling.

Tumor suppressor genes point mutations (such as *CTNNB1*) and alterations in the cortisol producing (*ARMC5* and *PKC* mutations) could lead to alterations in cAMP pathway.

Mutations in the genes *PDE11A*, *PDE8B*, and *PRKAR1A* have also been identified in patients with adrenal disorders related to cortisol production (14).

Nevertheless, lesions of the adrenal gland that are associated with adrenal Cushing syndrome, independent of their *GNAS*, *PRKAR1A*, *PDE11A*, and *PDE8B* mutations, have functional abnormalities in cAMP signaling. It is hypothesized that epigenetic events or additional genetic defects of the regulatory molecules in this pathway exist and have yet to be identified (41).

## CONCLUSION

The genetic analyses of the molecular pathogenesis of sporadic cortisol-secreting adrenocortical adenomas confirm the key role of the cAMP/PKA-signaling pathway in stimulating both the function and proliferation of adrenocortical cells. They provide insights into the development of adrenal hormonal autonomy and may provide the basis for novel advances in the diagnosis and therapy of adrenal Cushing's syndrome.

In conclusion, this review demonstrates that not all increased cAMP/PKA signaling has the same effect on adrenocortical tumor formation. The role of non-PKA-dependent functions of cAMP in the adrenal cortex has not been adequately investigated.

Based on strong previous evidence implying that increased cAMP signaling is sufficient for cell proliferation and cortisol production (38), we surmise that although really rare, these *gsp* mutations are probably sufficient for macronodule formation and the hypercortisolism status.

## AUTHOR CONTRIBUTIONS

MCBVF, IQW, IPC, BMPM: All authors contributed equally for the article.

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# Alterations of Phosphodiesterases in Adrenocortical Tumors

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Alterations in the cyclic (c)AMP-dependent signaling pathway have been implicated in the majority of benign adrenocortical tumors (ACTs) causing Cushing syndrome (CS). Phosphodiesterases (PDEs) are enzymes that regulate cyclic nucleotide levels, including cyclic adenosine monophosphate (cAMP). Inactivating mutations and other functional variants in *PDE11A* and *PDE8B*, two cAMP-binding PDEs, predispose to ACTs. The involvement of these two genes in ACTs was initially revealed by a genome-wide association study in patients with micronodular bilateral adrenocortical hyperplasia. Thereafter, *PDE11A* or *PDE8B* genetic variants have been found in other ACTs, including macronodular adrenocortical hyperplasias and cortisol-producing adenomas. In addition, downregulation of *PDE11A* expression and inactivating variants of the gene have been found in hereditary and sporadic testicular germ cell tumors, as well as in prostatic cancer. PDEs confer an increased risk of ACT formation probably through, primarily, their action on cAMP levels, but other actions might be possible. In this report, we review what is known to date about *PDE11A* and *PDE8B* and their involvement in the predisposition to ACTs.

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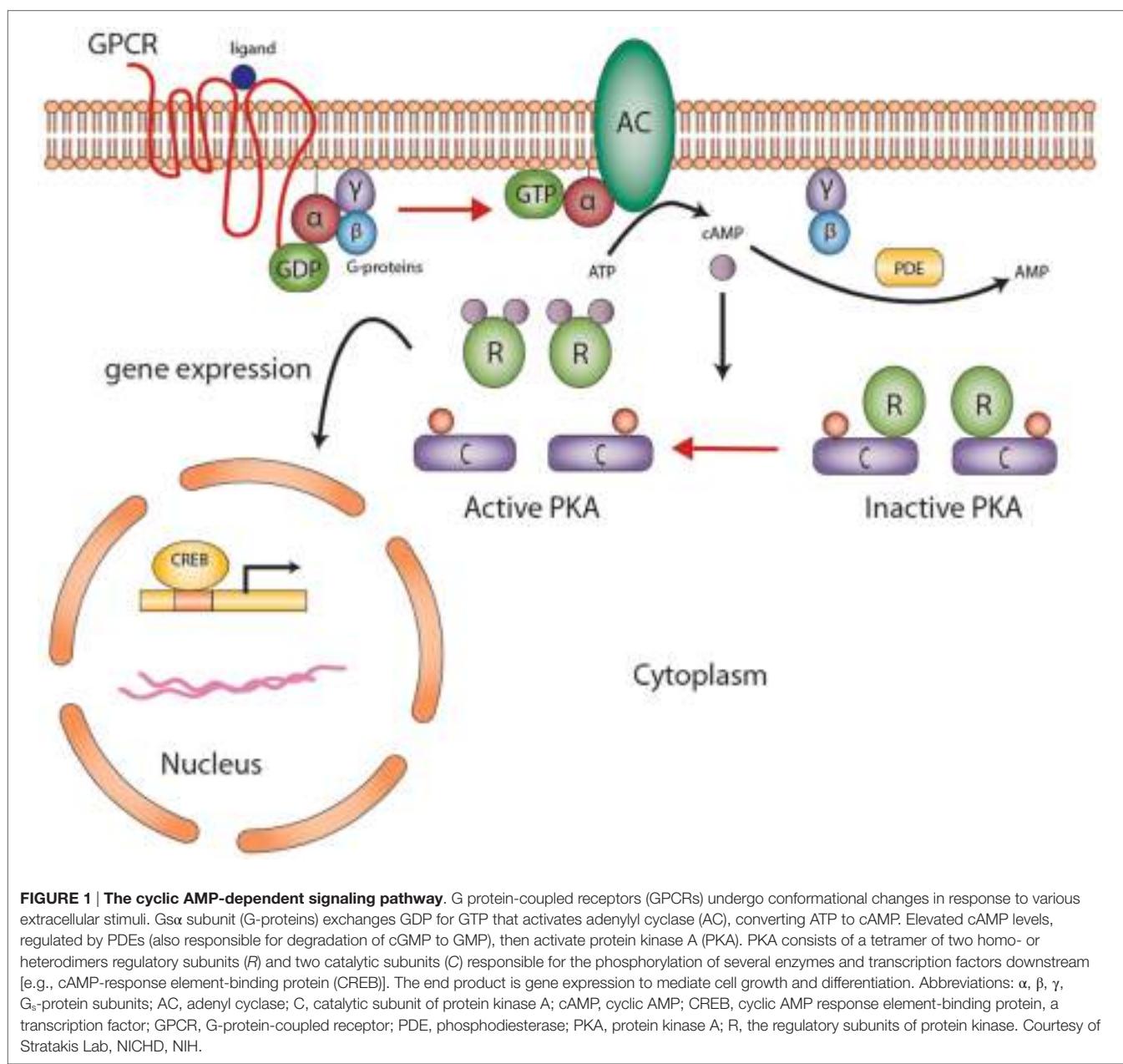
## INTRODUCTION

The development and function of the endocrine system is highly dependent on second messengers in hormonal signaling. Advances in molecular and genetic studies and appreciation for the impact of second messengers on endocrine physiology and disease sparked important discoveries in signal pathway research especially over the past decades. The first second messenger to be identified was cyclic adenosine monophosphate (cAMP), described in 1958 by Earl Sutherland (1). The production and degradation of cAMP is regulated by adenyl cyclases (AC) and phosphodiesterases (PDEs),

**Abbreviations:** AC, adenyl cyclase; ACTH, adrenocorticotropic hormone; AIMAH, ACTH-independent macronodular adrenal hyperplasia; AMP/ATP, adenosine monophosphate/adenosine triphosphate; Alleles, alternative forms of a gene; cAMP, cyclic adenosine monophosphate; CNC, Carney complex; CS, Cushing syndrome; CREB, cAMP-response element-binding protein; GMP/GDP/GTP, guanosine monophosphate/guanosine diphosphate/guanosine triphosphate; Genes, units of inheritance at specific locations (loci) on a chromosome; GPCRs, G protein-coupled receptors; GWA, genome-wide association; heterozygous, a genotype with two different alleles of a gene for a particular trait; homozygous, a genotype with the same allele of a gene for a particular trait; MMAD, massive macronodular adrenocortical disease; mutations, alteration of genetic material producing a new variation; PBAD, primary bimorphic adrenocortical disease; PBMAH, primary bilateral macronodular adrenocortical hyperplasia; PDEs, phosphodiesterases; phenotype, detectable expression of a genotype; PKA, protein kinase A; PPNAD, primary pigmented micronodular adrenal disease; PRKAR1A, protein kinase A regulatory subunit type 1.

respectively (**Figure 1**) (1, 2). Recent studies have demonstrated a link between genetic alterations in PDEs and increased predisposition of tumor formation, particularly in the prostate, testis, and the adrenal cortex (3, 4). In primary bilateral macronodular adrenocortical hyperplasia (PBMAs), aberrant expression of several non-mutated G-protein-coupled receptors (GPCRs) showed that cAMP signaling could be increased without genetic mutations (5). In primary pigmented nodular adrenocortical disease (PPNAD), germline inactivating mutations of the protein kinase A regulatory subunit type 1 (*PRKAR1A*)-linked cAMP-dependent protein kinase (PKA) to adrenocortical tumors (ACTs) (6). Indeed, cAMP signaling dysregulation through expression defects or mutations appears to underlie the pathogenesis of most benign ACTs (5, 7).

Phosphodiesterases exist in over 100 isoforms and are derived from 21 genes separated into 11 *PDE* gene families (summarized in **Table 1**) (8–10). PDEs function through the hydrolyzation of cAMP (PDEs isoform 4, 7, and 8) and cyclic guanosine monophosphate (cGMP) (PDEs isoforms 5, 6, and 9) into AMP and GMP, respectively (8, 9). Dual-specificity PDEs (acting on both cAMP and cGMP with varying affinities) include the PDE1, PDE2, PDE3, PDE10, and PDE11 enzymes. All PDEs share a major structural feature; a conserved catalytic domain with about 300 amino acids located near the C-terminal regions, and a variable regulatory domain located in the N-terminal regions. PDEs vary in a number of ways, including a difference in substrate selectivity, tissue distribution, kinetic, and tissue expression (**Table 1**). The adrenal cortex expresses several isoforms of PDEs, including PDE2A



**TABLE 1 | Characteristics of phosphodiesterases (PDEs).**

PDEs	Gene(s)	Locus	Substrate	Major functions/regulations
PDE1	<i>PDE1A</i>	2q32.1	cAMP > cGMP	• Vascular smooth muscle contraction, sperm function
	<i>PDE1B</i>	12q13.2	cAMP > cGMP	• Dopaminergic signaling, immune cell activation
	<i>PDE1C</i>	17p14.3	cAMP = cGMP	• Vascular smooth muscle cell proliferation, sperm function
PDE2	<i>PDE2A</i>	11q13.4	cAMP = cGMP	• Aldosterone and ACTH secretion, long-term memory
PDE3	<i>PDE3A</i>	12p12.2	cAMP > cGMP	• Cardiac contractility, platelet aggregation, vascular smooth muscle contraction, oocyte maturation, and regulation of renin release
	<i>PDE3B</i>	11p15.2		• Impact on lipolysis, glycogenolysis, insulin secretion, and cardiac function
PDE4	<i>PDE4A</i>	19p13.2	cAMP	• Brain function, monocyte and macrophage activation, neutrophil infiltration, vascular smooth muscle proliferation, fertility
	<i>PDE4B</i>	1p31.3		• Regulate $\beta$ -adrenergic signaling and excitation–contraction coupling in the heart and thus play a role in vasodilatation and cardiac contractility
	<i>PDE4C</i>	19p13.11		
	<i>PDE4D</i>	5q11.2–q12.1		
PDE5	<i>PDE5A</i>	4q26	cGMP > cAMP	• Modulate NO/cGMP effects in vascular smooth muscles, platelets, and lower urinary tract organs • Cardiac stress response
PDE6	<i>PDE6A</i>	5q32	cGMP > cAMP	• Primary effector enzyme in the phototransduction cascade
	<i>PDE6B</i>	4p16.3		• Regulate cGMP concentration in rod and cone photoreceptors
	<i>PDE6C</i>	10q23.33		
PDE7	<i>PDE7A</i>	8q13.1	cAMP	• Play a critical role in the regulation of the human T-cells function
	<i>PDE7B</i>	6q23.3		
PDE8	<i>PDE8A</i>	15q25.3	cAMP	• Play a role in T-cell activation
	<i>PDE8B</i>	5q13.3		• Regulate adrenal steroidogenesis • Regulate TSH levels • Control of LH signaling and steroidogenesis in Leydig cells
PDE9	<i>PDE9A</i>	21q22.3	cGMP > cAMP	• Energy balance
PDE10	<i>PDE10A</i>	6q27	cAMP > cGMP	• Play a role in striatal activation and behavioral activity
PDE11	<i>PDE11A</i>	2q31.2	cAMP = cGMP	• Only the A4 splice variant is expressed in adrenal tissue • Sperm production

ACTH, adrenocorticotropic hormone; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanine monophosphate; LH, luteinizing hormone; PDE11A, phosphodiesterase 11A gene; NO, nitric oxide; TSH, thyroid-stimulating hormone.

Adapted from Ref. (10).

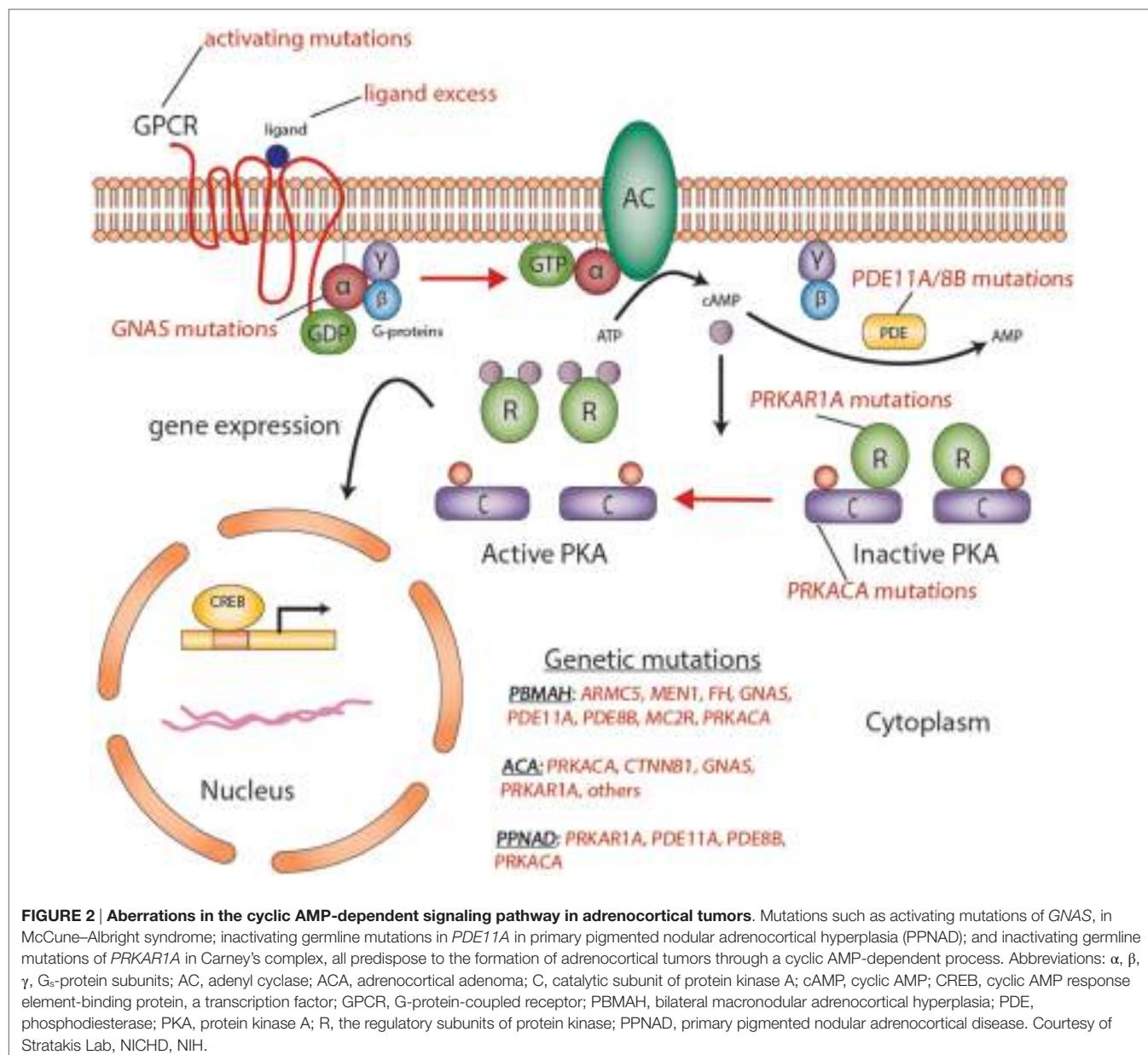
that has been implicated in the downregulation of aldosterone production in adrenal zona glomerulosa cells and the regulation of the adrenocorticotropic hormone (ACTH)-induced increase in intracellular cAMP in the zona fasciculata cells (11).

Recent studies have demonstrated inactivating mutations and other germline variants in *PDE11A* and *PDE8B* in ACT causing Cushing syndrome (CS) (12–17). Here, we present a brief overview of the alterations of PDEs in ACTs. Given the breadth of this topic, we begin with a discussion of the cAMP-dependent signaling pathway in physiology, describe the current classification of ACTs, and then proceed with a discussion of PDE alterations in ACTs.

## THE cAMP-DEPENDENT SIGNALING PATHWAY

Briefly, GPCRs undergo conformational changes in response to various extracellular stimuli, such as ACTH (Figure 1). The first step in cAMP activation in adrenal cortex is the action of ACTH on its seven-transmembrane receptor, ACTHR [e.g., melanocortin 2 receptor (MC2R)]. This activation leads to the dissociation of the G $\alpha$  subunit (encoded by the *GNAS* gene) from the

heterotrimeric G-proteins, activation of AC, generation of cAMP, and activating PKA (Figure 1). PKA exists as a tetrameric complex of two regulatory subunits (alpha and beta type 1 or alpha and beta type 2, encoded by *PRKAR1A*, *PRKAR2A*, *PRKAR1B*, and *PRKAR2B*) and two catalytic subunits (catalytic alpha and catalytic beta, encoded by *PRKAC*A and *PRKAC*B, respectively); the latter subunits are responsible for the phosphorylation of several enzymes and transcription factors downstream, including the cAMP-response element-binding protein (CREB). Abnormalities in some of these genes predispose to the formation of cortisol-producing ACTs (Figure 2) and increased steroid hormone secretion. The latter is often mediated by secondary factors regulated by PKA; in one study, silencing p54(nrb)/NONO expression in H295R human adrenocortical cells decreased the ability of the cells to increase intracellular cAMP production and subsequent cortisol biosynthesis in response to ACTH (18). The expression of multiple PDE isoforms, including PDE2A, PDE3A, PDE3B, PDE4A, PDE4D, and PDE11A, was induced in p54(nrb)/NONO knockdown cells, which suggests that these proteins may be responsible for the splicing and degradation of *PDE* transcripts (18). Studies in knockout mice (Table 2) have also pointed out to the significance of cAMP signaling-regulating genes in ACT formation.



**FIGURE 2 | Aberrations in the cyclic AMP-dependent signaling pathway in adrenocortical tumors.** Mutations such as activating mutations of GNAS, in McCune–Albright syndrome; inactivating germline mutations in *PDE11A* in primary pigmented nodular adrenocortical hyperplasia (PPNAD); and inactivating germline mutations of *PRKAR1A* in Carney's complex, all predispose to the formation of adrenocortical tumors through a cyclic AMP-dependent process. Abbreviations:  $\alpha$ ,  $\beta$ ,  $\gamma$ , G $\alpha$ -protein subunits; AC, adenyl cyclase; ACA, adrenocortical adenoma; C, catalytic subunit of protein kinase A; cAMP, cyclic AMP; CREB, cyclic AMP response element-binding protein, a transcription factor; GPCR, G-protein-coupled receptor; PBMAD, bilateral macronodular adrenocortical hyperplasia; PDE, phosphodiesterase; PKA, protein kinase A; R, the regulatory subunits of protein kinase; PPNAD, primary pigmented nodular adrenocortical disease. Courtesy of Stratakis Lab, NICHD, NIH.

## A HISTORICAL PERSPECTIVE AND NOMENCLATURE OF ADRENOCORTICAL TUMORS

In 1912, Cushing described the pituitary tumors that cause the condition that today bears his name (19). The causes of CS are broadly divided into ACTH-dependent and ACTH-independent disease. In 1984, the first description of Carney complex, a multiple neoplasia syndrome associated with spotty skin pigmentation, cardiac myxomas, pituitary tumors, and CS caused by PPNAD, provided the first insight into the genetic forms of ACT (20). In 1991, Weinstein et al. (21) described G $\alpha$  subunit (GNAS) mutations in individuals with McCune-Albright syndrome (MAS); MAS is classically associated with polyostotic fibrous dysplasia, café-au-lait skin spots, and precocious puberty but

**TABLE 2 | Adrenocortical phosphodiesterases mouse models and phenotypes.**

Gene	Model	Phenotype
<i>PDE2</i>	<i>Pde2a</i> <sup>tm1Dgen</sup> <i>Pde2a</i> <sup>tm1Dist</sup>	Homozygous for knockout allele exhibit lethality early in gestation therefore difficult to study (58)
<i>PDE8</i>	<i>Pde8b</i> <sup>tm1Dgen</sup>	Homozygous for null allele with increased urine corticosterone, decreased serum ACTH, and decreased sensitivity to a PDE8-selective inhibitor (39)
<i>PDE11</i>	<i>Pde11a</i> <sup>tm1Lex</sup>	No adrenal phenotype. Homozygous for null allele have reduced sperm concentration (45)

also with adrenal hyperplasia and/or tumors (21). MAS, albeit rare, appears to be the most frequent cause of CS among ACTH-independent adrenal hyperplasia in the infantile period (22).

Kirschner et al. (23, 24) identified the regulatory subunit type 1A (R1 $\alpha$ ) of PKA (encoded by the *PRKAR1A* gene on chromosome 17q22-24) as the cause of PPNAD and CNC (25). PPNAD is the most frequent endocrine manifestation in CNC.

In 2006, the first association between *PDE* mutations and ACT, using a genome-wide association (GWA) study approach, was identified in patients with CS with PPNAD but without *GNAS* or *PRKAR1A* mutations (12). Three inactivating mutations in *PDE11A* were identified initially in patients, predominantly children, with micronodular adrenocortical hyperplasia (iMAD), a rare form of bilateral adrenocortical hyperplasia (BAH) leading to CS. Subsequent studies found other *PDE* mutations or functional variants, including alterations in *PDE8B*, in other ACTs, including PBMAH (14–17, 26, 27).

The new genetic findings influenced the diagnostic classification of these ACTs (5). In brief, three major ACTH-independent ACT subtypes exist: bilateral hyperplasias (BAH), adrenocortical adenomas (ACA), and adrenocortical cancer (ACC) (5). PBMAH, a form of BAH, is estimated to affect ~2% of patients with endogenous CS (5). This disease should be distinguished from secondary adrenocortical hyperplasia, which can occur after long-term stimulation by ACTH in Cushing disease (CS in the context of an ACTH-producing pituitary tumor) or ectopic ACTH secretion, predominantly from a neuroendocrine tumor (28). PBMAH is usually sporadic, but familial forms have been described (29). Cortisol-producing adenoma (CPA) is a benign subset of ACAs causing adrenal CS.

Bilateral adrenocortical hyperplasias can be broadly classified on the basis of the size of their nodules into micronodular (<1 cm in diameter) or macronodular (>1 cm in diameter) (5). The micronodular subtype is divided into pigmented (c-PPNAD, familial seen in CNC, or isolated, i-PPNAD) and not pigmented (e.g., iMAD) (5). PBMAH is the most common macronodular BAH [previously referred to as massive macronodular adrenocortical disease (MMAD) and ACTH-independent macronodular adrenocortical hyperplasia (AIMAH)] and is largely caused by mutations of the *ARMC5* gene (30); several genes have been implicated in other forms of macronodular BAH, including *GNAS*, *APC*, and *MEN1*. Patients with MAS may develop a form of macronodular bilateral adrenal hyperplasia, called primary bimorphic adrenocortical disease (PBAD).

## PDEs IN ADRENOCORTICAL TUMORS

Mutations and variants in ACTs that lead to functional abnormalities of cAMP signaling have been reported in the *GNAS*, *PRKAR1A*, *PDE11A*, and *PDE8B* genes (9). Mutation-negative disease with activation of the cAMP pathway has been reported (31), suggesting that additional genetic (or perhaps epigenetic) "hits" may play a role in the pathogenesis of ACT.

### PDE2A

The predominant PDE isoform in adrenal tissue is PDE2A (32). Three PDE2A isoforms exist: PDE2A1, PDE2A2, and PDE2A3, and exhibit higher affinity for cGMP than cAMP (33). PDE2A is implicated in the downregulation of aldosterone production

in adrenal zona glomerulosa cells and the regulation of the ACTH-induced increase in intracellular cAMP (11). This ACTH response is described as a rapid and sustained activation of AC followed by a biphasic effect of ACTH on PDE2 activity with an initial and rapid inhibition, followed by a delayed activation (11). In one study, PDE2 involvement was observed to be more important in rat than in human adrenal glomerulosa cells, whereas AC was more stimulated in human than in rat glomerulosa cells (34). Thus, PDE2 activity is involved in the regulation of cAMP accumulation induced by ACTH and suggests that ACTH inhibits this activity. However, no studies to date have reported an association between alterations in PDE2 and ACTs. PDE2A has been shown to be upregulated in beta-catenin (*CTNNB1*)-mutated ACTs (35). However, it has not been studied in individuals with PPNAD that also have somatic mutations in *CTNNB1* (36, 37). Importantly, *Pde2a* knockout mice do not survive past 17–18 days gestation (38); *Pde2a* heterozygote mice are not known to develop ACTs or even hypertension.

### PDE8

The PDE8 family of proteins includes two genes, *PDE8A* and *PDE8B*, which encode for two highly specific enzymes responsible for the highest affinity of the PDEs to degrade cAMP (32). Through negative modulation, these isomers play an important role in adrenal, ovarian, and testicular steroidogenesis (32, 39). Although homologous in structure and function, *PDE8B* is the major regulator of one or more pools of cAMP in steroidogenesis and carries the highest expression across zona fasciculata compared to other PDEs (14, 32, 39). *PDE8A* is expressed from a small population of zona fasciculata cells that lie adjacent to zona glomerulosa (32), while *PDE8B* is expressed throughout the zona fasciculata. The *PDE8B* locus, like that of other PDEs, is quite complex and encodes multiple isoforms, arising mainly from alternative splicing and displaying tissue-specific expression (32, 40, 41). Tsai et al. (39) demonstrated that *Pde8b* knockout mice showed elevated urinary corticosterone as a result of adrenal hypersensitivity toward ACTH (39), pointing to PDE8B's possible role in regulating steroidogenesis. However, the investigators also demonstrated that these mice do not develop adrenal hyperplasia or increased adrenal size.

A GWA study identified a link between the 5q13 locus harboring the *PDE8B* gene and iMAD (12). A novel missense mutation was found in *PDE8B* (c.914A > C, p.P305H) in a 2-year-old girl with iMAD where her father carried the same genetic defect with subclinical disease (15); this pattern of an unaffected male passing on the disease to an affected female was also seen in other alterations of PDEs (12, 42). The p.P305H mutation led to higher levels of cAMP when introduced in HEK293 cells (15).

A more recent study of ACTs found several variations in *PDE8B*: missense substitutions p.H391A, p.P660L, and p.V697I and the c.1365-5G>A splice variant in *PDE8B* were identified (43). Interestingly, one patient with ACC had both the missense p.R121H and the splice c.1365-5G>A variations, while the other germline *PDE8B* mutations were found in samples including PBMAH, PPNAD, and secreting and even non-secreting ACAs (43).

Perhaps, the most important recent finding confirming PDE8B's role in ACT pathogenesis was the genome-wide transcriptomic work by Wilmot Roussel et al. (44). Among over 3000 genes that showed correlation with cortisol secretion in 22 unilateral ACAs (5 non-secreting, 6 subclinical cortisol producing, and 11 cortisol producing), *PDE8B* showed the strongest positive correlation (44). Accordingly, there was marked increase of the PKA activity to cAMP ratio in secreting adenomas compared to non-secreting adenomas (44).

## PDE11A

*PDE11A* is located on chromosome 2q31.2 and encodes a dual-specificity PDE that degrades both cAMP and cGMP (32). This gene is highly polymorphic in the general population (42) and was the first of the PDEs to be linked with an inherited condition associated with ACTs. Four different transcript variants exist (Table 1), with only the *PDE11A4* detected in adrenal tissues (32). *Pde11a* knockout mice show impaired sperm function and spermatogenesis (45), but no adrenal phenotype has been described for the knock out or heterozygote mouse. Several studies have reported conflicting results with regard to the adrenal expression of *PDE11A*, suggesting that its expression may only be driven in the diseased adrenal gland (46, 47). The exact role of *PDE11A* in regulating adrenocortical cAMP levels also remains largely unknown.

Horvath et al. (12) published the first GWA single-nucleotide polymorphism (SNP) association between *PDE11A* mutations and ACTs in patients with CS from PPNAD or iMAD without known genetic defects (12). Three inactivating mutations in *PDE11A* were identified. These tumors showed 2q31–2q35 loss of heterozygosity (LOH) and elevated cAMP levels (12), supporting *PDE11A*'s role in tumor formation. Three of the four patients had PPNAD; a mother and her daughter with the same *PDE11A* gene mutation, and a third unrelated patient with a different *PDE11A* mutation, in which the adrenal glands were described as small (largest total adrenal weight = 6.9 g and normal = 8–9 g) with very minor involvement of the superficial cortex evidenced by a few transcapsular cortical extensions into the peri-adrenal fat (48). The fourth patient's adrenal glands were slightly enlarged owing to hyperplasia of the superficial cortex with a few PPNAD-type nodules in the deep cortex (48). The *PDE11A* mutation was inherited from her father who had an enlarged right adrenal gland but no CS (48).

In another study, Horvath et al. (42) examined two relatively frequent variants of *PDE11A* in ACTs and the general population (42). Twelve of 745 controls had these variants, with a lower frequency in patients with ACTs (1.6%;  $\chi^2 = 14.62$ ,  $P < 0.0001$ ). *In vitro* data demonstrated elevated cAMP levels in HeLa and HEK293 cells, particularly when the p.R804H mutation was studied (42). Another study showed the p.R867G *PDE11A* gene variant in one patient with familial PBMAH (49). The mechanism by which partially inactivated *PDE11A* causes adrenocortical overgrowth is largely unclear; the most likely explanation is chronic (albeit modest) elevations of cAMP levels in adrenocortical tissues. Collectively, these experiments suggest that genetic variations in *PDE11A* may be low-penetrance alleles that occur

relatively frequently in the general population and may predispose to the development of ACTs.

The association of *PDE11A* variants and ACTs was studied further in larger cohorts. Libé et al. (27) examined the role of the *PDE11A* in a large cohort of ACT, and found an inactivating mutation (p.R307\*) in one ACC, with a significant difference between ACC and controls for a polymorphism in exon 6 (p.E421E; OR, 2.1;  $P = 0.03$ ) (27). Three associated polymorphisms located in intron 10–exon 11–intron 11 were also significant in these tumors (OR, 0.5;  $P = 0.01$ ) (27). Other variants in the study included 22 germline missense variants (18.8%) in ACA, compared to only 11 missense variants (5.7%) in controls (16 versus 10% in ACC, 19 versus 10% in ACA, and 24 versus 9% in PBMAH; OR, 3.53;  $P = 0.05$ ) (27). This study suggested a higher frequency of mutations in ACTs, especially PBMAH, when compared to controls. In another study that examined a large cohort of patients with PBMAH, the frequency of all *PDE11A* variants (e.g., p.D609N or p.M878V) was significantly higher among patients with PBMAH (28%) than controls (7.2%) ( $P = 5 \times 10^{-5}$ ) (17). These variants were also studied in HEK293 cells, where the mutant *PDE11A*-transfected cells had higher cAMP levels than the wild-type ones ( $P < 0.05$ ), suggesting that these mutants exhibit diminished cAMP hydrolytic activity (17).

These experiments pointed to several important points about the possible *PDE11A*'s role in adrenocortical tumorigenesis. First, the spectrum of ACTs varies from benign to malignant. Second, bilateral disease is favored. Third, the allelic losses of the wild-type allele in ACC with missense mutations supports *PDE11A* role as a tumor suppressor gene. Fourth, *PDE11A* sequence defects may underlie at least part of the commonly found adrenocortical incidentalomas.

Alterations in PDEs may also be involved in modifying the expression of syndromic diseases associated with ACTs. CNC is caused by *PRKAR1A* mutations, as discussed earlier (23). In one study of 150 patients with CNC, a higher frequency of *PDE11A* variants was observed when compared with healthy controls (25.3 versus 6.8%,  $P < 0.0001$ ), particularly in men (30.8 versus 13%,  $P = 0.025$ , PPNAD subgroup) (16). Importantly, these men had a higher incidence of large-cell calcifying Sertoli cell tumors, as well (16). Moreover, simultaneous *in vitro* inactivation of *PRKAR1A* and *PDE11A* by small inhibitory RNA led to increased PKA activity and/or cAMP signaling (16). Thus, it is conceivable that *PDE11A*-inactivating variants act in concert with other genes in disease predisposition and/or progression.

## ABERRATIONS IN THE cAMP-DEPENDENT SIGNALING PATHWAY IN CORTISOL-PRODUCING ADENOMAS

Several genetic aberrations in the cAMP-dependent signaling pathway have been implicated in CPA. The most common genetic aberration in CPA is a somatic-activating mutations of *PRKACA* (c.617A>G/p.L206R) with an estimated incidence of ~42% (86 of 206 tumors studied to date) (50–52), with a predilection to younger patients with overt CS, suggesting a driver mutation role

in tumorigenesis (44). Somatic mutations in *GNAS* were identified in 5–17% of CPA (53). The somatic allelic losses of *PRKAR1A* were described in 23% of CPA; these tumors were smaller in size and had a paradoxical increase in urinary cortisol levels after dexamethasone suppression (54), due to increased glucocorticoid receptor expression in ACT (55), as often observed in patients with c-PPNAD. Defects in *Wnt*-signaling have been reported in CPA, with *CTNNB1* (p.S45P, p.S45F) in ~23% of cases (56). There are still many unknown genetic defects that lead to CPA formation.

## FUTURE DIRECTIONS

There has been significant progress in PDE-related research over the past two decades. Genetic testing has uncovered several adrenocortical conditions that were linked to aberrations in *PDE*, often preceded by a long and insidious pre-diagnostic course. This has allowed earlier identification and better management of these lesions. However, there are several unanswered questions. PDE-related research is hampered by inherent (i.e., complexity of their structures, many intracellular interactions, and largely unknown function) and technical issues (e.g., there is lack of specific antibodies for the multiple isoform of each PDE). In addition to improving the characterization of PDE expression and function, future studies should also focus on the characterization of patients with various ACT phenotypes and PDE genotypes.

Preliminary results in using recombinant compounds to activate or inhibit the PDE11A structure may have important implications for drug development. Jäger et al. (47) produced approximately fourfold to fivefold increase in PDE11A-mediated hydrolysis of both cAMP and cGMP, with some degree of PDE11A specificity, with a cGMP analog (Rp-8-pCPTPET-cGMPS) bound to the PDE11A4 GAF domain (47). Furthermore, Ceyhan et al. (57) showed that BC11-28 and BC11-38 (potent and selective PDE11A inhibitors) in both yeast-based and enzyme assays had a >350-fold selectivity for inhibiting PDE11's cGMP hydrolytic activity versus all other PDEs, while only BC11-38 inhibited PDE11A cAMP hydrolytic activity in H295R cells (57). Thus, a targeted molecular

therapy approach for lesions related to defects in PDE may aid in the future management of affected or at risk patients.

## CONCLUSION

Alterations in PDEs that lead to dysregulation of the cAMP-dependent signaling pathway have been linked to the development of ACT. These lesions are usually benign and represent an important group of genetic disorders causing CS. As genetic technology continues to revolutionize the field of endocrine genetics and as we continue to discover novel disease-causing genes on an unprecedented scale, new methods to rapidly assess the functional significance of *PDE* variants singly, or in combination, will evolve. In this review, we focused our discussion on the various genotypes and phenotypes of ACT due to alterations in *PDE*, particularly mutations in *PDE8B* and *PDE11A*. Although there has been significant progress in PDE-related research over the past two decades, there are as yet unidentified molecular causes for all of these lesions. We hope that one day targeted molecular therapies will replace adrenalectomy as the treatment of choice for these lesions.

## AUTHOR CONTRIBUTIONS

All authors contributed equally to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work; drafting the work or revising it critically for important intellectual content; final approval of the version to be published; agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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# Mouse Models Recapitulating Human Adrenocortical Tumors: What Is Lacking?

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Adrenal cortex tumors are divided into benign forms, such as primary hyperplasias and adrenocortical adenomas (ACAs), and malignant forms or adrenocortical carcinomas (ACCs). Primary hyperplasias are rare causes of adrenocorticotropin hormone-independent hypercortisolism. ACAs are the most common type of adrenal gland tumors and they are rarely “functional,” i.e., producing steroids. When functional, adenomas result in endocrine disorders, such as Cushing’s syndrome (hypercortisolism) or Conn’s syndrome (hyperaldosteronism). By contrast, ACCs are extremely rare but highly aggressive tumors that may also lead to hypersecreting syndromes. Genetic analyses of patients with sporadic or familial forms of adrenocortical tumors (ACTs) led to the identification of potentially causative genes, most of them being involved in protein kinase A (PKA), Wnt/β-catenin, and P53 signaling pathways. Development of mouse models is a crucial step to firmly establish the functional significance of candidate genes, to dissect mechanisms leading to tumors and endocrine disorders, and *in fine* to provide *in vivo* tools for therapeutic screens. In this article, we will provide an overview on the existing mouse models (xenografted and genetically engineered) of ACTs by focusing on the role of PKA and Wnt/β-catenin pathways in this context. We will discuss the advantages and limitations of models that have been developed heretofore and we will point out necessary improvements in the development of next generation mouse models of adrenal diseases.

**Keywords:** adrenal, tumor, mouse models, PKA, WNT

## INTRODUCTION

Adrenocortical tumors (ACTs) are classified as benign adrenocortical adenomas (ACAs) and malignant adrenocortical carcinomas (ACCs). Most ACTs are benign, unilateral, and non-secreting adenomas, often discovered incidentally during abdominal imaging for reasons unrelated with adrenal gland (adrenal “incidentalomas”). Although less frequently, ACAs may be secreting tumors associated

**Abbreviations:** ACA, adrenocortical adenoma; ACC, adrenocortical carcinoma; ACT, adrenocortical tumor; ACTH, adrenocorticotropin hormone; APA, aldosterone-producing adenoma; CPA, cortisol-producing adenoma; CS, Cushing’s syndrome; GIP, gastric inhibitor polypeptide; GPCR, G-protein-coupled receptors; LH/hCG, luteinizing hormone/choriogonadotropin; PA, primary aldosteronism; PBMAH, primary bilateral macronodular adrenal hyperplasia; PKA, protein kinase A; PPNAD, primary pigmented nodular adrenal hyperplasia; SF-1, steroidogenic factor 1.

with endocrine hyperfunction that leads to several symptoms and significant morbidity. Indeed, clinical manifestations of secreting ACAs differ depending on their secretion profile. Cortisol-producing adenomas (CPAs) lead to Cushing's syndrome (CS). Notably, hypercortisolism associated with unilateral ACAs is the most common form of adrenocorticotropin hormone (ACTH)-independent CS (1, 2). Aldosterone-producing adenomas (APAs) lead to primary aldosteronism (PA). APAs, together with bilateral hyperplasia, comprise 95% of all PA cases (3).

Although bilateral forms of ACTs are less frequent, several adrenal pathological conditions converge in the group of diseases termed adrenocortical hyperplasia, characterized by bilateral adrenal enlargement. Primary bilateral macronodular adrenal hyperplasia (PBMAH) is the most common and is a rare cause of CS. The report of familial forms and the bilateral nature suggest a genetic origin for PBMAH (4). Unlike PBMAH, primary pigmented nodular adrenal hyperplasia (PPNAD) is rarer but it may cause overt Cushing (5).

Contrary to ACAs, ACCs are extremely rare, with an annual incidence of 0.5–2 cases per million. However, they are highly aggressive tumors associated with poor prognosis and often diagnosed at an advanced stage (6, 7). They can occur at any age but the incidence in children is particularly high in southern Brazil due to the high prevalence of a specific TP53 mutation (8). Besides tumor growth and metastasis, clinical manifestations of ACCs are often the result of steroid hypersecretion caused by endocrine dysfunction, reminiscent of adrenal adenomas.

Over the last 5 years, genetic analyses of patients with sporadic or familial forms of ACTs has resulted in identification of alterations in a new set of genes, most of them being involved in cAMP/protein kinase A (PKA) and Wnt/β-catenin signaling pathways (9–11) (**Figures 1** and **2**). The major difference in the prevalence of ACA and ACC in patients suggests that adenomas are not precursors of malignant neoplasms. Moreover, the malignant transformation of a benign and non-functional adrenal tumor is very rare (12–14). Notably, the risk that an adrenal incidentaloma progresses to a malignant tumor has recently been estimated as almost zero by the *European Society of Endocrinology* (ESE). Consequently, European recommendations for the clinical management of patients with non-functional ACAs have been reconsidered and modified to avoid unnecessary procedures (European Congress of Endocrinology, May 2016, Munich, Germany – Symposium 5: ESE clinical guidelines: Management of adrenal incidentaloma: <http://www.ece2016.org/scientific-programme/>). By contrast, secreting ACAs are surgically removed, which prevents evaluation of a possible benign to malignant continuum in functional adenomas. In fact, the hypothesis that ACC could develop in a multistep process from normal adrenal to adenoma followed by malignant transformation relied on one case report in which a carcinoma emerged in the center of a surrounding benign ACT (15). However, some genome-wide approaches performed on independent cohorts to analyze genomic changes and gene regulation in ACTs suggests that cancers could result from pangenomic cumulative changes occurring in a multistep tumor progression (16–18). Although it is important to predict to what extent a benign lesion can be

considered as the precursor of malignancy, analysis of patients' data may not be sufficient to provide a definitive conclusion.

The use of small animals for modeling tumors in a controlled experimental manner is a valuable strategy to explore the functional significance of mutations, to dissect mechanisms underlying both adrenocortical tumorigenesis and endocrine disorders, and to provide *in vivo* tools to screen for novel therapeutic approaches. To date, several genetically modified and xenografted mouse models have been developed to investigate the involvement of specific pathways and the heterogeneous nature of ACTs, respectively. Although models established until now have shed light on important aspects of adrenocortical diseases, many of them failed to fully mimic tumors found in human adrenals (**Table 1**). Hence, there is a strong need to develop relevant mouse models to shed light on mechanisms involved in the initiation and progression of adrenal tumors. In this article, we provide an overview of the existing mouse models (xenografted and genetically engineered) of ACTs relevant to human ACTs, including adrenal hyperplasia. We will discuss limitations of models that have been developed heretofore and we will point out necessary improvements in the development of next-generation mouse models of adrenal diseases. Notably, these models should allow, on the one hand, to firmly establish the role of newly identified genes in adrenocortical tumorigenesis and, on the other hand, to explore the interplay between pathways shown to be associated with ACTs (e.g., cAMP/PKA and Wnt/β-catenin).

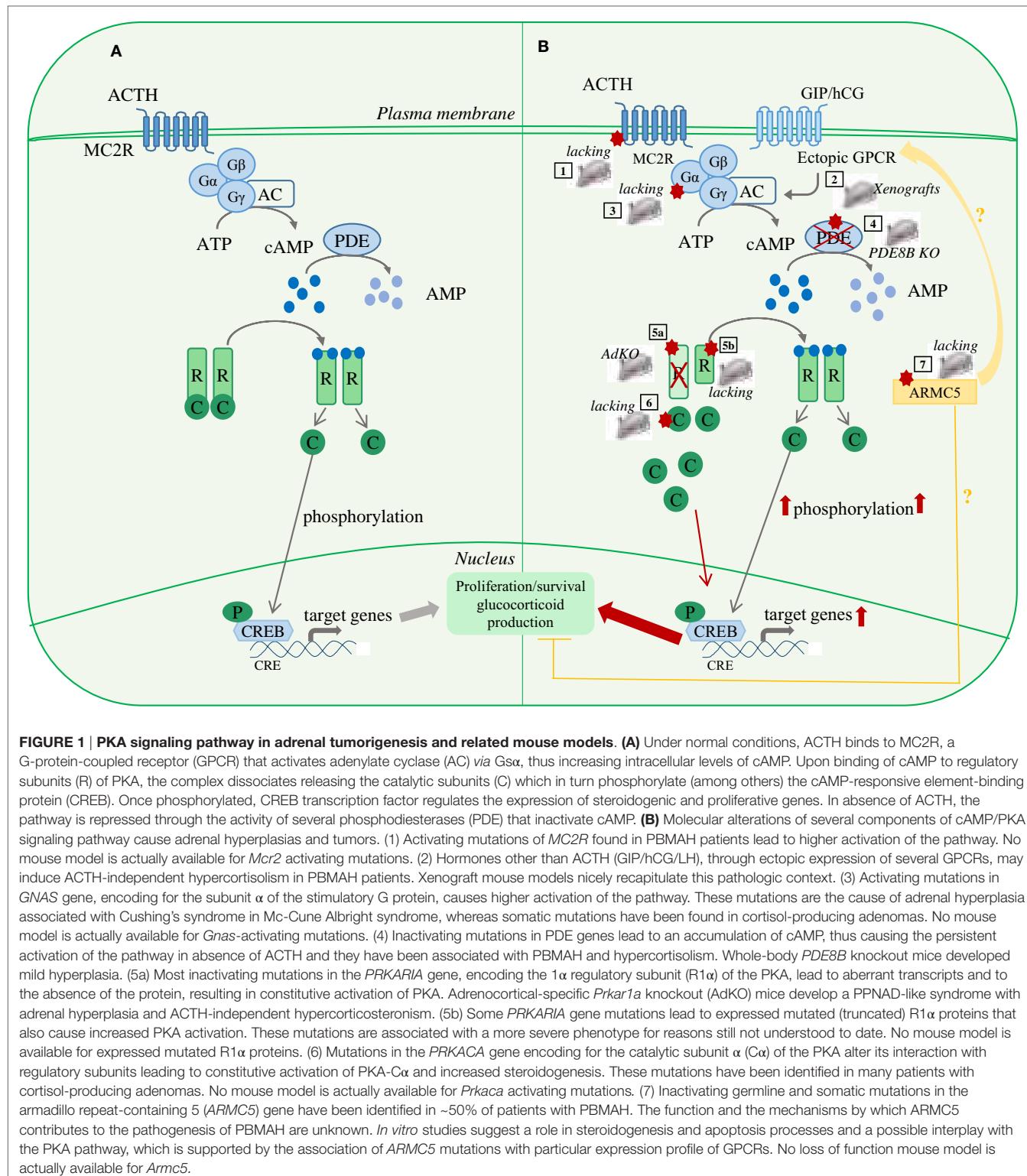
## BENIGN ADRENOCORTICAL TUMORS AND ASSOCIATED HYPERSECRETION SYNDROMES

### Primary Bilateral Macronodular Adrenal Hyperplasia

Primary bilateral macronodular adrenal hyperplasia is a rare cause of CS, accounting for <2% of all endogenous CS cases (33, 34). PBMAH is characterized by the presence of adrenocortical nodules larger than 10 mm and it is often diagnosed in patients between 40 and 60 years of age, with clinical signs of cortisol excess and suppressed levels of plasma ACTH. PBMAH was first described by Kirschner and colleagues (35) and it was named ACTH-independent macronodular adrenal hyperplasia (AIMAH). However, it has been recently found that cortisol production in PBMAH is not truly ACTH independent, since a population of adrenocortical cells in the hyperplastic tissue can produce ACTH that in turn stimulates cortisol secretion through autocrine and paracrine mechanisms (36). Hence, the term "ACTH independent" is not entirely appropriate for this disorder. Although in the majority of cases, PBMAH appears to be sporadic, several cases of familial clustering have been reported in the last years (37–39). However, the true prevalence of the familial vs. sporadic form is unknown, as systematic familial screening has not been conducted.

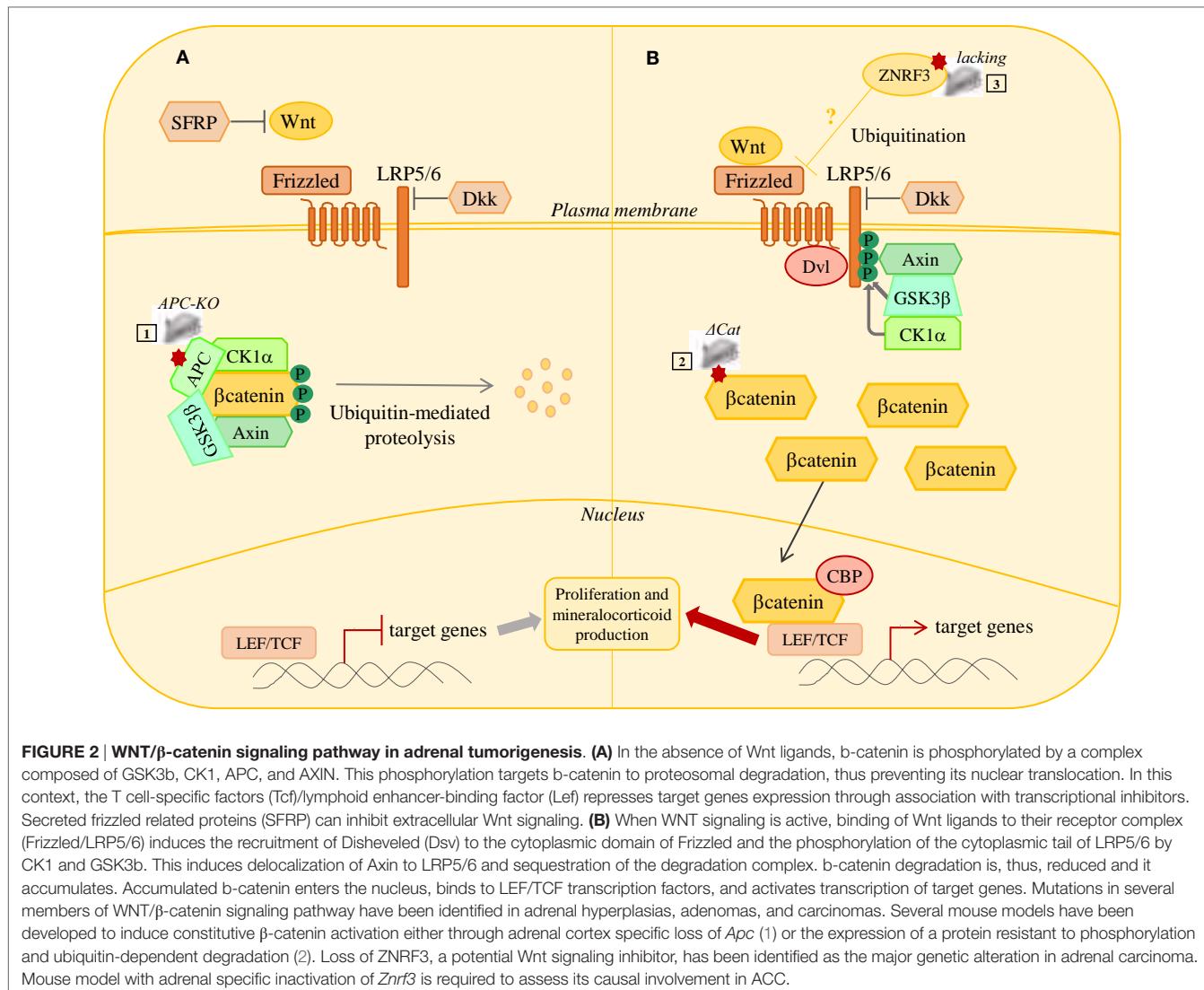
### Aberrant Hormone Receptors in PBMAH and Related Mouse Models

The mechanism by which cortisol production is stimulated in PBMAH, despite suppressed plasma ACTH, was previously



unknown and was referred to as being “autonomous.” Several groups have then shed light on the pathogenesis of hypercortisolism in PBMAH. They reported that in most patients with PBMAH and in some adenomas, cortisol secretion is regulated by hormones other than ACTH, through the aberrant expression

of several G-protein-coupled receptors (GPCRs) that are normally absent (ectopic) or expressed at lower levels in the adrenal cortex (40–42). The GPCRs for gastric inhibitory polypeptide (GIP), catecholamines, vasopressin, serotonin, and luteinizing hormone/human chorionic gonadotropin (LH/hCG) have been



shown to be involved in adrenal CS development (42). GIP-dependent CS has been reported in patients with PBMAH and with unilateral adenomas (33, 43, 44). In these patients, plasma cortisol levels were increased following meals, despite ACTH suppression, and paralleled postprandial elevation of GIP plasma concentrations. Hypercortisolism associated with aberrant LH/hCG receptors was first identified in a woman with transient CS during sequential pregnancies and persistent CS after menopause (45). Since then, several cases of PBMAH with aberrant LHR have been reported, alone or in association with GIPR (45, 46). The molecular mechanisms responsible for aberrant expression of these receptors are unknown as no genetic mutations have been found in the coding or regulatory regions of receptor genes. Furthermore, whether expression of these receptors is a primary or a secondary event in the pathogenesis of PBMAH is still a matter of debate. Several lines of evidence support the hypothesis that it is a causative factor. First, aberrant receptors are almost universally present in PBMAH and at early stages (47). Second, the same aberrant receptors were found in all members of some

affected families (37, 48), although this was not found in a known Brazilian family (39). Further data supporting a triggering role of ectopic receptors in PBMAH came from xenotransplantation mouse models. Indeed, to investigate the role of ectopic GIPR and LHR expression in the development of PBMAH, Mazzucco and colleagues used an *in vivo* model of cell transplantation and tissue reconstruction. Primary bovine adrenocortical cells were genetically engineered to express GIPR or LHR (retrovirus-mediated enforced expression) and transplanted under the kidney capsule of adrenalectomized immunodeficient mice (49, 50). Transplantation of GIPR- or LHR-expressing cells induced the formation of hyperplastic and hypertrophic adrenocortical tissues. The growth advantage provided by aberrant receptors expression at least relied on increased proliferation rates of transplants but the downstream mechanisms supporting proliferation were not explored. These models nicely recapitulated the context of human PBMAH with GPCR-dependent CS. Indeed, LHR-dependent CS is not exclusively observed during pregnancies or after menopause (41). Likewise, in the LHR xenotransplanted

**TABLE 1 | Current mouse models of adrenocortical tumors and their limitations.**

Model	Gene	Promoter/driver	Adrenocortical phenotype	Limitation	Reference
Men1 <sup>±</sup>	<i>Men1</i>	Whole-body KO	Hyperplasia, adenoma, carcinoma (lack of tumor grade definition)	Multiple tumors	(19, 20)
Prkar1a <sup>Δ/+</sup>	<i>Prkar1a</i>	<i>EIIA-Cre</i>	No adrenal phenotype	No adrenal phenotype	(21)
tTA/X2AS	<i>Prkar1a</i>	Tet-Off system	Hyperplasia maintaining of X-zone	Technical limitations, reproducibility	(22)
AdKO	<i>Prkar1a</i>	0.5 <i>Akr1b7-Cre</i>	Expansion of X-like zone with <i>zona fasciculata</i> features, autonomous corticosterone secretion	Late phenotype	(23)
Pde8b <sup>-/-</sup>	<i>Pde8b</i>	Whole-body KO	Mild hyperplasia	No adrenal-specific ablation	(24)
ΔCat	<i>Ctnnb1</i>	0.5 <i>Akr1b7-Cre</i>	Hyperplasia, adenoma and ectopic <i>zona glomerulosa</i> , hyperaldosteronism	Rare carcinomas	(25)
APC-KO	<i>Apc</i>	<i>Sf1-Cre</i> <sup>low</sup>	Hyperplasia progressed to microscopic and macroscopic adenomas	Progression to carcinoma was never observed	(26)
APC KO-H19 <sup>ADM</sup>	<i>Apc Igf2/H19 ICR</i>	<i>Sf1-Cre</i> <sup>low</sup>	Hyperplasia and adenomas, more severe phenotype than APC-KO mice	One carcinoma	(26)
PEPCK-IGF	<i>Igf2</i>	<i>Pepck-Igf2</i>	Hyperplasia	No adrenocortical tumors	(27)
ΔCat; AdIgf2	<i>Ctnnb1 Igf2</i>	0.5 <i>Akr1b7-Cre</i> 0.5 <i>Akr1b7-Igf2</i> 4.5 <i>Scc-Igf2</i>	Hyperplasia, adenoma, slight increased proliferation compared to ΔCat mice	Moderate effect on tumor progression	(28)
Acd <sup>acd/acd</sup> ; p53 <sup>±</sup>	<i>Acd, Tp53</i>		Increased development of ACC compared to Acd <sup>acd/acd</sup>	Multiple tumors	(29)
Inhα/TAg	SV40 ( <i>large T antigen</i> )	6kb <i>inhibinα-TAg</i>	Malignant ACTs developing upon gonadectomy	Unrelated to human pathology	(30)
AdTAg	SV40 ( <i>large T antigen</i> )	0.5 <i>Akr1b7-TAg</i>	Rapidly evolving tumors		(31)
YAC TR	<i>NR5A1</i>	YAC transgene	Hyperplasia and tumors	Tumors with gonadal phenotype	(32)

*MEN1*, multiple endocrine neoplasia type 1; *Prkar1a*, Protein kinase cAMP-dependent regulatory subunit type I alpha; *Akr1b7*, aldo-keto reductase family 1, member b7; *Pde8b*, phosphodiesterase 8b; *Ctnnb1*, catenin (cadherin-associated protein), beta 1; *APC*, adenomatous polyposis coli; *KO*, knockout; *ICR*, imprinting control region; *PEPCK*, phosphoenolpyruvate carboxykinase; *IGF2*, insulin-like growth factor 2; *Acd*, adrenocortical dysplasia; *TP53*, tumor protein P53; *TAg*, tumor antigen; *SV40*, simian virus 40; *YAC*, yeast artificial chromosome; *Nr5a1*, nuclear receptor subfamily; *tTA/X2AS*, transgenic mouse carrying an antisense transgene for *Prkar1a* exon 2 (X2AS) under the control of a tetracycline responsive promoter; *ΔCat*, *Ctnnb1*<sup>Δ/+</sup> line x 0.5*Akr1b7-Cre*.

model, the hyperplastic tissue formed in the absence of supra-physiological levels of plasma LH. This shows a direct role of aberrant LHR expression in the pathogenesis of PBMAH with LH-responsive CS, even though the molecular mechanisms leading to ectopic expression of GPCRs in adrenocortical cells are still unknown.

Xenotransplantation models developed by Mazzucco et al. are not easy to manage for long-term follow-up or for assessment of therapeutic strategies, because immunodeficient mice with CS have short life expectancy. However, this elegant approach combining genetic engineering and cell transplantation of bovine adrenal cells in mice may be a useful tool to test the cooperation of multiple genetic alterations in the tumorigenic process (51) or when genetic alterations may be not relevant in mouse (e.g., *KCNJ5* gene, see Aldosterone-Producing Adenomas: WNT Pathway, *KCNJ5*, and Lack of Mouse Models).

### Familial Forms of PBMAH, Genetic Alterations, and Related Mouse Models

Reports of rare familial forms and the bilateral nature of these tumors support a genetic origin of PBMAH. Many genes are associated with the development of PBMAH, including genes causing hereditary familial tumor syndromes, such as *APC* (52, 53), *MEN1* (54), and *FH* (55). Moreover, several reports

pointed out genes involved in the cAMP/PKA signaling pathway, such as *PDE8B* and *PDE11A* (56, 57), *MC2R* (58), *GNAS* (59), and *PRKACA* (9) (Figure 1). Mutations in members of cAMP/PKA pathway are predicted to over-activate the pathway but they have been observed in a limited number of patients (Figure 1).

Germline inactivating mutations of the *MEN1* gene cause a complex genetic syndrome named multiple endocrine neoplasia type 1 (MEN1) characterized by endocrine and non-endocrine tumors (60). PBMAH occurs in ~21% of MEN1 patients (54). Two whole-body *Men1* KO mouse models have been reported. They nicely recapitulate the spectrum of tumors of MEN1 syndrome, including adrenocortical lesions. Specifically, whereas homozygous whole-body *Men1* KO is embryonic lethal (19, 20), heterozygous mice are viable and develop tumors similar to those found in the human disease, including adrenocortical hyperplasia that seems to progress from adenoma to carcinoma (20). Of note, characterization of the histological phenotype, allowing establishment of tumor grade, was not detailed in this paper. Therefore, the conclusion that a multi-step tumor progression process occurs in *Men1* adrenal tumors should be taken with caution (Table 1). Since *MEN1* has recently been identified as a significantly mutated gene in ACC (11), *Men1* KO mice could be useful to identify novel actors and mechanisms underlying the evolution of benign ACTs to malignancy.

Although the bilateral nature and the multifocal nodules suggest an important role of genetic factors in PBMAH, genetic defects summarized above account for only a few cases of this adrenal disease. More recently, inactivating germline and somatic mutations in the armadillo repeat-containing 5 (*ARMC5*) gene have been identified in ~50% of patients with apparently sporadic PBMAH and also in a large family with genetically transmitted PBMAH (10, 39, 61, 62). *ARMC5* is a tumor suppressor gene with the typical “two-hit” pattern of mutations: a first germline mutation and a second somatic one. The function and the mechanisms by which *ARMC5* contributes to the pathogenesis of PBMAH are unknown. However, *in vitro* studies suggest a role in steroidogenesis and apoptosis processes (10). Indeed, *ARMC5* inactivation in cultured adrenocortical cells decreases the expression of *MC2R* and of various steroidogenic enzymes, both in basal conditions and after cAMP stimulation (10), suggesting that it may interfere with PKA pathway by impairing the stimulation of its target genes (Figure 1). Increased cell survival upon *ARMC5* inactivation is proposed to trigger hyperplasia while subclinical CS could be the result of the major increase in adrenal mass that would compensate for decreased per-cell steroidogenic activity. Furthermore, *ARMC5* mutations seem to be associated with particular expression profile of GPCRs, i.e., beta-adrenergic and dopamine receptors (10). This observation supports the hypothesis of a link between *ARMC5* and PKA signaling, as the abnormal expression of GPCRs leads to activation of PKA signaling, normally triggered by the ACTH receptor (*MC2R*) (Figure 1). Further *in vitro* studies and the development of knockout models are required to shed light on the *ARMC5*-dependent network that triggers development of PBMAH and CS. Notably, the combination of adrenal targeted GPCRs overexpression and *Armc5* knockout could provide information on a possible cooperation between *ARMC5* and PKA signaling.

## Primary Pigmented Nodular Adrenocortical Disease and Related Mouse Models

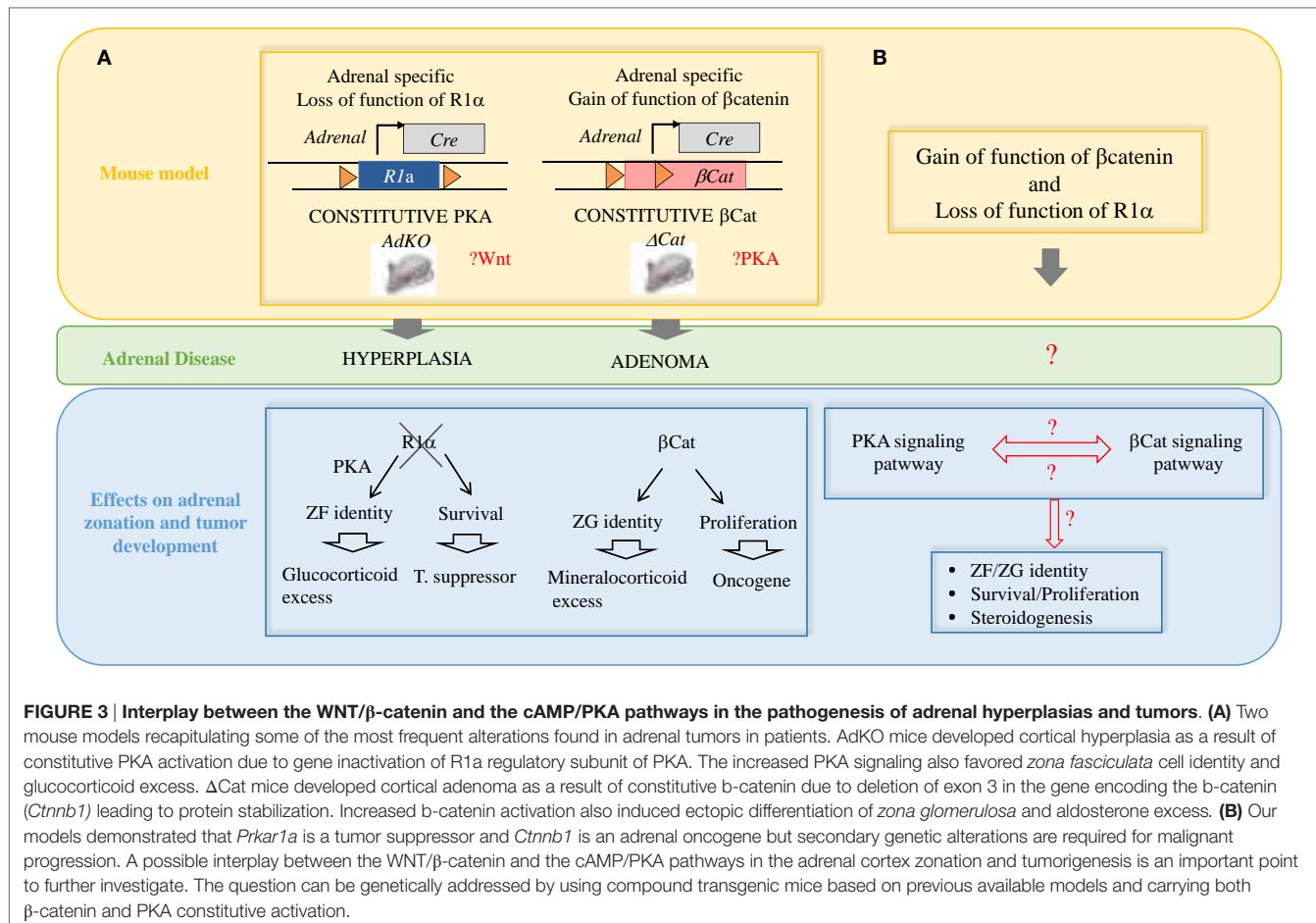
Primary pigmented nodular adrenocortical disease is a type of adrenal hyperplasia characterized by the presence of cortisol-secreting bilateral adrenal micronodules (<1 cm). PPNAD is the most common endocrine manifestation of Carney complex disease (CNC), an autosomal-dominant multiple neoplasia syndrome (63). More than 60% of CNC patients harbor mutations in the *PRKARIA* gene, encoding the regulatory subunit 1 $\alpha$  (R1 $\alpha$ ) of PKA (64) (Figure 1). Specifically, inactivating heterozygous germline mutations are observed in about two-thirds of Carney Complex patients and loss of heterozygosity (LOH) has been reported, indicating that *PRKARIA* acts as a tumor suppressor gene (64). Interestingly, somatic inactivating mutations have also been found in ACTs (65). Several mouse models have been developed to target *Prkar1a* gene inactivation by different strategies (Table 1). Total *Prkar1a* KO mice had severe defects in mesoderm development and died at E9.5 (66). Mice heterozygous for a null allele of *Prkar1a* developed several tumors but had no adrenal phenotype (21). An antisense RNA approach was developed to achieve a more severe loss of R1 $\alpha$  than in heterozygous

KO mice (22). This allowed a 70% decrease in *Prkar1a*, causing several neoplastic manifestations, including mild adrenocortical hyperplasia. Interestingly, adrenocortical manifestations in the antisense RNA model included maintenance of the X-zone, a normally transient zone of fetal origin. To achieve bi-allelic inactivation without compromising mouse survival, we generated an adrenocortical-specific *Prkar1a* knockout mouse model (AdKO) (23) by crossing *Prkar1a*-floxed strain with *Akr1b7-Cre* mice, thereby targeting specific genetic ablation in adrenocortical cells from E14.5 (67) (Figures 1 and 3; Table 1). These mice developed a PPNAD-like syndrome with ACTH-independent hypercortisolism and adrenal hyperplasia, composed of hypertrophic cells emerging from the innermost cortex. Endocrine overactivity was associated with unbuffered PKA catalytic activity, which resulted in overexpression of steroidogenic genes. Interestingly, fetal-like hyperplasia centrifugally expanded with aging at the expense of the normal adult cortex, which underwent progressive atrophy. This mouse model confirmed the important role of PKA pathway in adrenal hyperplasia and shed light on possible mechanisms responsible for PPNAD. The most likely mechanism to explain the defective zonal differentiation and cell renewal in AdKO mice is an impaired capacity of adult progenitor cells of the outer cortex to undergo centripetal differentiation and increased survival of cells in the inner cortex that would then cumulate both fetal-like and fasciculata cell features. We further showed that PKA-dependent induction of mTOR signaling was one of the mechanisms participating in the resistance to apoptosis, leading to both hyperplasia and cell hypertrophy, typical of PPNAD (68, 69). Altogether, these observations suggest that PPNAD should be considered as a developmental disease.

To further elucidate the molecular mechanisms by which PKA pathway contributes to the initiation and/or development of adrenal disorders, it is essential to provide deeper insight of its interplay with other signaling pathways (Figure 3). Interestingly, WNT pathway activation has been involved in both PBMAH and PPNAD (70, 71) (Figure 2). Furthermore, as we will discuss in next sections, WNT pathway is also associated with ACA and ACC. A possible interplay between WNT/ $\beta$ -catenin and cAMP/PKA pathways in the pathogenesis of adrenal hyperplasia and tumors would be an important point to further investigate with mouse models (Figure 3).

## Cortisol-Producing Adenomas: PKA Pathway, PRKACA Mutations, and Lack of Mouse Models

As discussed above (Sections “Familial Forms of PBMAH, Genetic Alterations, and Related Mouse Models” and “Primary Pigmented Nodular Adrenocortical Disease and Related Mouse Models”), a number of genetic defects in the cAMP/PKA pathway have been associated with adrenal hyperplasia and related to cortisol hypersecretion (Figure 1). Somatic mutations of *GNAS* (72–74) and *PRKAR1A* (75) have also been found in CPAs (Figure 1). However, these mutations only accounted for a small subset of CPAs, which represent a relevant cause of CS. Recently, Beuschlein and collaborators identified a hotspot mutation (L205R) in the *PRKACA* gene, encoding the catalytic subunit  $\alpha$  (Ca $\alpha$ ) of PKA, in



**FIGURE 3 |** Interplay between the WNT/β-catenin and the cAMP/PKA pathways in the pathogenesis of adrenal hyperplasias and tumors. **(A)** Two mouse models recapitulating some of the most frequent alterations found in adrenal tumors in patients. AdKO mice developed cortical hyperplasia as a result of constitutive PKA activation due to gene inactivation of R1α regulatory subunit of PKA. The increased PKA signaling also favored zona fasciculata cell identity and glucocorticoid excess. ΔCat mice developed cortical adenoma as a result of constitutive b-catenin due to deletion of exon 3 in the gene encoding the b-catenin (*Ctnnb1*) leading to protein stabilization. Increased b-catenin activation also induced ectopic differentiation of zona glomerulosa and aldosterone excess. **(B)** Our models demonstrated that *Prkar1a* is a tumor suppressor and *Ctnnb1* is an adrenal oncogene but secondary genetic alterations are required for malignant progression. A possible interplay between the WNT/β-catenin and the cAMP/PKA pathways in the adrenal cortex zonation and tumorigenesis is an important point to further investigate. The question can be genetically addressed by using compound transgenic mice based on previous available models and carrying both β-catenin and PKA constitutive activation.

more than one-third of patients with CPA (9). Four other groups subsequently reported the same mutation (72–74, 76). The PRKACA L205R mutation results in constitutive PKA activation (9, 74), which is a very likely cause of ACT formation (Figure 1). Supporting this hypothesis, patients with somatic mutations had adenomas, whereas patients with germline duplications had bilateral hyperplasias. Hence, PRKACA mutations, together with the previously identified GNAS and PRKAR1A inactivating mutations, strongly support a crucial role of cAMP/PKA pathway in the tumorigenesis of CPAs. The development of knock-in mouse models bearing *PRKACA* activating L205R mutation is required to evaluate its driver potential and to provide new insights into the mechanisms underlying PKA-dependent tumorigenesis in the context of cortisol-producing ACAs. In addition, adding extra copies of *Prkaca* in mouse, by additive transgenesis or targeted transgenesis at the *Rosa26* locus, would provide an opportunity to explore the pathogenic, maybe oncogenic, potential of PKA signaling. Such complementary mouse models would help understanding if gain-of-function mutations and gain of copy number alterations result in distinct adrenal lesions. Activating mutations in *CTNNB1*, the gene encoding β-catenin, have also been identified in CPAs (77). Interestingly, the recently identified PRKACA mutations were shown to be mutually exclusive with *CTNNB1* mutations (72–74, 76). Therefore, a possible interplay

between WNT/β-catenin and cAMP/PKA pathways in CPAs remains to be investigated. Once again, mouse models should be invaluable tools to address this question *in vivo* (Figure 3).

### Aldosterone-Producing Adenomas: WNT Pathway, KCNJ5, and Lack of Mouse Models

In recent years, high throughput next-generation sequencing technologies have allowed major advances in the knowledge of the genetic bases of APAs. By comparing the APA exome to the germline exome, recurrent somatic mutations have been identified in genes coding for ion channels and transporters regulating the cell membrane potential. Specifically, mutated genes encoding ion channels include *KCNJ5*, which encodes the G-protein activated potassium channel GIRK4, and is mutated in about 26–40% of APAs (78, 79) and *CACNA1D* and *CACNA1H* genes encoding for voltage-dependent calcium channels (80, 81). Genes encoding regulators of the cell membrane potential include two ATPases, *ATP1A1* and *ATP2B3* (82). All these mutations ultimately lead to increased intracellular calcium and abnormal activation of calcium-calmodulin-dependent kinase (Ca<sup>2+</sup>-CAMK) signaling, which plays a central role in aldosterone production. Before identification of these novel APA-associated genes, several mouse

models of potassium channels inactivation (*KCNK3/KCNK9* inactivation models) allowed understanding the effects of calcium homeostasis disruption and of some important aspects of PA (83–85). However, none of these models recapitulated hyperplasia and/or tumor development observed in human disease. Although one cannot exclude that mouse adrenal context may not be relevant to reproduce pathophysiological conditions associated with APA formation in humans, this suggests that in APAs, the tumorigenic potential could rely on a yet unidentified alteration. Among the newly identified genes, *KCNJ5* mutations represent the most frequent genetic defects in APAs, with higher prevalence in the Japanese population (79). Thus, animal models of *KCNJ5* inactivation are warranted to confirm a central role for this gene in the initiation of APAs. However, *Kcnj5* mRNA and *KCNJ5* protein are not expressed in the rat adrenal cortex, suggesting that it does not play a role in adrenal steroid production in this species and very likely in mice (86). Therefore, the demonstration that loss of *KCNJ5* is sufficient to initiate both hyperaldosteronism and tumor development will require genetic approaches in non-mouse systems, such as primary bovine adrenocortical cells and tissue reconstruction in xenografted mice.

Both cAMP/PKA and WNT/β-catenin pathways have been involved in the development of CPAs. Similarly, WNT/β-catenin pathway plays an important role in APA development, in addition to calcium signaling. Indeed, by generating a mouse model with constitutive β-catenin activation in the adrenal gland (ΔCat model), we found that these mice developed progressive dysplasia and hyperplasia, ectopic differentiation of *zona glomerulosa* (ZG), and increased aldosterone production (Table 1). The ΔCat model will be further discussed in the ACC section. In a subsequent paper, Berthon and colleagues reported that WNT/β-catenin pathway was aberrantly activated in 70% of a series of 47 patients, which was the most frequent alteration reported in APAs (87). Furthermore, a recent study has reported *CTNNB1* mutations in 5.1% of a cohort of 198 APAs (88). These mutations were associated with stabilized β-catenin, suggesting activation of WNT pathway (Figure 2). Because of the higher frequency of WNT/β-catenin activation than of *CTNNB1* mutations, it is essential to expand our knowledge of the other causes of aberrant WNT/β-catenin activation, by investigating other members of the pathway or possible crosstalks with other pathways. Interestingly, decreased expression of the WNT inhibitor *SRFP2* was shown to contribute to deregulation of WNT/β-catenin pathway in the adrenal (87) (Figure 2).

## ADRENOCORTICAL CARCINOMAS: THE LACK OF MOUSE MODELS

Adrenocortical carcinomas are extremely rare, with an annual incidence of 0.5–2 cases per million in adults. However, they are highly aggressive tumors associated with poor prognosis and often diagnosed at an advanced stage for which available treatments are rarely curative. The overall 5-year survival rates range from 10 to 40% (6, 7). ACCs can occur at any age, but the incidence in children is particularly high in southern Brazil due to the high prevalence of a specific germline mutation (p.R337H) of the *TP53* tumor suppressor gene (8). Pediatric and adult ACCs

differ in genetics and many other ways (89, 90), as will be further discussed in Section “Pediatric ACCs: TP53, SF1, and Related Mouse Models.” Although most ACCs arise sporadically, an increased incidence of ACCs has been reported in some genetic syndromes, such as familial adenomatous polyposis (FAP), characterized by mutations in *APC* and elevated Wnt/β-catenin signaling, and Beckwith–Wiedemann syndrome (BWS), characterized by elevated expression of insulin-like growth factor-2 (IGF2) (91, 92).

The developmental context of the disease is crucial for the treatment and management of adrenal tumors. Therefore, there is a strong need to generate mouse models resembling human pathology, to identify the mechanisms involved in benign to malignant progression. Up to now, the three most frequent alterations that have been reported in ACC patients include overexpression of IGF2 (93, 94), activation of WNT/β-catenin signaling pathway (11, 77, 95), and inactivation of TP53/RB pathway (96, 97). Several mouse models of deregulation of these pathways have been generated, which allowed better understanding of adrenal tumorigenesis. However, none of these models was able to recapitulate full-fledged ACC development (Table 1). In the following sections, we summarize mouse models of ACC generated up to now, and focus on what is still lacking for a better understanding of adrenal tumor initiation and progression.

## Mouse Models of WNT/β-Catenin and IGF2 Signaling Activation Are Insufficient to Trigger ACC Formation

The WNT/β-catenin pathway is essential for embryonic development and cell renewal in adult adrenal cortex where β-catenin is expressed and active in ZG (98) (Figure 3). Several tumor profiling studies have reported mutations in β-catenin gene (*CTNNB1*) in both ACAs and ACCs (77, 99, 100), suggesting that β-catenin activating mutations could be involved in adrenal tumor initiation and progression to malignancy. To assess this hypothesis, we have generated a mouse model of constitutive β-catenin activation in the adrenal cortex (ΔCat mice) (25) (Figures 2 and 3; Table 1). ΔCat mice were generated by mating mice harboring a floxed allele of β-catenin [*Catnb*<sup>lox/lox3</sup>] (101) with mice expressing the Cre recombinase in steroidogenic cells of the adrenal cortex, through the *Akr1b7* promoter region (67). Cre-mediated excision of the third exon of *Ctnnb1* gene prevents β-catenin phosphorylation and ubiquitin-dependent degradation, which induces accumulation of the protein and constitutive activation of its target genes. ΔCat mice showed adrenal hyperplasia and ectopic differentiation of ZG. However, aggressive tumor formation was only observed in a subset of 17-month-old animals. Our model demonstrated that *Ctnnb1* was an adrenal oncogene but it also suggested that secondary genetic alterations were required for malignant progression. Through a different approach, Hammer and colleagues obtained similar results by generating mice with adrenal cortex specific loss of *Apc* (APC KO mice) (26), a component of the multi-protein destruction complex of β-catenin, to target WNT pathway activation (Figure 2). As ΔCat mice, APC KO mice displayed hyperplasia. Furthermore, hyperplasia progressed to microscopic and macroscopic adenomas as early as

15 weeks of age, but progression to carcinoma was never observed (**Table 1**). Results from our two groups suggest that constitutive activation of  $\beta$ -catenin initiates benign tumor development but is not sufficient to trigger malignant evolution.

Insulin-like growth factor-2 is a growth factor involved in the control of cell proliferation and inhibition of apoptosis and it was hypothesized to interact with the Wnt/ $\beta$ -catenin pathway. In sporadic adrenal tumors, IGF2 is overexpressed in 80–90% of ACCs but not in ACAs (93, 94). Several transgenic mouse models have been generated to explore the role of IGF2 in adrenal tumorigenesis (**Table 1**). Mice with overexpression of IGF2 under the control of phosphoenolpyruvate carboxykinase (PEPCK) promoter showed four- to sixfold elevation of serum IGF2 levels and mild adrenocortical hyperplasia but did not develop ACTs (27), suggesting that IGF2 overexpression was not involved in initiation of adrenal tumorigenesis. We have generated transgenic mice with adrenal cortex specific overexpression of *IGF2* (AdIgf2 mice) (28) (**Table 1**). These mice had up to sevenfold higher basal levels of IGF2 (up to 87-fold higher with ACTH stimulation), but again they did not show tumor formation despite a mild increase in cortical cell proliferation. These results indicated that IGF2 alone could stimulate adrenal cortex proliferation but it was not able to induce oncogenic transformation. The mitogenic effect of IGF2 overexpression suggests a role in tumor maintenance rather than initiation, which would rather be triggered by other actors, such as WNT pathway. This two-step model is supported by the fact that IGF2 overexpression is found only in ACCs, whereas mutations in  $\beta$ -catenin are reported in both ACAs and ACCs. To test the hypothesis of cooperation between IGF2 and WNT pathways, we generated a mouse model presenting both genetic alterations, by mating  $\Delta$ Cat mice with IGF2 overexpressing mice (28) (**Table 1**). Our analysis of this model clearly showed that IGF2 overexpression in the context of constitutive WNT/ $\beta$ -catenin pathway activation only had a moderate effect on tumor progression. In another approach, Hammer and collaborators generated a mouse model with both loss of *APC*, to achieve WNT activation, and loss of imprinting at the *Igf2/H19* region, to achieve elevated *IGF2* expression (26) (**Table 1**). These mice displayed adrenocortical hyperplasia, microscopic and macroscopic adenomas, and cancer formation. Although the phenotype was more severe than that observed in mice presenting loss of *APC* alone, only one cancer formation was observed. Taken together, these results have clearly shown that genetic alterations in WNT pathway and IGF2 overexpression are not sufficient to trigger malignant adrenocortical tumorigenesis. Interestingly, we recently reported that expression of enhancer of zeste 2 (EZH2), a histone methyl transferase of the polycomb repressive complex 2 (PRC2), positively correlated with malignancy and poor prognosis in three different cohorts of patients with ACTs (102). In this study, we provided evidence that in the H295R human adrenal cancer cell line, EZH2 down-regulation or pharmacological inhibition significantly decreased cell proliferation and aggressive behavior and induced apoptosis. Interestingly, EZH2 overexpression was shown to be the result of P53/RB/E2F pathway deregulation in good agreement with ACC omic studies. Although this remains to be demonstrated in a relevant animal model, these data suggest that EZH2 could

be involved in malignant progression. Mouse models with various ability to express EZH2 (loss- and gain-of-function) are, thus, required to decipher the molecular mechanisms involved in EZH2-mediated malignant progression, i.e., transcription repression through PRC2 recruitment or activation (103) and to identify the actors that cooperate with WNT pathway in this process. Altogether, these mouse models indicate that malignant tumors may arise from typical adenomas even if this progression only affects a small proportion of benign tumors and requires additional alterations, such as EZH2 overexpression. One challenge of these studies is the evaluation of tumor staging in mouse models. Until now, mouse tumors have been evaluated with the same criteria as human tumors, i.e., essentially Weiss's scoring. However, the transposability of these histologic criteria is unclear and the only undoubtful proof of malignant progression is metastatic dissemination, which was not observed in any of the models discussed above. Irrespective of this issue, inheritable tumors in mouse genetic models provide an invaluable tool to follow tumor initiation and progression. Whole genomic analyses in such models would provide a unique opportunity to demonstrate or invalidate the existence of a normal adrenal adenoma–carcinoma continuum in adrenocortical tumorigenesis.

## Pediatric ACCs: TP53, SF1, and Related Mouse Models

In addition to overexpression of IGF2 and activation of WNT pathway, the third most frequent genetic alteration in ACCs is inactivation of TP53/RB pathway. Germline mutations in the *p53* tumor suppressor gene are associated with the development of Li–Fraumeni syndrome (LFS), an autosomal-dominant cancer syndrome resulting in multiple malignancies, including ACCs (104, 105). The rate of germline TP53 mutations in ACCs is age-dependent, ranging from up to 80% in pediatric ACCs (97) to 3–7% in adults (96). A 10-fold increased incidence of ACCs is observed in Southern Brazil due to a germline mutation within the oligomerization domain of *p53* (p.R337H). This germline mutation was first identified in 98% of children with ACCs (106). The p.R337H mutation was also present in 78% of children with sporadic ACTs in another series and in 13% of adult patients with ACCs (107). In adult ACTs, *TP53* mutations are mostly somatic and were considered to represent a later step in tumorigenesis (108, 109). Several mouse models of *p53* dysfunction have been generated, including targeted mutations, replicating proteins identified in humans with LFS (110, 111). These mice developed a large spectrum of tumors but not ACCs. Mouse models of adrenal-specific *TP53* loss have not been generated to date, despite the high prevalence of *TP53* mutations in ACCs. However, the adrenocortical dysplasia (*Acd*) mouse model (112), carrying an inactivating mutation in *Tpp/Acd*, which normally functions to protect telomerase, was used to explore the consequences of *TP53* loss during adrenal tumorigenesis (**Table 1**). Interestingly, ablation of *p53* (obtained by mating *Acd* mice with *p53* null mice) rescued a number of characteristics of the *Acd* phenotype, including adrenal hypoplasia. This indicates that hypoplasia in *Acd* mice results from *p53*-mediated senescence. Moreover, the loss of *p53* in *Acd* mice leads to development of

ACC, suggesting that p53-mediated escape from senescence may contribute to adrenocortical carcinogenesis (29). Another approach to explore the consequences of p53 ablation during adrenal tumorigenesis consists in adrenal targeting of the Simian Virus 40 (SV40) large T antigen (TAg), a potent oncogene acting in part by inactivating p53 [reviewed in (113)]. TAg expressed under the control of several promoters has been used to induce ACTs in transgenic mice. In the *inhibin- $\alpha$*  (*inha*) promoter-TAG mouse, ACCs are induced by gonadectomy (30) (Table 1). This mouse model will not be discussed in details here as mouse models of gonadectomy-induced ACTs are beyond the scope of this review, being unrelated to the context of human adrenal tumors. In order to better recapitulate the context of human adrenal tumor development, we have generated a model in which TAg is expressed under the control of the adrenal cortex specific *Akr1b7* promoter (31) (Table 1). In this AdTAG model 2 of 3 surviving founder mice developed adrenal tumors that were used to generate cell lines for further *in vitro* analysis (114). As exome sequencing and SNP array recently confirmed that p53/RB is one of the most frequently altered pathways in ACCs (11), future mouse models are needed to further explore the role of TP53 in adrenal tumor formation and progression. Considering the well-characterized antagonism of large TAg toward p53 and Rb tumor suppressors, one would predict that the AdTAG model could provide one of the most relevant and simple models to explore adrenal tumorigenic processes.

Transcriptional profiling has demonstrated distinct signatures of adult and pediatric ACCs with the pediatric tumor transcriptome displaying similarities to that of fetal adrenal tissue (115). Moreover, pediatric and adult ACCs differ in several clinical, pathological, and molecular aspects [reviewed in (90)], suggesting that they may represent genetically distinct entities. In addition to *TP53*, the genetic locus containing steroidogenic factor 1 gene (*SF-1*) is commonly amplified and overexpressed in pediatric ACCs (116, 117). *SF-1* is a nuclear receptor transcription factor that plays an important role in the regulation of steroidogenic genes, in development and function of the adrenal cortex, and in male sexual differentiation (118, 119). In contrast to children, the frequency of amplification and overexpression in adult patients is very low (120), but it is associated with poor outcome (121). A transgenic mouse model, harboring multiple copies of a yeast artificial chromosome, including the *SF-1* genetic locus, termed YACTR mice, has been generated (32) (Table 1). Although these mice developed adrenocortical hyperplasia that further progressed to adrenal tumors in a dose-dependent manner, the morphology of adrenal tumors differed from human ACTs and displayed a gonadal phenotype that was reminiscent of ACTs occurring in gonadectomized mice. These tumors are thought to arise from pluripotent adreno-gonadal precursor cells lying beneath the outer adrenal connective capsule, which have the potential to differentiate into cells of gonadal phenotype (122). Similarly, in YACTR mice, high levels of *SF-1* may trigger proliferation of pluripotent cells and the development of adrenal tumors with a gonadal phenotype. Despite the differences in tumor phenotypes in human and mice, *SF-1* appears to play an important role in adrenal tumorigenesis in both species. Recently, it has been proposed that *SF-1* overexpression induces alterations

of redox mechanisms, which may contribute to adrenal tumorigenesis. Indeed, inactivation of SF1 target gene *Vnn1*, encoding the Vanin-1 protein involved in the response to oxidative stress, was found to antagonize the development of adrenocortical neoplasia in *SF-1* transgenic mice (123).

## Novel Identified Genes in ACCs: Interplays with WNT Pathway and Need for In Vivo Studies

Recent OMIC studies confirmed that the most frequent genetic alterations in ACCs affect the tumor suppressor gene *TP53* and the oncogene *CTNNB1* (each being altered in ~16% of ACCs) (11, 124). In addition to pointing out alterations in already known drivers (*CTNNB1* and *TP53*), these studies identified new genes not previously reported in ACCs. Among these new genes, *ZNRF3* was found to be the most frequently altered (21% of ACCs). *ZNRF3* encodes a protein with E3 ubiquitin ligase activity, supposed to act as a negative regulator of the WNT/ $\beta$ -catenin pathway (125) (Figure 2). ACCs with alterations in *ZNRF3* locus showed activation of  $\beta$ -catenin target genes, but this activation was weaker than in tumors with *CTNNB1* mutations (11). Interestingly, *ZNRF3* and *CTNNB1* alterations were mutually exclusive. Further functional studies and novel mouse models are needed to further explore the role of *ZNRF3* in ACCs. Recurrent mutations have also been found in several known cell cycle regulators, including *CDKN2A*, *CDK4*, *RB1*, and *CCNE1*, confirming the notion that release from p53-sensitive checkpoints is a critical step in the process of adrenal tumorigenesis, a notion that previously emerged from analysis of p53 ablation in *Acd* mice.

Recently, mutations have also been found in *PRKARIA* (126), a gene mutated in PPNAD (as discussed in Section “Primary Pigmented Nodular Adrenocortical Disease and Related Mouse Models”) and much more rarely in ACAs (as discussed in Section “Cortisol-Producing Adenomas: PKA Pathway, PRKACA Mutations, and Lack of Mouse Models”). These recent reports of *PRKARIA* mutations expand the role of PKA signaling in ACC. If any functional interplay exists between the WNT/ $\beta$ -catenin and the cAMP/PKA pathways in the pathophysiology of adrenal cortex, this is an important point that remains to be clarified *in vivo* (Figure 3). As reviewed in Drelon’s study (127) data from the literature are in favor of such an interplay. This could influence normal adrenal cortex renewal/zonation as well as the pathophysiology of human adrenal tumors. However, whether these pathways cooperate or antagonize each other remains to be determined. This question could be genetically addressed by generating compound transgenic mice based on already available models carrying  $\beta$ -catenin and PKA constitutive activation (Figure 3) (23, 25).

## FUTURE DIRECTIONS

The understanding of mechanisms of adrenal tumor progression is crucial for the management and treatment of the disease. Indeed, almost half of ACC patients present with metastatic disease and although mitotane alone or in combination with chemotherapy

can improve patients' survival, there is no efficient treatment for advanced disease. There is, thus, a strong need to generate mouse models resembling human pathology to identify actors involved in adrenal carcinogenesis. Especially, mouse models testing the role of newly identified genes in pediatric and adult ACCs are warranted. Moreover, as recent OMIC approaches confirmed that p53/RB is one of the most frequently altered pathways in ACCs (11), the AdTAg mouse model, previously used to generate cell lines for *in vitro* analyses (114), now becomes a relevant model for the study of ACCs.

The study of molecular mechanisms underlying malignant tumor progression represents a crucial step to develop novel specific drugs. The next step is to test their efficacy through *in vitro* and *in vivo* experiments. The only available "*in vivo*" model is the ACC xenograft obtained by subcutaneous injection of the H295R cell line in nude mice (128). Although this model has been extensively used to evaluate new and established drugs, it is important to take its limitations into account. These include deregulated antitumoral response resulting from immunodeficiency and abnormal or deficient microenvironment associated with subcutaneous injection of cells. Therefore, the AdTAg mouse model could be a useful experimental platform to assess *in vivo* the role of newly identified candidates potentially involved in malignant progression, such as EZH2 (102), using genetic or pharmacological approaches. Considering pediatric adrenal cancers, knock-in mouse models reproducing TP53 p.R337H mutation found in Brazilian families will be a major advance in our understanding of the pathogenesis of these destructive tumors. There is, thus, a strong need for novel mouse models for preclinical studies.

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Finally, high throughput next-generation sequencing technologies have allowed major advances in the knowledge of the genetic bases of ACAs. Among the newly identified genes, ARMC5, KCNJ5, and PRKACA mutations represent the most frequent genetic defects in PBMAH, APAs, and CPAs, respectively (10, 39, 61, 62, 72–74, 79). Mouse models are required to confirm the driving potential of these genes in the initiation and/or progression of ACAs. In conclusion, we expect that the progress in gene editing methods and the recent identification of new recurrently mutated genes, will soon allow the development of novel mouse models capable of faithfully reproducing human adrenal diseases, overcoming the limitations of current models. These models will be useful, on the one hand, to investigate the mechanisms underlying malignant progression of adrenal tumors and, on the other hand, to develop novel therapeutic approaches.

## AUTHOR CONTRIBUTIONS

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# Intracellular Molecular Differences in Aldosterone- Compared to Cortisol-Secreting Adrenal Cortical Adenomas

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The adrenal cortex is a major site of steroid hormone production. Two hormones are of particular importance: aldosterone, which is produced in the zona glomerulosa in response to volume depletion and hyperkalemia, and cortisol, which is produced in the zona fasciculata in response to stress. In both cases, acute stimulation leads to increased hormone production, and chronic stimulation causes hyperplasia of the respective zone. Aldosterone- and cortisol-producing adenomas (APAs and CPAs) are benign tumors of the adrenal cortex that cause excess hormone production, leading to primary aldosteronism and Cushing's syndrome, respectively. About 40% of the APAs carry somatic heterozygous gain-of-function mutations in the  $K^+$  channel *KCNJ5*. These mutations lead to sodium permeability, depolarization, activation of voltage-gated  $Ca^{2+}$  channels, and  $Ca^{2+}$  influx. Mutations in the  $Na^+/K^+$ -ATPase subunit *ATP1A1* and the plasma membrane  $Ca^{2+}$ -ATPase *ATP2B3* similarly cause  $Na^+$  or  $H^+$  permeability and depolarization, whereas mutations in the  $Ca^{2+}$  channel *CACNA1D* directly lead to increased calcium influx. One in three CPAs carries a recurrent gain-of-function mutation (L206R) in the *PRKACA* gene, encoding the catalytic subunit of PKA. This mutation causes constitutive PKA activity by abolishing the binding of the inhibitory regulatory subunit to the catalytic subunit. These mutations activate pathways that are relatively specific to the respective cell type (glomerulosa versus fasciculata), and there is little overlap in mutation spectrum between APAs and CPAs, but co-secretion of both hormones can occur. Mutations in *CTNNB1* (beta-catenin) and *GNAS* ( $G_{s\alpha}$ ) are exceptions, as they can cause both APAs and CPAs through pathways that are incompletely understood.

**Keywords:** *KCNJ5*, *CACNA1D*, *ATP1A1*, *ATP2B3*, *CTNNB1*

## INTRODUCTION

Adrenal masses are common tumors in humans. Adrenal incidentalomas may be found in more than 4% of computed tomography series (1), and about 7% are malignant (2). Among hormone-producing lesions, besides pheochromocytomas, cortisol-producing and aldosterone-producing adenomas (CPAs and APAs) of the adrenal cortex are frequently diagnosed (2). Aldosterone and cortisol are physiologically synthesized in the two outer layers of the adrenal cortex (zona glomerulosa and fasciculata, respectively) from their common precursor cholesterol. The two main stimuli of aldosterone production are angiotensin II (ATII) and hyperkalemia. ATII levels rise in states of

volume depletion, *via* activation of the renin–angiotensin system. Binding of ATII to the AT1 receptor, a G protein-coupled receptor in the glomerulosa membrane, leads to the inhibition of potassium channels, depolarization and activation of voltage-gated calcium channels, and the release of calcium from intracellular stores (Figure 1). Other factors that physiologically regulate aldosterone release in concert with ATII and K<sup>+</sup> are corticotropin (ACTH, stimulatory) and atrial natriuretic peptide (ANP, inhibitory) (3). Binding of aldosterone to the mineralocorticoid receptor leads to the increased activity of downstream effectors, such as the Na<sup>+</sup>/K<sup>+</sup>-ATPase or the epithelial sodium channel (ENaC) (4). The increased activity of these pumps and channels in kidney and intestine causes increased sodium and water reabsorption and an increase in systemic blood pressure.

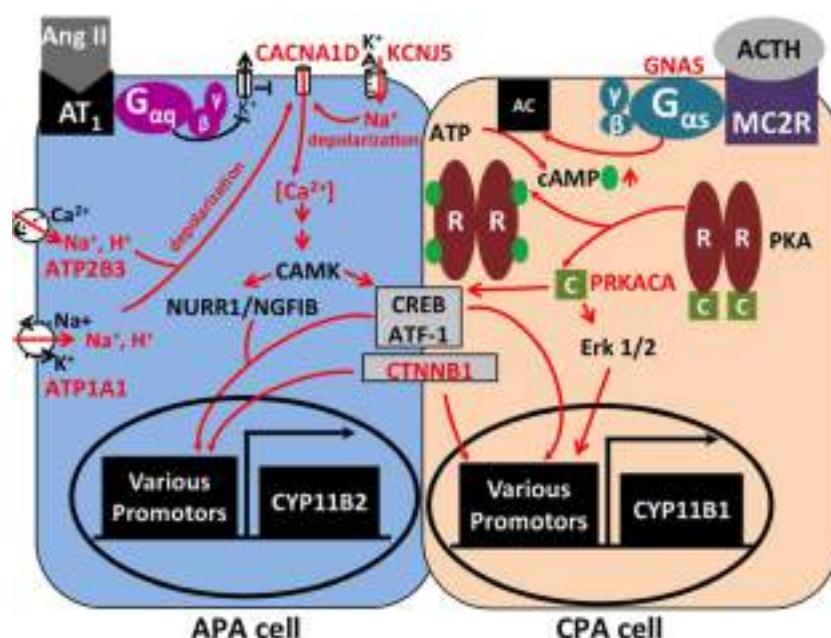
Cortisol is released from the zona fasciculata upon stimulation by pituitary ACTH, in response to stress. ACTH binds to the melanocortin receptor 2, a G protein-coupled receptor, which activates adenylate cyclase (6). As a result, cAMP is produced, which binds to the regulatory subunit of protein kinase A (PKA), causing release of its catalytic subunit. The catalytic subunit then phosphorylates target proteins, such as CREB and ATF, which lead to cortisol production and proliferation (7) (Figure 1).

Cortisol influences a variety of biological processes, including skeletal growth, immune response, glucose and lipid metabolism, cognition, and reproduction (8–10).

Cortisol-producing adenomas and APAs feature the compelling combination of both hormone production and proliferation, suggesting that they carry genetic changes that activate both processes. Such changes have been identified over the past 5 years through exome sequencing. Comparing DNA sequences from tumor specimens and corresponding normal tissue (such as blood or adjacent tissue) can reveal tumor-specific (somatic) mutations, which are candidates for disease causation (11). This review will discuss recent genetic discoveries in APAs and CPAs and the underlying pathways.

## KCNJ5 MUTATIONS IN PRIMARY ALDOSTERONISM

Primary aldosteronism (PA) features autonomous production of aldosterone from the adrenal gland and accounts for about 10% of hypertension in referral centers. The two most common causes are APAs and bilateral adrenal hyperplasia. Other causes, such as unilateral hyperplasia, malignant tumors, or familial hyperaldosteronism, are rare (12–16).



**FIGURE 1 | Signaling pathways affected by mutations in APAs and CPAs.** In zona glomerulosa, binding of angiotensin II (AngII) to its receptor inhibits potassium channels *via* G protein signaling. This leads to depolarization and opening of voltage-gated calcium channels. Increased intracellular calcium results in the activation of Ca<sup>2+</sup>/calmodulin-dependent protein kinase (CAMK) and the activation of transcription factors, such as NURR1/NGFIB, CREB, and ATF-1. As a consequence, genes involved in proliferation and aldosterone production (e.g., aldosterone synthase, CYP11B2) are activated (5). Mutations in KCNJ5, ATP1A1, and ATP2B3 lead to abnormal permeability for sodium or protons, which causes cellular depolarization and activation of the same pathways. Similarly, mutations in the calcium channel gene CACNA1D lead to increased calcium influx. In the zona fasciculata, binding of corticotropin (ACTH) to the melanocortin receptor (MC2R) causes activation of adenylate cyclase (AC) by the G<sub>αs</sub> subunit (encoded by GNAS). Binding of cAMP to the regulatory subunit ("R") of protein kinase A (PKA) leads to release of the catalytic subunit ("C," encoded by PRKACA) from the complex. Transcription factors CREB, ATF-1, and Erk 1/2 cause increased expression of genes involved in proliferation and cortisol production, such as 11 $\beta$ -hydroxylase (CYP11B1). Hypercortisolism can occur due to activating mutations in GNAS and PRKACA. Activating mutations in  $\beta$ -catenin (CTNNB1) are found in both APAs and CPAs; the underlying mechanisms are incompletely understood.

In the first exome sequencing study of APAs, Choi et al. analyzed four tumors and corresponding blood samples (11). This revealed only two to three somatic mutations per tumor. One gene (*KCNJ5*) was mutated in two tumors, with one tumor carrying a heterozygous G151R mutation, and the second carrying a heterozygous L168R mutation. By Sanger sequencing, these two mutations were found in 6 of 18 additional APAs. *KCNJ5* encodes an inward rectifier potassium channel, Kir3.4, or GIRK4. The G151 and L168 residues are located within or close to the selectivity filter of the channel (17), which allows only potassium, but not the smaller sodium ions, to pass through the channel. This suggested an effect of the variants on potassium selectivity. Accordingly, by electrophysiology, mutant channels were found to be permeable to sodium and cause cellular depolarization. These effects were inferred to contribute to aldosterone production and proliferation through the activation of voltage-gated calcium channels and calcium entry (3, 11) (Figure 1). Additional support for the notion that *KCNJ5* mutations are sufficient to cause aldosterone production and proliferation came from the discovery of heterozygous germ line *KCNJ5* mutations in families with early-onset PA and massive bilateral adrenal hyperplasia (11, 18–20). The high frequency of *KCNJ5* mutations in APAs (about 35% in European cohorts, more than 60% in Asian cohorts) has subsequently been confirmed in large cohorts (21–29) (Table 1). A higher prevalence in Asian cohorts may be due to selection bias; individuals with *KCNJ5* mutations tend to have a more florid presentation at least in some cohorts. Interestingly,

*KCNJ5* mutations are more prevalent in females than in males, which could account for the higher overall prevalence of APAs in females, a finding that remains unexplained. *In vitro* studies in the aldosterone-producing human adrenocortical cancer cell line HAC15 have demonstrated that gain-of-function mutations in *KCNJ5* lead to increased expression of aldosterone synthase and increased aldosterone production (30–32). Lastly, a recent study confirmed the role of *CYP11B2* transcriptional regulators NURR1 and ATF2 in mutant *KCNJ5*-induced aldosterone production (33) (Figure 1).

## KCNJ5 MUTATIONS AND GLUCOCORTICOIDS

Interestingly, tumors with *KCNJ5* mutations tend to be larger than other tumors and have fasciculata-like features by histopathology and gene expression analysis, which may have implications for the radiological diagnosis of these tumors (34–36). Another line of evidence pointing to a more fasciculata-like or mixed glomerulosa-fasciculata phenotype of *KCNJ5*-positive APAs is the finding that heterologous expression of a *KCNJ5* variant in HAC15 cells causes not only upregulation of *CYP11B2* expression but also increased expression of *CYP11B1* and synthesis of hybrid steroids 18-hydroxycortisol and 18-oxocortisol, as well as corticosterone (31, 33). This raises the question whether *KCNJ5*-positive APAs produce clinically relevant amounts of glucocorticoids. Interestingly, hypersecretion of cortisol and aldosterone are not

**TABLE 1 |** Mutation frequencies in APAs, A/CPAs, and CPAs.

Reference	N	APA						A/CPA			CPA		
		<i>CACNA1D</i>	<i>KCNJ5</i>	<i>ATP2B3</i>	<i>ATP1A1</i>	<i>GNAS</i>	<i>CTNNB1</i>	<i>KCNJ5</i>	<i>GNAS</i>	<i>GNAS</i>	<i>PRKACA</i>	<i>CTNNB1</i>	
Beuschlein et al. (66)	99	–	–	–	–	–	–	–	–	N/A	22.2	N/A	
Goh et al. (69)	55	–	–	–	–	–	–	–	–	5.5	23.6	16.4	
Cao et al. (67)	87	–	–	–	–	–	–	–	–	N/A	65.5	N/A	
Sato et al. (68)	65	–	–	–	–	–	–	–	–	16.9	52.3	N/A	
Di Dalmazi et al. (71)	100	–	–	–	–	–	–	–	–	N/A	22.0	N/A	
Thiel et al. (41)	52	–	–	–	–	–	–	–	–	–	7.7	23.1	25.0
Thiel et al. (41)	4	–	–	–	–	–	–	50.0	NA	–	–	–	
Yamada et al. (40)	3	–	–	–	–	–	–	66.7	NA	–	–	–	
Nakajima et al. (42)	10	–	–	–	–	–	–	60.0	20.0	–	–	–	
Xekouki et al. (84)	53	N/A	30.2	N/A	N/A	N/A	N/A	–	–	–	–	–	
Taguchi et al. (28)	23	N/A	65.2	N/A	N/A	N/A	N/A	–	–	–	–	–	
Kitamoto et al. (85)	108	1.9	69.4	2.8	N/A	N/A	–	–	–	–	–	–	
Boulkroun et al. (86)	380	N/A	33.9	N/A	N/A	N/A	N/A	–	–	–	–	–	
Azizan et al. (87)	73	N/A	41.1	N/A	N/A	N/A	N/A	–	–	–	–	–	
Cheng et al. (88)	69	N/A	37.7	N/A	N/A	N/A	N/A	–	–	–	–	–	
Kuppusamy et al. (89)	195	N/A	24.6	N/A	N/A	N/A	N/A	–	–	–	–	–	
Zheng et al. (27)	168	0.6	76.8	0.6	2.4	N/A	N/A	–	–	–	–	–	
Scholl et al. (36)	97	10.3	37.1	3.1	8.2	N/A	2.1	–	–	–	–	–	
Scholl et al. (45)	64	7.8	32.8	3.1	1.6	N/A	3.1	–	–	–	–	–	
Nakajima et al. (42)	33	N/A	72.3	N/A	N/A	6.1	N/A	–	–	–	–	–	
Beuschlein et al. (47)	308	N/A	38.3	1.6	5.2	N/A	N/A	–	–	–	–	–	
Williams et al. (24)	112	N/A	39.3	0.9	6.3	N/A	N/A	–	–	–	–	–	
Akerstrom et al. (22)	348	N/A	45.1	N/A	N/A	N/A	N/A	–	–	–	–	–	
Fernandes-Rosa et al. (23)	474	9.3	38.0	1.7	5.3	N/A	N/A	–	–	–	–	–	
Akerstrom et al. (83)	198	1.5	46.5	1.5	3.0	N/A	5.1	–	–	–	–	–	
Hong et al. (29)	66	0.0	71.2	0.0	0.0	N/A	N/A	–	–	–	–	–	
Wu et al. (25)	148	0.0	59.5	0.7	1.4	N/A	N/A	–	–	–	–	–	

N, number of study subjects; N/A, not available.

mutually exclusive in adrenal adenomas, and cases of aldosterone and cortisol co-secreting adenomas (A/CPAs) have been reported (37–40). This phenotype may be underdiagnosed due to incomplete screening for subclinical Cushing's syndrome (CS) in patients with APAs; many of these patients will not receive dexamethasone suppression tests. Yamada et al. reported three female patients with hypertension and hypokalemia who were diagnosed with A/CPAs. Two had *KCNJ5* mutations (G151R and L168R) (40). Thiel et al. reported *KCNJ5* mutations (G151R and L168R) in two of four A/CPAs, and no mutations in *PRKACA*, *ATP1A1*, *ATP2B3*, and *CACNA1D* were found (41). Lastly, Nakajima et al. demonstrated *KCNJ5* mutations in 6 of 10 A/CPAs (42). This suggests that *KCNJ5* mutations may cause excess secretion of not only aldosterone but also glucocorticoids, leading to PA with discrete features of CS. Potential explanations include the overlapping role of transcriptional regulators CREB and ATF in the regulation of both aldosterone and cortisol production (Figure 1) as well as a potential role of  $\text{Ca}^{2+}$  in cAMP formation (43).

In summary, *KCNJ5* mutations have been extensively studied in the context of PA. However, the physiological role of *KCNJ5* in human adrenal glomerulosa remains largely undetermined, and animal studies have been hampered by extremely low or absent expression of *KCNJ5* in rodents (44).

## CACNA1D MUTATIONS IN PRIMARY ALDOSTERONISM

The gene with the second highest somatic mutation burden in APAs is *CACNA1D*, with frequencies of about 8–11% described in the initial exome sequencing studies and similar findings in a large follow-up study (23, 35, 45) (Table 1). Similar to *KCNJ5* mutations, *CACNA1D* mutations are heterozygous. However, mutations are more scattered throughout the protein. *CACNA1D* encodes an L-type voltage-gated calcium channel (Cav1.3). Mutant *CACNA1D* channels show activation at more hyperpolarized membrane potentials and, in some cases, reduced channel inactivation compared to wild-type channels (45). In line with the notion that these effects will lead to increased calcium entry, expression of mutant *CACNA1D* channels causes increased aldosterone production in the adrenocortical cancer cell line H295R (46). Again, similar to *KCNJ5* variants, additional evidence for a role of *CACNA1D* in PA came from the discovery of germ line variants at the same positions found to be mutated in tumors (45). Among 100 unrelated subjects with early-onset PA and hypertension, two carried *de novo* mutations in *CACNA1D*. Interestingly, these subjects had a multi-organ phenotype, including primary aldosteronism, seizures, and neurologic abnormalities (PASNA) (45). The discovery of mutations in calcium channels as a cause of PA may suggest that specific calcium channel blockers could be useful in patients carrying such mutations (46).

## ATPase MUTATIONS IN PRIMARY ALDOSTERONISM

Additional somatic mutations in APAs without corresponding germ line mutations have been identified. Beuschlein et al. first

described heterozygous or hemizygous somatic mutations in the *ATP1A1* and *ATP2B3* genes in 5.2 and 1.6% of APAs, respectively. *ATP1A1* encodes a sodium/potassium ATPase subunit, whereas *ATP2B3* encodes the plasma membrane calcium ATPase. Mutations in both ATPases cluster within the M4 helix, again suggesting a gain-of-function mechanism (47). Azizan et al. subsequently demonstrated that *ATP1A1* mutations cause an ouabain-sensitive, voltage-dependent inward  $\text{Na}^+$  or  $\text{H}^+$  current, respectively. Heterologous expression of mutant *ATP1A1* in human adrenocortical H295R cells led to increased aldosterone production and *CYP11B2* expression levels (35), consistent with a role of mutant *ATP1A1* in cellular depolarization and activation of voltage-gated calcium channels, as with mutated *KCNJ5*. Similarly, a mutation in *ATP2B3* was shown to induce a pathological  $\text{Na}^+$  permeability, with increased intracellular  $\text{Ca}^{2+}$  levels and aldosterone production in H295R cells (48).

## SPECIFIC FEATURES AND ORIGIN OF CACNA1D- AND ATPase-MUTANT APAS

Azizan and colleagues first suggested an association of *CACNA1D* and *ATP1A1* mutations with a glomerulosa-like phenotype (35), whereas other groups reported mixed histological phenotypes (23, 36). Glomerulosa-like features in *CACNA1D* and *ATP1A1*-positive tumors could suggest that these tumors are derived from zona glomerulosa cells. Indeed, Nishimoto et al. recently studied 42 normal adrenal glands from kidney donors and identified so-called aldosterone-producing cell clusters (APCCs), nests of cells just below the adrenal capsule that feature high expression of aldosterone synthase and protrude into cortisol-producing cells (49). Remarkably, targeted next-generation sequencing of DNA from 23 APCCs identified known somatic *CACNA1D* mutations in six cases and known somatic *ATP1A1* mutations in two cases, suggesting that APCCs may represent precursors of a subtype of APAs. These results also support the presence of APCCs and potentially subclinical PA in a substantial number of apparently healthy individuals, which is interesting, given that prior clinical studies identified a higher risk of developing hypertension in individuals with increased aldosterone levels within the physiologic range (50). No somatic *KCNJ5* mutations were identified in APCCs, suggesting that APAs carrying such mutations may arise from cells of the zona fasciculata or may grow more rapidly, with precursors evading detection in apparently healthy individuals.

## INVESTIGATIONS OF MULTINODULAR TUMORS

Even though the classical presentation of aldosterone-producing adenoma is that of a uninodular lesion, many cases feature associated hyperplasia or multiple secondary nodules, many of which do not show increased expression of aldosterone synthase. Investigations of individual nodules revealed the presence of characteristic APA mutations in aldosterone-producing nodules, whereas non-producing nodules do not carry such mutations (51). Some individuals carry different

aldosterone-driver mutations in different nodules, suggesting that independent mutation events account for the development of multiple nodules (52, 53). Whether germ line susceptibility variants promote the formation of multiple tumors remain to be determined. Interestingly, some adenomas appear to show intra-tumoral heterogeneity, indicating that the somatic events underlying APA formation can also occur in the context of preexisting nodules (53). This has led to the proposal of a two-hit model of adenoma development, with one hit being responsible for proliferation and another hit causing hormone production (54). However, the rarity of such findings and the absence of second hits explaining proliferation in the exomes of tumors carrying aldosterone-driver mutations suggest that APA driver mutations alone are sufficient to cause proliferation and hormone production in the majority of APAs.

## CACNA1H MUTATIONS IN FAMILIAL HYPERALDOSTERONISM

One additional ion channel gene implicated in PA to date has been found to be mutated in the germ line only, but not in APAs. A novel germ line heterozygous variant in the *CACNA1H* gene (M1549V) was found in 5 of 40 unrelated subjects with PA and hypertension diagnosed at age 10 or below (55). Microscopic glomerulosa hyperplasia without macroscopic enlargement was demonstrated in one subject who had undergone unilateral adrenalectomy, suggesting a limited proliferative effect of the variant. *CACNA1H* encodes the low-voltage-activated T-type calcium channel Cav3.2 (56). Cav3.2 has been hypothesized to be responsible for fine adjustments in the aldosterone production when activated by small changes in potassium or ATII levels and appears to be necessary for glomerulosa membrane potential oscillations (55, 57, 58). The observed M1549V variant causes impaired channel inactivation and a slight shift of activation to more hyperpolarized potentials (55), as well as increased *CYP11B2* expression (59), suggesting a pathophysiology similar to that of *CACNA1D* variants.

## SOMATIC MUTATIONS IN ADRENAL CUSHING'S SYNDROME

Cushing's syndrome features hypercortisolism and is associated with a plethora of signs and symptoms, including weight gain, hypertension, diabetes mellitus, lethargy, acne, depression, hirsutism, and increased mortality (60, 61). CPAs are less frequent than ACTH-secreting pituitary tumors (62), but still account for up to 10% of endogenous CS (60, 63, 64). Somatic *PRKAR1A* loss-of-function mutations were identified as a cause of sporadic CPAs in a hypothesis-driven approach (65).

Following the description of somatic mutations in PA, using exome sequencing, four groups independently identified somatic mutations in the *PRKACA* gene as a cause of CS (66–69). *PRKACA* encodes the catalytic subunit of protein kinase A involved in the regulation of adrenal cortisol production (see Introduction and Figure 1). Beuschlein and colleagues sequenced the exomes of 10 CPAs and identified heterozygous somatic *PRKACA*

mutations in eight, with a frequency of 37% in the entire cohort of CPAs associated with overt CS. No *PRKACA* variants were found in CPAs associated with subclinical CS, APAs, or inactive adenomas, and the presence of *PRKACA* variants was associated with a more severe phenotype (66). All but one tumor carried a single variant, L206R, suggesting a gain-of-function effect. L206 is located in the highly conserved interaction site between the regulatory and the catalytic subunits of PKA, and binding of the regulatory subunit at this position prevents substrate phosphorylation. Molecular modeling and functional analysis of PKA activity suggested that the L206R mutation would lead to a steric hindrance and prevent inhibition of catalytic activity by the regulatory subunit (70). Somatic *PRKACA* variants other than L206R are exceedingly rare (71). Further support for the causative role of increased PKA activity in CS came from the discovery of germ line *PRKACA* duplications in subjects with bilateral adrenal hyperplasia and CS (66).

These results were confirmed in independent cohorts. Cao et al. reported an L205R variant (equivalent to L206R in the initial report) in the *PRKACA* gene in 27 of 39 CPAs. Further, two *GNAS* ( $G_{\alpha s}$ ) mutations and a *CTNNB1* ( $\beta$ -catenin) mutation were found (see below) (67). Sato et al. screened tumors of 65 patients with ACTH-independent CS. They identified *PRKACA*<sup>L206R</sup> mutations in 52.3% and *GNAS* mutations in 16.9% of the tumors. In addition, they provided evidence of an association of *PRKACA*<sup>L206R</sup> with smaller tumor size and a more severe phenotype (68). Lastly, Goh et al. reported a *PRKACA*<sup>L206R</sup> mutation in 24% of CPAs (35% of cases with overt CS). They also reported *CTNNB1* mutations in 16% and *GNAS* mutations in 6% of tumors (69). Similar results were found in additional cohorts (39, 41, 71) (Table 1). Functionally, L206R has been shown to enhance the phosphorylation of PKA downstream effectors CREB and ATF in cell culture and tumor tissue samples (69) (Figure 1).

## MUTATIONS IN GNAS AND CTNNB1 IN CUSHING'S SYNDROME AND PRIMARY ALDOSTERONISM

Mutations in *GNAS* have long been known to inhibit GTPase activity of the  $G_{\alpha s}$  subunit and thereby cause constitutive  $G_{\alpha s}$  activation, abnormal cAMP signaling, endocrine hyperfunction, and tumor formation; postzygotic *GNAS* mutations are found in McCune-Albright syndrome, which can be associated with CS (72, 73). The discovery of mutually exclusive somatic gain-of-function mutations of *PRKACA* and *GNAS* in CPAs (see above) has further demonstrated that increased cAMP signaling is sufficient to cause tumorigenesis and cortisol hypersecretion. However, somewhat unexpectedly, given the absence of *PRKACA* mutations in APAs, *GNAS* variants were also reported in A/CPAs in two instances (42). On a molecular level, given the accessory role of ACTH in stimulating aldosterone secretion, increased cAMP signaling may play a role.

*CTNNB1* encodes  $\beta$ -catenin of the Wnt/ $\beta$ -catenin pathway, which is known to play an important role in adrenocortical development and cancer (74). Activating mutations are not only found in benign and malignant adrenal tumors (75) but also

in tumors of other organs. Such mutations prevent  $\beta$ -catenin degradation and cause proliferation. Even though such events have been shown to trigger benign aldosterone-secreting and cortisol-secreting tumor development as well as malignancy in a mouse model and human tissue samples (36, 45, 69, 75–77), the exact mechanisms underlying hormone secretion in *CTNNB1* positive tumors remain to be determined.

In this context, a common pathway of *PRKACA*, *GNAS*, and *CTNNB1* has been suggested (78). However, it has been shown that *GNAS* and *CTNNB1* mutations are not always mutually exclusive in CPAs, and that mutations of *CTNNB1* are also present in non-secreting adrenal tumors (79). A recent study described an association with pregnancy in two of three cases with APAs and *CTNNB1* mutations and suggested that the manifestation may be mediated by *CTNNB1*-induced LHCGR expression and increased LH levels in pregnancy (80). However, the absence of an association with pregnancy in previously described female cases (81), the high prevalence of LHCGR overexpression in APAs (82), and the finding of *CTNNB1* mutations in male individuals with APAs (83) suggest a role of additional factors.

## CONCLUSION AND OPEN QUESTIONS

Taken together, the recent findings on the genetic causes of APAs and CPAs suggest that both result from gain-of-function mutations that concurrently lead to excess hormone hypersecretion and increased proliferation. In most cases, a single mutation is apparently sufficient for tumor formation and hormone hypersecretion. There is little overlap between CPAs and APAs

in terms of the mutational spectrum. While CPAs often carry mutations that lead to increased intracellular cAMP levels, mutations known to cause APAs mostly affect intracellular calcium signaling. Overlapping roles in the function of transcription factors ATF and CREB in glomerulosa and fasciculata function, as well as overlapping roles of signaling downstream of ACTH and calcium in cortisol and aldosterone synthesis, may explain the presence of *KCNJ5* and *GNAS* mutations in tumors secreting both cortisol and aldosterone (**Figure 1**). Open questions include the determinants of the histological phenotype of APAs with certain mutations, the molecular pathways involved in proliferation of both APAs and CPAs, potential additional factors that drive hormone production in tumors with *CTNNB1* mutations, and the pathogenesis of tumors without mutations in known driver genes. In summary, despite significant progress over the past few years, the pathophysiology behind CPAs and APAs has not been fully unraveled. Distinct and common molecular switches appear to exist in both disorders.

## AUTHOR CONTRIBUTIONS

All authors listed have made substantial, direct, and intellectual contribution to the work and approved it for publication.

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# The Role of ACTH and Corticosteroids for Sepsis and Septic Shock: An Update

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Sepsis is a common disorder associated with high morbidity and mortality. It is now defined as an abnormal host response to infection, resulting in life-threatening dysfunction of organs. There is evidence from *in vitro* and *in vivo* experiments in various animal models and in patients that endotoxin or sepsis may directly and indirectly alter the hypothalamic–pituitary–adrenal response to severe infection. These alterations may include necrosis or hemorrhage or inflammatory mediator-mediated decreased ACTH synthesis, steroidogenesis, cortisol delivery to tissues, clearance from plasma, and decreased sensitivity of tissues to cortisol. Disruption of the hypothalamic–pituitary–adrenal axis may translate in patients with sepsis into cardiovascular and other organ dysfunction, and eventually an increase in the risk of death. Exogenous administration of corticosteroids at moderate dose, i.e., <400 mg of hydrocortisone or equivalent for >96 h, may help reversing sepsis-associated shock and organ dysfunction. Corticosteroids may also shorten the duration of stay in the ICU. Except for increased blood glucose and sodium levels, treatment with corticosteroids was rather well tolerated in the context of clinical trials. The benefit of treatment on survival remains controversial. Based on available randomized controlled trials, the likelihood of survival benefit is greater in septic shock versus sepsis patients, in sepsis with acute respiratory distress syndrome or with community-acquired pneumonia versus patients without these conditions, and in patients with a blunted cortisol response to 250 µg of ACTH test versus those with normal response.

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Sepsis places a burden on health-care systems worldwide due to an annual incidence of about 100 per 100,000 inhabitants (1) and mortality rates between 15 and 40% (when shock is present) in the short term and up to 80% at 5 years (2). Moreover, roughly half of survivors may present with progressive decline in cognitive function (2, 3).

Sepsis is defined as an abnormal host response to infection, resulting in life-threatening dysfunction of organs (4). Host response to stress was originally described by Selye (5). The so-called general adaptation syndrome typically includes an early alarm phase, followed by a phase of resistance, and then a phase of exhaustion, which may result in death. Host response to stress relies on three major systems: the hypothalamic–pituitary–adrenal (HPA) axis, the autonomic nervous system, and the immune system (6). A correct balance between activation of these systems allows controlling infection while maintaining cardiovascular and metabolic homeostasis. A typical neuroendocrine response to stress includes (i) immediate increased secretion of catecholamines from

the sympathetic nervous system and adrenal medulla, release of corticotrophin-releasing hormone (CRH) and vasopressin from parvocellular neurons into the portal circulation, and secretion of oxytocin from the neural lobe of the pituitary, (ii) 5–10 s later, secretion of corticotrophin (ACTH) by anterior pituitary cells, (iii) followed a few seconds later by decreased secretion of pituitary gonadotropins and increased secretion of prolactin and growth hormone (in primates), and of renin and glucagon from the kidneys and pancreas, respectively, and (iv) a few minutes later, increased plasma levels of glucocorticoids and inhibition of gonadal steroids secretion. Any imbalance between neuroendocrine and immune responses favoring a proinflammatory state may trigger organ dysfunction and progression of infection to sepsis.

This review will summarize current knowledge on HPA axis and disruption during sepsis and the potential role of treatment with corticosteroids.

## ACTIVATION OF THE HYPOTHALAMIC–PITUITARY–ADRENAL AXIS DURING SEPSIS

During stress, the HPA axis is mainly activated by CRH-independent pathways, involving immune mediators. The hypothalamus and pituitary glands are protected from exogenous or endogenous toxic molecules by the blood–brain barrier (BBB). Invading pathogens are identified by various cells, including epithelial, endothelial, and immune cells, thanks to danger molecule associated patterns (DAMP) they express on their surface or cytosol (7). Subsequently, these cells produce factors promoting recruitment of additional cells and destruction and clearance of pathogens. Among them, proinflammatory cytokines, such as tumor necrosis factor (TNF), interleukin (IL)-1 and -6, and anti-inflammatory cytokines, such as IL-4 and -10, may contribute activating the HPA axis.

### Activation of the Hypothalamic–Pituitary Axis At the Hypothalamic–Pituitary Axis Level

There are three main routes for immune mediators to reach the hypothalamus and/or the pituitary gland. First, terminal nerve endings of autonomic nervous afferent fibers express pathogens or DAMP and receptors for many mediators (8). Then, sensing pathogens or related DAMP in tissues results in hypothalamic signaling *via* autonomic nuclei in the brainstem, which have projections to the hypothalamus, for example, between the locus ceruleus and the arcuate nucleus, and other structures of the limbic system as well. Then, efferent fibers, particularly of the vagus nerve, contribute to the attenuation of inflammation and in resuming homeostasis (9). Corticotrophin-releasing hormone is released upon acetylcholine stimulation of muscarinic receptor, an effect that is prevented by non-specific nitric oxide (NO) blockade (10). Second, inflammatory mediators released in blood from tissues can reach the portal circulation in the median eminence, located outside the BBB, *via* the anterior hypophyseal arteries. They are carried onto the brain structures, expressing

receptors for these mediators, either through areas lacking a BBB, i.e., the circumventricular organs or across it using specific transporters (11, 12). Third, systemic inflammation may cause breakdown to the BBB, facilitating blood-borne cytokines traffic to deep brain structures (13–16). Among the various factors that contribute to the disruption of tight junctions or swelling of the BBB, the complement system, particularly C5a anaphylatoxin expressed both by astrocytes and endothelial cells, may play a key role (17).

Dendritic and microglial cells may produce immune molecules. In animals, peripheral administration of endotoxin yielded expression of IL-1 (18) and TNF (19). Similarly, in patients with septic shock, postmortem examination suggested overexpression of IL-1 and TNF in hypothalamic nuclei (20). Different cytokines in different brain regions induce different brain responses. For example, IL-1 and TNF are likely the two main mediators of the so-called sickness behavior, whereas IL-6 may have no apparent direct effect on behavior (21). Experiments in animals suggest that TNF- and IL-1-induced release of corticosterone is CRH-dependent mechanism (22, 23), whereas IL-6 may stimulate adrenal function by both CRH-dependent and -independent mechanisms (24). IL-1-related activation of the HPA axis is mainly dependent on brain endothelial cells and is independent of hematopoietic cells and perivascular macrophages (25).

### At the Adrenal Gland Level

Tumor necrosis factor is produced in adrenal tissues by resident macrophages and by adrenocortical cells, particularly in the fasciculate and reticular layers (26). The presence within the adrenals of TNF and of its receptors suggests that this cytokine plays a role in adrenal function, even though experiments found variably stimulatory (27, 28) or inhibitory (26, 29) effects of TNF on steroidogenesis. Similarly, IL-1 and its receptor are also produced in adrenal tissues and may contribute to steroidogenesis at least partly by regulating prostaglandins pathways (30). Toll-like receptors (TLR) types 2 and 4 are expressed in human's adrenal cortex (31). TLR2 or TLR4 knockout mice showed impaired glucocorticoid response to LPS (32, 33). Recent data suggested that these DAMP molecules expressed by immune cells recruited in adrenal tissues play a major role in the local immune-adrenal crosstalk (34).

## Mechanisms of Disrupted Hypothalamic–Pituitary–Adrenal Axis in Sepsis

### Irreversible Damage to Neuroendocrine Cells

Sepsis is infrequently associated with necrosis or hemorrhage within the HPA axis. The venous drainage of the adrenals being limited, sepsis-associated massive increase in arterial blood flow to these glands results in enlarged glands (Table 1) (35). Then, adrenal necrosis and hemorrhage have been reported as a consequence of sepsis for more than a century (36, 37). Predisposing factors of the Waterhouse–Friderichsen syndrome include renal failure, disseminated intravascular coagulopathy, and treatment with anticoagulants or tyrosine kinase inhibitors. Ischemic lesions and hemorrhage have also been described within the hypothalamus or pituitary gland (38).

**TABLE 1 | Mechanism explaining hypothalamic–pituitary–adrenal axis disruption in sepsis.**

HPA axis level	Main mechanisms	Precipitating factors
Hypothalamus	Necrosis or hemorrhage	Anticoagulants, brisk variations in blood pressure, high dose of vasoressors Coagulopathy, severe hypoxia, hyperglycemia
	Decreased CRH/AVP synthesis/release	Treatment with corticosteroids, psychoactive drugs Increased brain levels of proinflammatory cytokines (mainly TNF and IL-1) Hypercortisololemia
Pituitary gland	Necrosis or hemorrhage	Anticoagulants, brisk variations in blood pressure, high dose of vasoressors Coagulopathy, severe hypoxia, hyperglycemia
	Decreased ACTH synthesis/release	Treatment with corticosteroids, psychoactive drugs, anti-infective drugs, megestrol acetate medroxyprogesterone Increased blood levels of proinflammatory cytokines (mainly TNF and IL-1) Coagulopathy, severe hypoxia, hypercortisololemia
Adrenals	Necrosis or hemorrhage	Anticoagulants, brisk variations in blood pressure, high dose of vasoressors Coagulopathy, severe hypoxia
	Decreased steroidogenesis	Cholesterol-lowering drugs
	Depletion of lipid droplets	
	Decreased expression of scavenger receptor B1	Proinflammatory mediators
	Enzymes inhibition	Aminoglutethimide, ketoconazole, fluconazole, etomidate, dexmedetomidine Proinflammatory mediators Circulating and adrenals proinflammatory mediators (e.g., corticostatins)
Tissue resistances	Decreased sensitivity of ACTH receptors	
	Decreased cortisol delivery to tissues	Proinflammatory mediators, liver failure, severe denutrition
	Accelerated glucose clearance	Phenobarbital, phenytoin, rifampin
	Decreased binding capacity and affinity of glucocorticoid receptor	Proinflammatory mediators

HPA, hypothalamic–pituitary–adrenal.

### Altered CRH/ACTH Synthesis

Hypothalamic/pituitary stimulation by cytokines, particularly IL-1, induced a biphasic response with initial proportional increase followed by progressive decline in anterior pituitary ACTH concentrations (39, 40). Sepsis is associated in animals (41, 42) and in humans (20) with marked overexpression of the inducible isoform of NO synthase (iNOS) in hypothalamic nuclei that is partly triggered by TNF and IL-1. Subsequent abundant release of NO may cause apoptosis of neurons and glial cells in

the neighborhood. In both rodents and humans, sepsis decreased ACTH synthesis, though its secretagogues remained unaltered (43). Then, the suppression in ACTH synthesis following sepsis may be mediated by NO (11).

ACTH synthesis can also be inhibited by various treatments (44). Opioids are the main component of patients' sedation regimen in ICU worldwide. In animals, depending on dose, timing, and duration, opioids have been shown to variably stimulate or inhibit the CRH/ACTH axis, whereas in humans, they predominantly inhibited it (45). In animals, sepsis is associated with early marked increase in ACTH levels that returned to baseline values around 72 h (46). Clinical studies have found ACTH levels to be significantly lower in critically ill patients (47, 48) and particularly in septic shock (48) than in controls. However, altered ACTH synthesis in response to metyrapone was observed in roughly half of septic shock, and very occasionally in patients without sepsis (48).

### Altered Steroidogenesis

The adrenals storage of cortisol is very limited. Therefore, adequate response to stress relies almost entirely on cortisol synthesis. The normal HPA axis response to sepsis remains unknown. Cortisol production rate is increased in critically ill patients (47). The main finding in this study was an average 50% reduction in cortisol clearance from plasma, mainly resulting from a loss in cortisol inactivation through suppressed liver and renal cortisol to cortisone shuttle. About half of septic-shock patients have decreased cortisol synthesis (48). Following administration of metyrapone, 60% of septic shock had 11 $\beta$  deoxycortisol concentrations <7  $\mu$ g/dl, suggesting decreased cortisol synthesis. The alteration may occur at various steps in the cortisol synthesis chain. First, histological examination of the adrenal cortex of both animals and humans with sepsis found marked depletion in lipid droplets, suggesting deficiency in esterified cholesterol storage (49). This loss in lipid droplets is likely mediated by annexin A1 and formyl peptide receptors (50). In normal conditions, both increased plasma ACTH concentrations and depletion in adrenal cholesterol stores upregulate adrenals scavenger receptor B1 (SRB1), an HDL receptor, which captures esterified cholesterol from blood (51). SRB1-mediated cholesterol uptake is considered an essential protective mechanism against endotoxin (52). Then, sepsis-induced deficiency in SRB1 expression by the adrenal cortex, was associated with increased mortality (53). Second, a number of environmental factors may inhibit adrenal steroidogenesis (54). Steroidogenesis may be inhibited at various enzymatic steps by drugs, including P-450 aromatase, hydroxysteroid dehydrogenase, or mitochondrial cytochrome P-450-dependent enzymes (44). In critically ill patients, etomidate, which inhibits the last enzymatic step in cortisol synthesis, increased the risk of adrenal insufficiency, 4–6 h (OR 19.98; 95% CI 3.95–101.11) and 12 h (OR 2.37; 95% CI 1.61–3.47) post-dosing (55). This effect was associated with organ dysfunction, but the ultimate effects on mortality remained unclear. Finally, inflammatory mediators, such as corticostatins, may bind to ACTH receptors in the adrenal cortex, thus preventing ACTH stimulation of cortisol synthesis (56).

## Tissues Resistance to Glucocorticoids

A number of factors may prevent cortisol bioactivity in tissues. First, cortisol clearance may be accelerated, particularly following administration of various drugs, for example, psychoactive drugs (barbiturates, phenytoin) or antibiotics (rifampicin) (52). Second, sepsis is often associated with marked reduction in corticosteroid-binding globulin (CBG) and albumin (48, 57). On the one hand, the reduction in cortisol carriers increased free cortisol concentrations in plasma. On the other hand, cortisol bound to CBG is specifically released at the level of inflamed tissues, *via* neutrophils elastase-dependent mechanisms (58, 59). Thus, the net effect of sepsis-associated reduced CBG and albumin levels is reduced cortisol delivery to local sites of inflammation. Third, at tissue levels, T-helper 2 cell-derived cytokines, for example, IL-2 or IL-4, may inactivate cortisol to cortisone by upregulating the 11 $\beta$ -hydroxysteroid dehydrogenase (11 $\beta$ -HSD) type 2 enzyme (60). Finally, downregulation of the glucocorticoid receptor (GR)- $\alpha$  is a well-known complication of sepsis (61). The decrease in GR binding and affinity may be at least partly related to exaggerated release of NO in tissues (62). Sepsis may also cause alteration in the translocation of the GR- $\alpha$  (63). The loss in the dimerization of the GR- $\alpha$  caused resistance to glucocorticoids and lethality in septic animals (64).

## CORTICOSTEROIDS FOR SEPSIS AND SEPTIC SHOCK

Corticosteroids have been used for more than 60 years in the management of patients with severe infections. There is a strong rationale (as described earlier) for exogenous administration of glucocorticoids in sepsis. Nevertheless, their use in practice still remains controversial. There is a general agreement that corticosteroids improve sepsis-associated comorbidities, such as shock, organ dysfunction, and length of hospital stay. Their effects on survival and on the risk of secondary infections are controversial.

### Corticosteroids Improve Cardiovascular Function

Corticosteroids contribute to restoring effective blood volume, notably *via* sodium and water retention by binding to mineralocorticoid receptors in the kidney. They also contribute to restoring systemic vascular resistance. First, increase in sodium and water content in a vessel's interstitium results in increased stiffness of the vessel wall. Second, corticosteroids enhance vascular contractile (65) and blood pressure (66) responses to  $\alpha$ -1 agonists. This effect occurs within minutes following corticosteroid administration and is likely a non-genomic effect *via* modulation of the  $\alpha$ -1 agonists' receptors second messenger (65) and ATP-sensitive potassium channels (67). The endothelial GR is crucial for preventing prolonged activation of NO and NF- $\kappa$ B, following sepsis (68). Thus, prolonged improvement in vascular responsiveness to corticosteroids is likely a genomic transpressive effect. Patients with septic shock and blunted response to 250  $\mu$ g ACTH bolus, i.e., increase in total cortisol of <9  $\mu$ g/dl, have more depressed systemic vascular resistance and a greater effect of hydrocortisone

bolus on blood pressure response to norepinephrine than patients with intact HPA axis (65). Corticosteroids also improved microcirculation and tissue perfusion in septic shock (69). This effect may be mediated by upregulation of endothelial NO synthase *via* activation of the mitogen-activated protein kinase and protein Akt pathway (70).

A recent systematic review found 12 trials ( $n = 1561$  patient) and reported the effects of corticosteroids on shock reversal (weaned off vasopressor therapy) by 1 week (71). In this review, the relative risk (RR) of having shock reversed by day 7 was 1.31 (95% CI 1.14–1.51;  $P$  value = 0.0001, random effects model), in favor of corticosteroids.

### Corticosteroids Decrease Organ Failure

There is strong evidence that corticosteroids attenuate inflammation in various organs in sepsis. For example, they have been shown to dramatically decrease NF- $\kappa$ B activity in peripheral immune cells (72) or in the lung (73). Corticosteroids have been shown to inhibit iNOS activation in the renal cortex, preventing hypoxic injuries and restoring an adequate oxygen delivery to oxygen balance (74). They also improve glomerular function (75), free water clearance, and sodium renal excretion (76). Corticosteroids may attenuate sepsis-associated brain inflammation particularly by preventing the breakdown of the BBB (77, 78). A total of eight trials ( $n = 1132$  patients) (71) reported a dramatic reduction in the number and degree of severity of failing organs, with a mean reduction in the SOFA score – a measure of organ dysfunction (79) – of  $-1.53$  ( $-2.04$  to  $-1.03$ ;  $P$  value < 0.00001), in favor of corticosteroids. Corticosteroids also reduced ICU length of stay by  $-1.68$  days ( $-3.27$  to  $-0.09$ ;  $P$  value = 0.04) and  $-2.19$  days (95% CI  $-3.93$  to  $-0.46$ ;  $P$  value = 0.01), in ICU survivors (71).

### Corticosteroid Tolerance

#### Secondary Infections

Corticosteroids shift the recruitment of T cells from T-helper type 1 to T-helper type 2 and thus to favor the production of anti-inflammatory cytokines. Data from 19 trials ( $n = 2567$  patients) found that the RR for superinfection was 1.02 (0.87–1.20;  $P$  value = 0.81) (71). Corticosteroids may blunt febrile response to infection and alter leukocyte count and most inflammatory biomarkers. Thus, it may become difficult to recognize secondary infections in corticosteroid-treated patients. In practice, physicians should systematically screen on a daily basis any potential source of infection and draw samples for bacterial culture.

### Metabolic Complications

Corticosteroids induce hyperglycemia by stimulating neoglycogenesis, glycogenolysis, and by insulin resistance in skeletal muscles and adipocytes. In septic shock, corticosteroids are associated with hyperglycemia ( $P$  value < 0.00001) and hypernatremia ( $P$  value < 0.00001) (71). As compared with bolus administration, continuous infusion of corticosteroids may ease the control of glycemia in sepsis (79). However, preventing hyperglycemia by intensive insulin therapy did not improve morbidity or mortality (80).

## Acquired Neuromyopathy

Myopathy is a common complication of prolonged or acute exposure to corticosteroids, particularly high doses of fluorinated derivatives (e.g., dexamethasone). They induce myonecrosis, diffuse atrophy of fibers, cumulated sarcoplasmic glycogen vesicles, myofibril disorganization, and selective depletion of thick myosin filaments (81, 82). Upregulation of the calpain pathway suggests that altered calcium metabolism and/or increased proteolysis may contribute to corticosteroid muscle toxicity (82). The risk of myopathy associated with corticosteroids may be potentiated by hyperglycemia, hypoxia, or non-depolarizing neuromuscular drugs.

## Other Complications

In theory, corticosteroids may be associated with psychotic disorders or gastroduodenal bleeding. In practice, data from 19 trials ( $n = 2382$ ) found that the RR of gastroduodenal bleeding was of 1.24 (95% CI 0.92–1.67;  $P$  value = 0.15) (71). ICU studies variably found that exposure to systemic corticosteroids increased (83) or not (84) the risk of transition to delirium. Corticosteroid weaning may be associated with psychiatric manifestations, including depressive state and apathy.

## Corticosteroids' Effects on Survival

Most experiments, in both small and large animals, based on endotoxin challenges or live bacteria-induced sepsis, found survival benefit from various doses and durations of corticosteroids (85). At least 33 trials have evaluated corticosteroids for severe infection with or without septic shock (71). Data from 27 trials ( $n = 3176$  patients) found a RR of dying at 28 days of 0.87 (0.76–1.00,  $P$  value = 0.05). The survival benefit was more remarkable ( $P$  value = 0.01) in 22 trials of prolonged (>96 h) treatment with a moderate (<400 mg daily of hydrocortisone or equivalent) dose of corticosteroids. In this Cochrane review,

meta-regression found a significant dose effect of corticosteroids, i.e., the lower the dose, the lower the RR of dying. This review also suggested that septic shock, ARDS, or community-acquired pneumonia were more likely to draw a survival benefit. Finally, data from eight trials ( $n = 583$  patients), reporting subgroups based on the response to 250 µg ACTH test, found a RR of dying of 0.88 (0.88–1.02,  $P$  value = 0.09), in favor of corticosteroids. Depending on trial selection and definition of outcomes, different meta-analyses variably found (86), or did not find (87, 88), survival benefit from corticosteroids. Current international guidelines recommend restricting the use of hydrocortisone to vasopressor-dependent septic shock (89).

## CONCLUSION

There are numerous experimental and clinical data establishing the paramount importance of an appropriate activation of the HPA axis to respond to severe infection. Similarly, experiments in animals and clinical observations strongly support the role of an inadequate HPA axis response in the physiopathology and outcome of sepsis. In most animal studies, corticosteroid administration consistently protected against lethal sepsis. In contrast, clinical trials in sepsis found much less consistency in survival benefits from corticosteroids, though most trials demonstrated faster resolution in shock and organ dysfunction. Thus, physicians should consider corticosteroids mainly in septic shock who do not respond rapidly to fluid therapy and vasopressors. Trials also consistently found that corticosteroids should be given at doses of 200 mg of hydrocortisone equivalent per day for at least 3 days at full dose.

## AUTHOR CONTRIBUTIONS

DA is the sole author and is responsible for the whole content.

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# Glucocorticoid Regulation of Food-Choice Behavior in Humans: Evidence from Cushing's Syndrome

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The mechanisms by which glucocorticoids regulate food intake and resulting body mass in humans are not well-understood. One potential mechanism could involve modulation of reward processing, but human stress models examining effects of glucocorticoids on behavior contain important confounds. Here, we studied individuals with Cushing's syndrome, a rare endocrine disorder characterized by chronic excess endogenous glucocorticoids. Twenty-three patients with Cushing's syndrome (13 with active disease; 10 with disease in remission) and 15 controls with a comparably high body mass index (BMI) completed two simulated food-choice tasks (one with "explicit" task contingencies and one with "probabilistic" task contingencies), during which they indicated their objective preference for viewing high calorie food images vs. standardized pleasant, unpleasant, and neutral images. All participants also completed measures of food craving, and approximately half of the participants provided 24-h urine samples for assessment of cortisol and cortisone concentrations. Results showed that on the explicit task (but not the probabilistic task), participants with active Cushing's syndrome made fewer food-related choices than participants with Cushing's syndrome in remission, who in turn made fewer food-related choices than overweight controls. Corroborating this group effect, higher urine cortisone was negatively correlated with food-related choice in the subsample of all participants for whom these data were available. On the probabilistic task, despite a lack of group differences, higher food-related choice correlated with higher state and trait food craving in active Cushing's patients. Taken together, relative to overweight controls, Cushing's patients, particularly those with active disease, displayed a reduced vigor of responding for food rewards that was presumably attributable to glucocorticoid abnormalities. Beyond Cushing's, these results may have relevance for elucidating glucocorticoid contributions to food-seeking behavior, enhancing mechanistic understanding of weight fluctuations associated with oral glucocorticoid therapy and/or chronic stress, and informing the neurobiology of neuropsychiatric conditions marked by abnormal cortisol dynamics (e.g., major depression, Alzheimer's disease).

**Keywords:** Cushing's syndrome, choice behavior, decision-making, reward processing, glucocorticoids, cortisol, food craving

## INTRODUCTION

Hypothalamic-pituitary-adrenal (HPA) axis activity is essential for ensuring body homeostasis and survival during stress. Stress initiates a cascade of neuroendocrine responses including secretion of corticotropin-releasing hormone (CRH) from the hypothalamus, which stimulates adrenocorticotrophic hormone (ACTH) secretion from pituitary corticotrophs, culminating in glucocorticoid (GC) secretion from the adrenal glands (Smith and Vale, 2006). This stress response precipitating the release of GCs has downstream effects on multiple regulatory systems such as appetite control (La Fleur, 2006), thereby helping to regulate eating and satiety (Uchoa et al., 2014). Chronic stress, which results in persistent excess GC exposure, has been associated with increased hepatic glucose production, decreased glucose transport and utilization, decreased protein synthesis, enhanced protein degradation in muscles, and adipose tissue expansion, culminating in obesity and insulin resistance (Nieuwenhuizen and Rutters, 2008; Lacroix et al., 2015). However, although GCs regulate food intake and resulting body mass, the mechanisms for these relationships in humans are not well-understood.

One potential mechanism of how GCs orchestrate eating behavior could involve reward processing (Adam and Epel, 2007). This hypothesis follows from research literatures that have examined modulation of reward processing via transiently (experimentally) increased cortisol (e.g., experimental administration of cortisol or laboratory stressors) or chronically enhanced cortisol (e.g., due to early life stress). Studies have reliably shown decreased *non-food* reward processing in individuals exposed to stress. For example, experimental administration of cortisol or laboratory stressors (e.g., cold pressors, watching aversive movie clips, or trying to solve unanswerable math problems) are associated with lower eagerness to receive task-related rewards or lower functional magnetic resonance imaging (fMRI) activity [e.g., during a card-guessing task or the anticipation phase of the monetary incentive delay (MID) tasks] in corticolimbic regions relevant to reward (e.g., striatum, amygdala, hippocampus, and/or medial prefrontal cortex) (Ossewaarde et al., 2011; Porcelli et al., 2012; Montoya et al., 2014). In addition, individuals characterized as highly stress-reactive (i.e., showing high cortisol reactivity and self-reported negative affect after a stress induction) showed diminished sensitivity to reward, but not punishment, under stress (Berghorst et al., 2013). Individuals who experienced childhood maltreatment or early life stress, relative to individuals who did not experience these stressors, also rated reward cues as being less pleasant and showed blunted fMRI response in the globus pallidus, a striatal dopaminergic region, to reward cues during a MID task (Dillon et al., 2009); showed less ventral striatal fMRI response to happy faces (Goff et al., 2013); and responded less to money as an incentive to improve task performance (Mueller et al., 2012). Results of studies examining the effects of stress on *food* reward have been less consistent. A large body of literature suggests that presence of stress typically elevates food reward and consumption, often for unhealthy foods with high caloric densities (Tomiyama et al., 2011; Groesz et al., 2012; Hoffman et al., 2012; Talbot et al., 2013; Tryon et al.,

2013; Aschbacher et al., 2014; Ferreira de Sa et al., 2014; Pursey et al., 2014; Maier et al., 2015). However, one study in healthy individuals showed that stress was associated with decreased activation in the reward circuitry including the orbitofrontal cortex and putamen specifically during a food-choice procedure (i.e., when participants selected foods to be consumed later) (Born et al., 2010). Moreover, animal models (typically with the aim of examining a depressive phenotype) have shown that chronic administration of corticosterone or laboratory stress are associated with reduced eating behavior (Kvarta et al., 2015) or a diminished ability to establish preference for sweets (Mateus-Pinheiro et al., 2014).

A disease in humans characterized by excessive cortisol production is Cushing's syndrome (CS), which provides a unique model of chronic GC exposure (i.e., that is independent of precipitating chronic life stressors that could potentially have additional downstream functional consequences to cloud interpretations). CS is a rare endocrine disorder (1.2–2.4/million/year) characterized by chronic excess endogenous GCs due to an ACTH pituitary adenoma or a cortisol-producing adrenal adenoma (Lacroix et al., 2015); left untreated, CS results in increased mortality and multiple morbidities including obesity, diabetes, hypertension, cardiovascular disease, and overall lower health-related quality of life (Webb et al., 2008; Feelders et al., 2012; Carluccio et al., 2015). Individuals with CS also exhibit persistent neurocognitive impairments, including in memory and learning (Whelan et al., 1980; Martignoni et al., 1992; Mauri et al., 1993; Forget et al., 2000; Starkman et al., 2001, 2003; Leon-Carrion et al., 2009; Michaud et al., 2009; Pereira et al., 2010; Ragnarsson et al., 2012; Resmini et al., 2012); such deficits are quite anticipated in light of the wide distribution of GC receptors in brain areas (e.g., hippocampus, amygdala, and PFC) that are important for these respective executive functions (McEwen et al., 2016). Such deficits (e.g., in verbal memory) and associated improvement with treatment have been correlated with salivary or urinary cortisol concentrations (Grillon et al., 2004; Hook et al., 2007), supporting the idea that GC abnormalities underlie these deficits. More recently, and of greater importance for the current study, CS patients have also exhibited differences from controls in task-related decision-making (Crespo et al., 2014) and dispositional novelty-seeking (Dimopoulou et al., 2013). Such findings are consistent with a more general role of GCs in healthy individuals in potentially modulating risk-taking behavior during conditions of uncertainty (Coates and Herbert, 2008; Putman et al., 2010).

In the present study, we examined the effects of chronic GC exposure resulting from CS on laboratory food-choice tasks in which participants chose to view high-caloric food images vs. viewing standardized pleasant (e.g., smiling babies), unpleasant (e.g., disfigurement), or neutral (e.g., household objects) images; members of our team originally developed this task for use in cocaine addiction (i.e., evaluating choice for cocaine images) (Moeller et al., 2009). Given data suggesting that, on balance, chronic GC exposure is associated with blunted reward processing, we hypothesized that CS patients would choose to view fewer food-related and/or fewer pleasant-related images than a sample of healthy controls with similar body

mass index (BMI). Moreover, given data suggesting that chronic GC exposure drives a metabolic memory that results in long-term mortality risk, cognitive and emotional impairments, and reduced quality of life even after normalization of cortisol levels (Tiemensma et al., 2010a,b; Geer et al., 2012; Pereira et al., 2012; Lambert et al., 2013; Andela et al., 2015; Pivonello et al., 2015), we further hypothesized that such blunted choice, although accentuated in CS participants with active disease, would also be present in CS in remission, who would show improved but not normalized reward processing.

## METHODS

### Participants

Participants included 23 patients with CS and 15 overweight healthy controls (OHC) with similar BMI (independent *t*-test:  $p = 0.12$ ), such that results could be more plausibly attributed to differences in GCs between the groups, rather than to more general metabolic disturbances and food-seeking behaviors that often characterize obesity. Of these patients with CS, 13 were pre-surgery with active disease, and 10 were post-surgery with disease in remission; these groups were considered separately in all analyses reported below. All participants provided written informed consent, and the Mount Sinai Institutional Review Board approved the study procedures. Participants were required to be age 17 or older. Enrolled CS participants were those who were eligible for surgery within 3 months of test administration, or who had achieved endocrine remission from previous surgical treatment (range of time since endocrine remission was achieved in the treated cohort was 6–60 months). Exclusion criteria for both groups were pregnancy; untreated hypothyroidism; use of exogenous GCs, in the 1-week prior to testing (for the active CS and OHC cohorts); and being unable or unwilling to comply with the study procedures or give informed consent. Exclusion criteria for the OHC were underlying endocrine or metabolic disorders, history of alcohol abuse, or unstable weight.

Active CS participants had elevated cortisol levels, as measured by 24 h urine free cortisol (UFC) (see also **Table 1**), and normal or elevated plasma ACTH concentrations (if the participant had Cushing's disease: CD;  $N = 22$ ), or low plasma ACTH (if the participant had adrenal CS;  $N = 1$ ), as previously defined (Nieman et al., 2008). An MRI scan or inferior petrosal sinus sampling, which was indicated for 10 patients, confirmed pituitary source of CD. For CS participants studied after surgical treatment, endocrine remission was confirmed by resolution of CS features, 1–2 day post-operative hypocortisolism (serum cortisol < 5 mcg/dL) and/or normal 24-h UFC after physiologic oral GC replacement had been discontinued, as previously defined (Nieman et al., 2015). Among all CS patients, two active patients were taking diabetes medications, five active and three remitted patients were taking psychiatric (including anti-anxiety, antidepressant, mood stabilizing, or sedating) medications, and one active patient was taking analgesic medications (for OHC, two participants were taking diabetes medications, and one was taking oral stimulant medication). Four remitted CS patients were taking physiologic oral hydrocortisone replacement (hydrocortisone 15–20 mg daily): two of these patients had

required bilateral adrenalectomy in order to achieve endocrine remission after unsuccessful transsphenoidal surgery, and the remaining two were still transiently hypocortisolemic due to successful surgical treatment within the past 6 months. Two remitted patients, who underwent two transsphenoidal surgeries, developed hypopituitarism and were taking pituitary endocrine replacement, including physiologic levothyroxine in both and testosterone in one. All were ambulatory with normal renal function and no liver disease.

**Table 1** provides demographics, self-reports, and clinical characteristics of the study sample, split by disease status (active CS, CS in remission, OHC). The groups did not differ on gender, age, race, childhood trauma, or trait impulsivity (Patton et al., 1995). However, there was a higher proportion medication usage in all CS patients than controls. Other group differences were between active CS patients vs. the other two groups: active CS patients had a higher BMI and more dysphoric symptoms, including higher scores on the Beck Depression Inventory (BDI) (Beck, 1996), the State-Trait Anxiety Inventory (STAI) (Spielberger et al., 1983), and the Perceived Stress Scale (PSS) (Cohen et al., 1983); such dysphoric symptoms are consistent with neuropsychiatric comorbidities commonly reported in this population (Pereira et al., 2010; Pivonello et al., 2015).

### Picture Choice Tasks

Two picture choice tasks, which have been previously validated in cocaine addiction as extensively described elsewhere (Moeller et al., 2009, 2010, 2012a,b, 2013, 2014), were completed by all study participants at the Mount Sinai Clinical Research Unit in the morning after an overnight fast  $\geq 8$  h duration. The tasks—one with explicit contingencies and one with probabilistic contingencies—use standardized pleasant, unpleasant, and neutral images selected from the International Affective Picture System (IAPS) (Lang et al., 2008). For the current study, we incorporated images of food in lieu of cocaine, matched on size and ratio of human to non-human content. These food images depicted palatable “junk” foods (e.g., hamburgers, pizza, or ice cream), and people eating these foods. These two tasks assess complementary notions of choice as described below. **Table 2** provides the raw means and standard deviations of both choice tasks by study group.

### Explicit Choice Task (Figure 1A)

Participants chose via continued button pressing between two fully-visible side-by-side images from different picture categories (pleasant, unpleasant, neutral, and food) [we note that a fifth picture category, blank (black) screens containing no image, was also included on this task, but these null images were not analyzed in this study to better equate behavior on the two tasks]. Choice for a desired image enlarged this chosen image to fully cover the screen, which participants could view for the trial duration of 5 s by continued button pressing; 0.5 s of non-response, however, returned the side-by-side image display. After each trial, a new trial with new images ensued. Button pressing (i.e., “working”) for images was an important design feature of this task, meant to model “working” for a desired reward; from a behavioral economic perspective, choice on the explicit task can be thought to index breakpoint (i.e., preference for one category of image

**TABLE 1 | Demographics, self-report measures, and clinical characteristics by study group.**

	Statistical test	Active Cushing's (N = 13)	Cushing's in remission (N = 10)	Overweight controls (N = 15)
Gender (women/men)	$\chi^2_{(2, N = 38)} = 3.86$	10/3	4/6	7/8
Medication (any) (yes/no)	$\chi^2_{(2, N = 38)} = 13.07^*$	12/1 <sup>c</sup>	7/3 <sup>c</sup>	4/11 <sup>a,b</sup>
Age (years)	$F_{(2, 35)} = 1.09$	$42.1 \pm 12.9$	$38.4 \pm 16.5$	$34.4 \pm 12.4$
Race (White/non-White)	$\chi^2_{(2, N = 38)} = 7.90$	12/1	10/0	9/6
Ethnicity (non-Hispanic, Hispanic)	$\chi^2_{(2, N = 38)} = 1.23$	11/2	9/1	11/4
Body mass index (kg/m <sup>2</sup> )	$F_{(2, 35)} = 4.85^*$	$33.1 \pm 4.6^{b,c}$	$28.7 \pm 2.9^a$	$28.9 \pm 4.2^a$
Barrett impulsiveness scale	$F_{(2, 35)} = 0.28$	$58.9 \pm 8.4$	$62.0 \pm 10.7$	$59.7 \pm 10.9$
Childhood trauma questionnaire	$F_{(2, 35)} = 0.53$	$40.3 \pm 9.3$	$39.3 \pm 15.5$	$45.5 \pm 21.7$
Perceived stress scale	$F_{(2, 35)} = 6.68^*$	$47.0 \pm 8.5^{b,c}$	$36.5 \pm 8.4^a$	$36.4 \pm 8.4^a$
Beck depression inventory	$F_{(2, 34)} = 8.78^*$	$23.7 \pm 11.6^{b,c}$	$12.2 \pm 12.3^a$	$6.7 \pm 8.3^a$
State-trait anxiety inventory: state	$F_{(2, 34)} = 6.65^*$	$30.5 \pm 14.8^{b,c}$	$16.0 \pm 15.6^a$	$12.9 \pm 9.8^a$
State-trait anxiety inventory: trait	$F_{(2, 34)} = 8.50^*$	$33.5 \pm 12.9^{b,c}$	$15.7 \pm 14.7^a$	$15.1 \pm 11.6^a$
State food craving	$F_{(2, 35)} = 0.14$	$42.4 \pm 14.6$	$45.1 \pm 11.2$	$43.1 \pm 10.9$
Trait food craving	$F_{(2, 35)} = 4.75^*$	$136.8 \pm 32.8^c$	$114.9 \pm 42.5$	$101.4 \pm 15.4^a$
Urine free cortisol ( $\mu\text{g}/24\text{ h}$ ) <sup>d</sup>	$F_{(2, 21)} = 8.55^*$	$279.0 \pm 172.3^{b,c}$	$54.7 \pm 45.8^a$	$81.0 \pm 25.7^a$
Urine cortisone ( $\mu\text{g}/24\text{ h}$ ) <sup>d</sup>	$F_{(2, 21)} = 6.31^*$	$380.0 \pm 210.5^c$	$216.7 \pm 146.1$	$140.7 \pm 51.3^a$

Numbers are mean  $\pm$  standard deviation.

\*omnibus  $p < 0.05$ , with significant follow-up pairwise comparisons.

<sup>a</sup>Differs significantly from active Cushing's patients.

<sup>b</sup>Differs significantly from Cushing's patients in remission.

<sup>c</sup>Differs from overweight controls.

<sup>d</sup>Sample sizes are 11, 3, and 10 for active Cushing's, Cushing's in remission, and overweight controls, respectively.

**TABLE 2 | Descriptive statistics of choice behavior by study group.**

	Active Cushing's (N = 13)	Cushing's in remission (N = 10)	Overweight controls (N = 15)
<b>EXPLICIT TASK (TOTAL BUTTON PRESSES)</b>			
A. Pleasant pictures	$170.9 \pm 147.1$	$243.8 \pm 205.8$	$186.9 \pm 135.2$
B. Unpleasant pictures	$6.9 \pm 8.5$	$4.3 \pm 8.9$	$36.3 \pm 61.9$
C. Neutral pictures	$127.4 \pm 129.6$	$147.9 \pm 128.1$	$140.3 \pm 104.2$
D. Food pictures	$132.3 \pm 111.8$	$195.5 \pm 164.2$	$207.1 \pm 157.1$
<b>PROBABILISTIC TASK (TOTAL SELECTIONS)</b>			
A. Pleasant pictures	$20.2 \pm 5.8$	$19.4 \pm 5.3$	$18.5 \pm 9.2$
B. Unpleasant pictures	$11.2 \pm 7.6$	$11.1 \pm 7.2$	$8.3 \pm 5.4$
C. Neutral pictures	$17.5 \pm 8.0$	$16.6 \pm 5.7$	$8.9 \pm 6.2$
D. Food pictures	$18.5 \pm 9.4$	$24.1 \pm 5.1$	$19.5 \pm 8.8$

Numbers are mean  $\pm$  standard deviation, without correction for any covariates.

over another when there is a cost of effort expenditure) (e.g., Mackillop et al., 2010; Moeller et al., 2013). We summed the number of button presses (across 70 total trials), indexing vigor of responding, per picture category.

### Probabilistic Choice Task (Figure 1B)

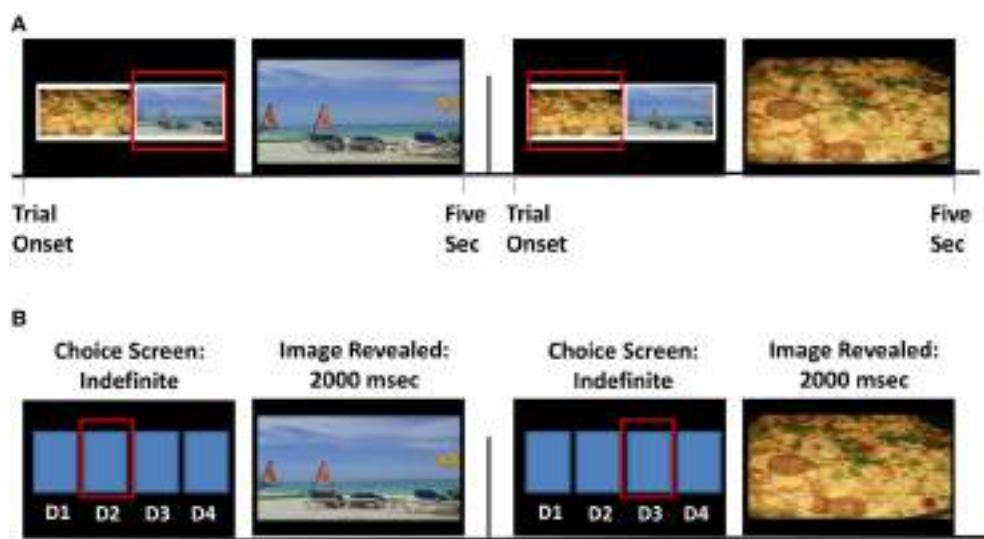
On each trial, participants chose via a single button press to view pictures hidden under flipped-over cards, arranged in four decks. Immediately after choosing from a particular “deck,” an image was revealed that covered the entire screen for 2 s

of passive viewing. The images were arranged probabilistically: each deck contained 26 (out of a total of 30) pictures from a particular category (e.g., pleasant), allowing pictures from other categories to be interspersed within each deck (two pictures from a secondary category, e.g., food; and one picture from each of the two remaining categories, e.g., unpleasant and neutral). After participants selected from a particular deck eight total times (corresponding to one task run), deck location of the four picture categories shifted. Thus, this task contained a “seeking” component, rather than a “working” component: throughout, participants needed to seek (and re-seek, once task contingencies changed) their preferred deck(s). In contrast to the explicit task, choice on the probabilistic task was meant to model more standard notions of choice; here, from a behavioral economic perspective, probabilistic choice can be thought to reflect intensity (i.e., preference for one category of image over another when there is no cost associated with the choice) (e.g., Mackillop et al., 2010; Moeller et al., 2013). We summed the total number of cards selected per picture category across four task runs.

## Food Craving Questionnaires

### State Food Craving

Current food craving was measured with the Food Craving Questionnaire-State [FCQ-S (Cepeda-Benito et al., 2000; Nijs et al., 2007)]. The FCQ-S consists of 15 items assessing: desire to eat, anticipation of positive reinforcement from eating, anticipation of negative reinforcement (reduction of negative affect) from eating, lack of control over eating, and



**FIGURE 1 | Task schematics of the explicit and probabilistic food choice tasks. (A)** The explicit task included training and one block, consisting of 70 trials. Two sample trials are displayed. Continuous button pressing for a given image (indicated by the red box) enlarged the corresponding image, for the 5-s trial duration; no response (for 0.5 s) after initial response returned the side-by-side display. **(B)** The probabilistic task included training and four task runs. In each trial within a run, participants pressed one of four buttons corresponding to their chosen deck (D1, D2, D3, or D4; also indicated by the red box).

(physiological) hunger; higher scores reflect stronger food craving. Participants respond, using a Likert-type scale, how much each item is true of them *right now*. Demonstrating applicability of this questionnaire to the current study, FCQ-S scores were increased after exposure to food images in “high cravers” of chocolate (Meule et al., 2012), and were correlated with increased attention allocation (that interfered with response inhibition) to food vs. neutral pictures (Meule et al., 2014a).

### Trait Food Craving

Trait food craving was assessed with the Food Craving Questionnaire-Trait (FCQ-T) (Cepeda-Benito et al., 2000; Nijs et al., 2007). The FCQ-T consists of 39 items assessing: intentions/plans to eat, anticipation of positive reinforcement from eating, anticipation of negative reinforcement from eating, lack of control over eating, preoccupation with food, (physiological) hunger, emotions preceding or following food cravings or eating, environmental cues that may elicit food cravings, negative emotions including guilt experienced as a consequence of food cravings, and/or indulging such cravings; higher scores again indicate higher trait craving. Individuals are asked to respond, using a Likert-type scale, how much each item is true of them *in general*. Unlike the FCQ-S, and appropriately for a trait measure, the FCQ-T was unaffected by food deprivation (Meule et al., 2014b). In the current study, active CS had higher trait (but not state) food craving than OHC (Table 1).

### Urine Cortisol and Cortisone Measures

Twenty-four hour UFC and cortisone were quantified by gas chromatography-mass spectroscopy (GC-MS) as previously described (Yehuda et al., 2009a,b). The limit of sensitivity is 2

ng, the inter-assay coefficients of variations are <10%, and the normal range for UFC is 10–100 µg/24 h. As to be expected, these measures were highest in active CS (Table 1).

## Statistical Analyses

### Effects of Disease Status on Food Picture Choice

We analyzed both choice tasks using a 4 (Picture Type: pleasant, unpleasant, neutral, food) × 3 (Disease Status: active CS, CS in remission, OHC) mixed analysis of covariance (ANCOVA), with the total number of presses (explicit task) or selections (probabilistic task) entered as covariates in the appropriate model to control for individual differences in response frequency. Significant interactions were followed by paired (within-group) and independent (between-group) comparisons that similarly controlled for response frequency. For the latter, a core interest was in testing for between-group linear contrasts (i.e., stepwise patterns in the effects as a function of Disease Status). In all ANCOVAs and follow-up comparisons,  $p < 0.05$  was considered significant.

### Correlation Analyses

For both tasks, we examined associations between the food choice variables and the food vs. pleasant choice difference scores (food > pleasant) with the variables of interest specified below. The focus on this specific food > pleasant difference score follows from our prior results in individuals with cocaine use disorder, in which cocaine vs. pleasant (cocaine > pleasant) choice was particularly useful in predicting drug-relevant outcomes (Moeller et al., 2012a, 2013). Correlations were conducted across all participants and split by the three study groups. Due to the presence of some outliers across various measures, correlations were conducted using non-parametric (Spearman) analyses. In

all correlational analyses,  $p < 0.01$  was considered significant to protect against Type I error. After satisfying this initial criterion, however, we retained significant correlations if they achieved a significance level of  $p < 0.05$  when accounting for covariates.

#### **Between-task reliability and initial validity**

Because these tasks were new to a CS population, and because they included a new image category (food), we tested for intercorrelations between the explicit and probabilistic task scores. To provide evidence of construct validity of these tasks in CS, we also examined correlations between the task variables with BMI and self-reported food craving (state and trait).

#### **Associations with cortisol levels**

To provide further attribution of our effects to GCs, we performed correlations between the two food-choice variables and the two food > pleasant choice scores with urine markers of cortisol inclusive of free cortisol and cortisone in the subsample of participants for whom these data were available.

#### **Effects of Covariates**

Because medication use, BMI, and dysphoric symptoms (i.e., state and trait anxiety, perceived stress, and depression symptoms) differed between the groups (Table 1), these variables were covaried in subsequent ANCOVAs or multiple regressions as appropriate. Note that food craving and urine cortisol/cortisone were considered dependent variables, not covariates.

## **RESULTS**

### **Effects of Disease Status on Food Picture Choice**

Results of the mixed 4 (Picture Type)  $\times$  3 (Disease Status) ANCOVAs revealed main effects of Picture Type [ $F_{(3, 32)} > 36.50$ ,  $p < 0.001$ ]: on both tasks, unpleasant choice was lowest, followed by neutral choice, and these were both lower than pleasant and food choice (all pairwise comparisons,  $p < 0.01$ ). There were no main effects of Disease Status (both  $p > 0.096$ ). Of greater interest, on the explicit task, the Picture Type  $\times$  Disease Status interaction reached significance [ $F_{(6, 64)} = 3.12$ ,  $p = 0.010$ ] (Figure 2A). Planned linear contrast analyses showed that active CS participants made fewer food choices than CS participants in remission, who in turn made fewer food choices than OHC ( $p = 0.021$ ); linear contrasts for the other picture categories did not reach significance (all  $p > 0.074$ ), indicating specificity to food choice. Follow-up within-group comparisons showed that both active CS and CS in remission pressed for fewer food pictures than pleasant pictures (both  $p < 0.012$ ) but not neutral pictures (both  $p > 0.063$ ). OHC showed an opposite pattern of results, pressing for food pictures more than neutral pictures ( $p = 0.003$ ) but not pleasant pictures ( $p = 0.099$ ). These collective analyses suggest that CS patients displayed reduced vigor for responding to food reward, particularly in those with active disease. Because the Picture Type  $\times$  Disease Status interaction did not reach significance on the probabilistic task [ $F_{(6, 64)} = 1.18$ ,  $p = 0.34$ ], no follow-up comparisons were performed.

## **Correlation Analyses**

### **Between-Task Reliability and Initial Validity**

Correlations did not reach significance at  $p < 0.01$  across the whole sample. Nevertheless, after splitting the three groups, a positive correlation emerged between the food > pleasant choice variables on the two tasks in OHC ( $r_s = 0.74$ ,  $p = 0.001$ ) but not in either of the CS groups ( $r_s < 0.39$ ,  $p > 0.19$ ). In addition, higher food > pleasant choice, this time on the probabilistic task, positively correlated with higher state ( $r_s = 0.82$ ,  $p = 0.001$ ) and trait ( $r_s = 0.79$ ,  $p = 0.001$ ) food craving in active CS but not in CS in remission or OHC ( $r_s < |0.38|$ ,  $p > 0.28$ ) (Figures 2B,C). This correlation suggests that food-related choice on this task is a valid metric of the desire to eat, both currently and in general, in a CS patient population with active disease.

### **Associations with Cortisol Levels**

Across all participants, food > pleasant choice on the explicit task negatively correlated with urine free cortisone (the higher the cortisone, the lower the food-related choice) ( $r_s = -0.56$ ,  $p = 0.004$ ) (Figure 2D). A similar trend was observed for UFC ( $r_s = -0.42$ ,  $p = 0.041$ ), which, although not reaching nominal significance, increases confidence in the cortisone effect.

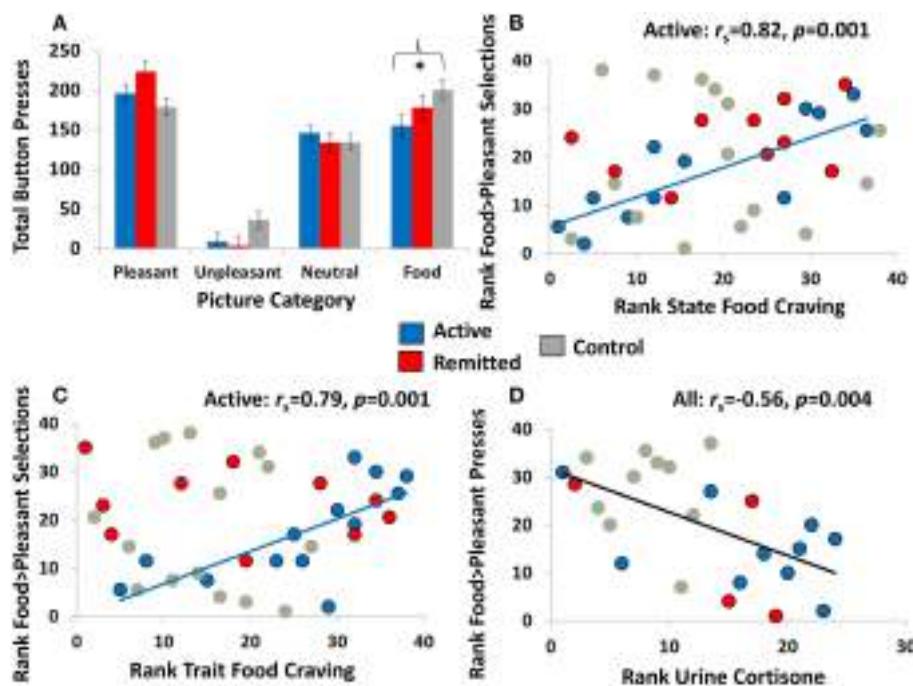
### **Effects of Covariates**

The Picture Type  $\times$  Disease Status interaction remained significant after controlling for medication use ( $p = 0.048$ ), BMI ( $p = 0.018$ ), and dysphoric symptoms (state and trait anxiety, perceived stress, and depression symptoms:  $p < 0.022$ ). In addition, results of multiple regression analyses controlling for these same covariates showed that correlations were still detected between the food > pleasant scores on both tasks in OHC ( $p < 0.004$ ), between food > pleasant probabilistic choice with state ( $p < 0.007$ ) and trait ( $p < 0.032$ ) food craving in active CS, and between food > pleasant explicit choice and urine cortisone in all participants ( $p < 0.025$ ). Thus, it is unlikely that these potential confounds drove the current results.

## **DISCUSSION**

In this study, CS with active disease, CS with disease in remission, and OHC participants completed two behavioral tasks that tested the choice to view food pictures vs. non-food-related pleasant, unpleasant, and neutral pictures. We hypothesized that, as a model of chronic exposure to excessive GCs, individuals with CS (and especially those individuals with active disease) would make fewer food-related choices (and fewer pleasant-related choices) than OHC. Results supported the hypothesis that individuals with higher endogenous GC levels, independent of BMI, exhibited decreased food (but not pleasant) choice on the explicit (but not probabilistic) task.

The primary result of this study was that, when task contingencies were explicit (i.e., fully certain) and choice required working (continuously pressing buttons) to view the respective stimuli, individuals with active CS chose to view fewer food images than individuals with CS in remission, who in turn chose to view fewer food images than OHC; thus, CS patients, especially those with active disease, were less apt to exert effort to view



**FIGURE 2 | Relevant choice task results and scatterplots showing associations between food-related choice with state food craving and cortisol. (A)**

Results of the explicit task showing total button presses (estimated marginal means) for each of the four picture categories (pleasant, unpleasant, neutral, and food) for individuals with active Cushing's syndrome ( $N = 13$ ), individuals with Cushing's syndrome in remission ( $N = 10$ ), and overweight comparison participants ( $N = 15$ ). The asterisk indicates a significant linear contrast among the groups at  $p < 0.05$ , and error bars represent standard error of the mean. Note that results of the probabilistic choice are not shown, given the nonsignificant Picture Category  $\times$  Diagnosis interactions (for descriptive statistics of this task, see Table 2). **(B,C)** In individuals with active Cushing's syndrome but not the other two study groups, higher probabilistic food choice (compared with probabilistic non-food pleasant choice) correlated with higher state and trait food craving. **(D)** Across all participants for whom urine cortisol markers were available ( $N = 24$ ), higher explicit food choice (compared with explicit non-food pleasant choice) correlated with lower urine cortisone.

food cues. Although at first blush this decreased food choice may be unexpected given that individuals exposed to chronic stress or GCs often consume more calories and/or eat less healthfully (Tomiyama et al., 2011; Groesz et al., 2012; Hoffman et al., 2012; Talbot et al., 2013; Aschbacher et al., 2014), it is critical to note that the “control” group was also overweight (i.e., not differing from the overall CS group on BMI, which was a goal in our recruitment; see Methods). Moreover, our results are consistent with prior work showing that chronic excessive exposure to GCs, here attributable to active CS but potentially also generalizable to chronic stress or chronic GC administration, is associated with blunted reward sensitivity in human- (Dillon et al., 2009; Goff et al., 2013) and animal models (Mateus-Pinheiro et al., 2014; Kvarta et al., 2015). Because in the current study results were specific to the food-choice task that required “working,” it is possible that active CS patients have a higher threshold for perceiving food as reinforcing, potentially needing to consume more to achieve the same hedonic effect. An important parallel can be made to addiction literature, whereby reduced drug sensitivity is associated with increased or uncontrolled drug use [e.g., whether because of putatively drug-mediated adaptations to dopamine neurotransmission in drug addiction (Volkow et al., 1997, 2010; Martinez et al., 2009; Peechatka et al., 2015) or because of genetics that elevate susceptibility to developing

addiction later in life (Edenberg, 2007; de Wit and Phillips, 2012; Gubner et al., 2013)]. More broadly, our results support and extend prior research in CS on deficits in memory, learning, and decision-making (Whelan et al., 1980; Martignoni et al., 1992; Mauri et al., 1993; Forget et al., 2000; Starkman et al., 2001, 2003; Leon-Carrion et al., 2009; Michaud et al., 2009; Pereira et al., 2010; Ragnarsson et al., 2012; Resmini et al., 2012; Crespo et al., 2014); and that such neurocognitive deficits may be ameliorated, but not fully normalized, even after surgical remission (Tiemensma et al., 2010a,b; Geer et al., 2012; Pereira et al., 2012; Lambert et al., 2013; Andela et al., 2015; Pivonello et al., 2015).

In further analyses, we correlated our behavioral choice results with self-reported food craving and *in vivo* GC levels; these analyses, respectively, were meant to validate these food-choice tasks as a model of food reward in a CS population and to provide a putative neurobiological correlate of the food-choice results. For the latter (neurobiological correlate), and corroborating our main between-group analyses above, correlations emerged between food-related choice on the explicit task and urine GCs: across all participants, 24-h urine cortisone correlated with explicit food-related choice, with similar albeit nonsignificant effects emerging for UFC. The underlying mechanism of these effects, which requires testing in further studies, could involve

GC modulation of the dopamine system that alters the incentive salience of food cues (Borges et al., 2013; Yau and Potenza, 2013; Soares-Cunha et al., 2014). For the former (task validity), probabilistic (though not explicit) food-related choice positively correlated with state and trait food craving within active CS. Thus, although between-group effects in the current study were not observed on this probabilistic task, correlations with food craving help to validate these food-choice tasks as a model of food reward in active CS.

Limitations of this study include the following. First, the current study did not include a sample of normal-weight controls, and without this reference group we cannot definitively conclude whether results are primarily driven by decreased food-choice in CS, increased food-choice in OHC, or both. Future studies will need to include a normal-weight control group. Nevertheless, our paramount concern in this initial study was to equate the groups on BMI, which indeed was achieved for the full CS sample. Although a BMI group difference emerged when splitting the groups by disease status (**Table 1**), it is important to note that our results were robust to statistical correction for BMI, indicating that this variable did not drive our results. Second, urine cortisol markers were unavailable in half the study sample. However, effects with cortisone were indeed observed using a relatively strict statistical threshold, reducing the potential for spurious effects; and, more importantly, in our main analyses we found stepwise effects as a function of remission status on behavioral choice in the entire sample. Third, we did not obtain self-report ratings of preference for the food stimuli prior to their use in the current tasks. However, main effects of Picture Type indicated that these food images were indeed palatable, such that all participants chose them for viewing more often than neutral images or unpleasant images. Fourth, it is possible that demand characteristics or participants' task-related motivation could have contributed to the lower food-related choice in CS participants. However, if results were driven by demand characteristics associated with the desire to reduce food intake and lose weight, we strongly suspect that OHC participants would have been similarly motivated to avoid choosing food images. If results were driven by reduced task-related motivation in CS (e.g., due to anhedonia), one could anticipate that choice for non-food-related rewarding stimuli (here, pleasant images)

would be reduced as well (Pizzagalli et al., 2009; Domschke et al., 2015; Fletcher et al., 2015), but this pattern of effects did not occur; moreover, dysphoric symptoms did not explain our findings.

In conclusion, chronic GC exposure from active CS was associated with reduced simulated food choice when compared with such choice in overweight individuals with intact GC functioning; CS patients in remission with remediated GC functioning showed an intermediate pattern of food choice, suggesting that prior GC exposure may exert lasting effects on brain reward systems. Future studies can use neuroimaging and/or neurochemical approaches in CS, OHC, and (importantly) normal-weight controls to test for commonalities and/or differences in food choice and its neurobiological underpinnings among these study groups. Longitudinal designs can also test whether GC-mediated food-choices have downstream consequences, such as increasing risk for weight gain and/or cardiovascular events. More broadly, beyond illuminating neurocognitive sequelae of CS (and possibly chronic stress), our findings can also inform the neurobiology of elevated and/or dysregulated cortisol as seen in aging, major depressive disorder, and/or Alzheimer's disease (Pereira et al., 2012; Notarianni, 2013; Gupta and Morley, 2014; Du and Pang, 2015; Furtado and Katzman, 2015).

## AUTHOR CONTRIBUTIONS

SM, RG, and EG designed research. LC, VC, and YL performed research. SM, IM, NW, RY, and EG analyzed data. SM and EG wrote the paper. All authors provided critical revisions of the paper.

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