Chapter 4
The PDE4 cAMP-Specific Phosphodiesterases: Targets for Drugs with Antidepressant and Memory-Enhancing Action

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Abstract The PDE4 cyclic nucleotide phosphodiesterases are essential regulators of cAMP abundance in the CNS through their ability to regulate PKA activity, the phosphorylation of CREB, and other important elements of signal transduction. In pre-clinical models and in early-stage clinical trials, PDE4 inhibitors have been shown to have antidepressant and memory-enhancing activity. However, the development of clinically-useful PDE4 inhibitors for CNS disorders has been limited by variable efficacy and significant side effects. Recent structural studies have greatly enhanced our understanding of the molecular configuration of PDE4 enzymes, especially the "long" PDE4 isoforms that are abundant in the CNS. The new structural data provide a rationale for the development of a new generation of PDE4 inhibitors that specifically act on long PDE4 isoforms. These next generation PDE4 inhibitors may also be capable of targeting the interactions of select long forms with their "partner" proteins, such as RACK1, β-arrestin, and DISC1. They would therefore have the ability to affect cAMP levels in specific cellular compartments and target localized cellular functions, such as synaptic plasticity. These new agents might also be able to target PDE4 populations in select regions of the CNS that are implicated in learning and memory, affect, and cognition. Potential therapeutic uses of these agents could include affective disorders, memory enhancement, and neurogenesis.

Keywords cAMP • Phosphodiesterase • PDE4 • Beta-arrestin • RACK1 • PKA • ERK1/2 • Learning • Memory • Depression

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4.1 Introduction 1

The cAMP-specific phosphodiesterases (PDE4) enzymes) hydrolyze the ubiquitous "second messenger" cAMP and thereby serve to regulate its abundance in specific sub-cellular compartments (Francis et al. 2011; Conti and Beavo 2007; Houslay 2010; Maurice et al. 2014; Baillie 2009; Menniti et al. 2006). They are an essential component of the cAMP signal transduction system, which also includes adenylyl cyclase, specific G-proteins, G-protein coupled receptors (GPCRs), the cAMP-dependent protein kinase (PKA) and the cAMP target, Epac (Beavo and Brunton 2002). The PDE4 family is a member of the cyclic nucleotide PDE super-family, which consists of 11 distinct families (PDE1 through PDE11, respectively) that can be distinguished by their substrate specificity (cGMP and/or cAMP), molecular structure, and their ability to be inhibited by family-selective inhibitors (Bolger 2007). Like all members of the PDE super-family, the PDE4s are important targets for drug discovery. Currently, three PDE4-selective inhibitors, roflumilast, apremilast and crisaborole, have been developed for clinical use, in COPD and inflammatory disorders (Fabbri et al. 2009; Calverley et al. 2009; Hatzelmann et al. 2010; Page and Spina 2012; Schafer et al. 2014; Kavanaugh et al. 2015; Papp et al. 2015; Murrell et al. 2015), and additional PDE4 inhibitors are being tested in a wide variety of pre-clinical models and in clinical trials (Page and Spina 2012; Zhang et al. 2005a; Bruno et al. 2011; Giembycz and Maurice 2014; Richter et al. 2013). PDE4s are expressed in many areas of the CNS and PDE4 inhibitors have been shown to have antidepressant, anti-psychotic, and memory-enhancing actions in both rodent models and in humans (Fleischhacker et al. 1992; Scott et al. 1991; Hebenstreit et al. 1989; Eckmann et al. 1988; Zeller et al. 1984; Bobon et al. 1988; Barad et al. 1998; Bach et al. 1999; Titus et al. 2013; Mueller et al. 2010; Nibuya et al. 1996; O'Donnell and Zhang 2004; Kanes et al. 2007; Halene and Siegel 2008). However, the development of clinically-effective PDE4 inhibitors in CNS disorders has been hampered by lack of effectiveness and significant side effects, such as nausea (Higgs 2010; Gavalda and Roberts 2013). 2

This review discusses recent advances in the PDE4 field that promise to greatly enhance our understanding of the biology of PDE4 isoforms and also to accelerate the development of PDE4-selective inhibitors with greater activity and selectivity in the CNS. It will first review the structure of PDE4 genes and their transcripts. It will then discuss recent advances in the structure and function of PDE4 proteins, with emphasis on dimerization of PDE4 isoforms, the role of phosphorylation, and the interactions of PDE4s with their "partner" proteins, such as DISC1, RACK1 and β-arrestin2. The focus will then change to the cellular functions of the PDE4s, with special emphasis on their differential effects on important PKA substrates in the CNS. It will then review briefly the functional roles of the PDE4s in the intact brain, with emphasis on both the CNS effects of PDE4-selective inhibitors and on the CNS phenotypes of PDE4-mutant mice, especially those of newer dominant-negative models. Finally, it will discuss the implications of all these developments for drug discovery, with special emphasis on the potential of PDE4-selective inhibitors for CNS disorders. 3

4.2 The Structure of the PDE4 Genes and Their Transcripts 1

One of the most important aspects of PDE4 biology is the marked diversity of PDE4 isoforms, with over 20 isoforms having been identified to date (Conti and Beavo 2007; Houslay 2010; Maurice et al. 2014; Bolger 2007; Bolger et al. 1993; Swinnen et al. 1989). The PDE4s are encoded by four different genes in mammals (called *PDE4A, PDE4B, PDE4C* and *PDE4D* in humans), with additional diversity being produced by alternative mRNA splicing and the use of several isoform-specific promoters within each gene (Conti and Beavo 2007; Houslay 2010; Maurice et al. 2014; Bolger 2007; Bolger et al. 1993; Swinnen et al. 1989). Each of the PDE4 isoforms has a distinct pattern of expression in cells and tissues and the vast majority of them has been demonstrated to have an isoform-specific pattern of expression in the CNS (Bolger et al. 1994; Cherry and Davis 1999; Miro et al. 2002; D'Sa et al. 2005; D'Sa et al. 2002; Reyes-Irisarri et al. 2008; Nishi et al. 2008; Mori et al. 2010; Kuroiwa et al. 2012; Ahmed and Frey 2003). These pronounced differences in regional expression in the CNS suggest that each isoform has a distinct function; a concept that will be discussed in more detail, below. 2

The PDE4 isoforms can be categorized into "long" forms, which possess both UCR1 and UCR2 regulatory domains, "short" forms that lack UCR1, and "super-short" forms that lack UCR1 and have a truncated UCR2 (Conti and Beavo 2007; Bolger 2007; Bolger et al. 1993). In addition, each isoform has a unique amino-terminal region, encoded by one or more exons specific to that isoform, that frequently has unique properties. For example, the unique amino-terminus of the widely-found PDE4D5 isoform (Fig. 4.1) is essential for its interaction with its "partner" proteins (Bolger et al. 1997; Perry et al. 2002; Bolger et al. 2003; Baillie et al. 2003; Shukla et al. 2014; Yarwood et al. 1999; Bolger et al. 2002; Steele et al. 2001; Li et al. 2009a; Bolger et al. 2006; Baillie et al. 2007; Smith et al. 2007). PDE4D5 interacts selectively with β-arrestin2, implicated in the regulation of GPCRs and other cell signaling components (Perry et al. 2002; Bolger et al. 2003; Baillie et al. 2003; Li et al. 2009a; Bolger et al. 2006; Baillie et al. 2007; Smith et al. 2007; Bradaia et al. 2005; Lynch et al. 2005), and also with the β-propeller protein RACK1 (Yarwood et al. 1999; Bolger et al. 2002; Steele et al. 2001; Bolger et al. 2006; Smith et al. 2007; Bird et al. 2010). In contrast, the PDE4B1 isoform, which has an amino-terminal region completely different from that of PDE4D5, interacts selectively with the DISC1 protein, implicated in affective disorders and schizophrenia (Millar et al. 2005; Murdoch et al. 2007; Bradshaw et al. 2011; Hayashi-Takagi et al. 2010). 3

The catalytic regions of all PDE4 isoforms encoded by any individual PDE4 gene are identical in amino acid sequence and, in general, the biochemical and pharmacologic properties of each of the isoforms encoded by any individual PDE4 gene differ only modestly. For example, five different isoforms encoded by the *PDE4D* gene have differ less than fivefold in their K_m for cAMP and in their IC_{50} for the prototypical PDE4-selective inhibitor rolipram (Bolger et al. 1997). The catalytic regions of the proteins encoded by the four different PDE4 genes are extremely 4

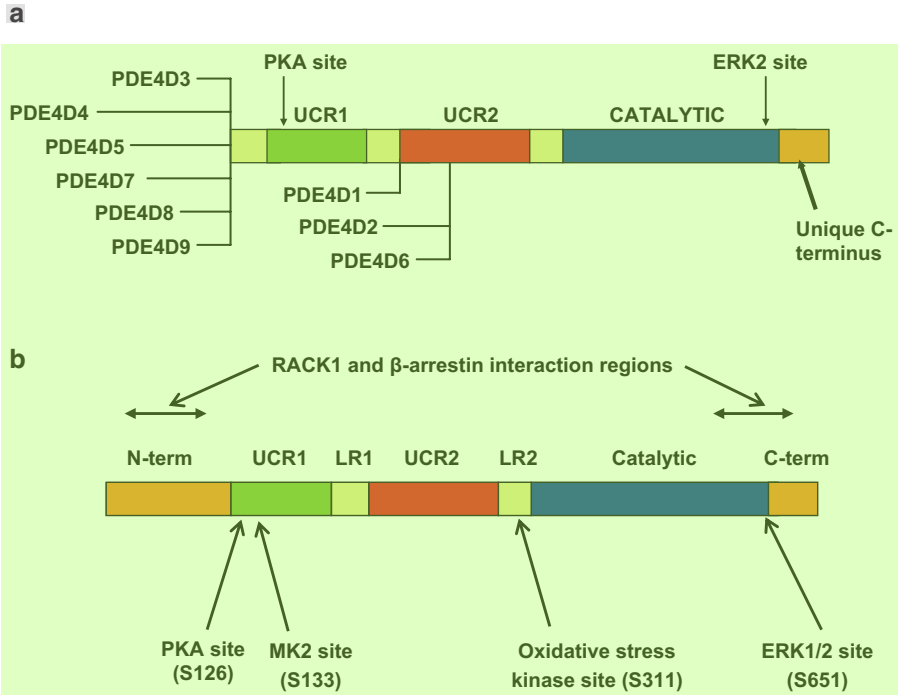

Fig. 4.1 Primary structures of PDE4D isoforms. (**a**) Schematic representation of the nine different isoforms encoded by the human *PDE4D* gene. The isoforms are divided into long isoforms, such as PDE4D5, that contain both UCR1 and UCR2, short isoforms, such as PDE4D1, that contain only UCR2, and super-short isoforms, such as PDE4D2, that contain only a truncated UCR2. Also shown is the C-terminal region, present in all PDE4D isoforms, but differing from the C-terminal regions of isoforms encoded by other PDE4 genes. (**b**) Schematic representation of human PDE4D5. PDE4D5 contains UCR1, UCR2, and catalytic domains, which are separated by the unstructured LR1 and LR2 regions. Also shown are the 88 amino acid unique N-terminal region (N-term), the C-terminus (C-term), and regions required for the interaction of PDE4D5 with RACK1 and β-arrestin2. The locations of PKA, ERK1/2, MK2, and oxidative stress kinase sites are also shown

similar (approximately 90% sequence identity). As all PDE4-selective inhibitors act, at least in part, at the catalytic sites of the PDE4 enzymes (Lee et al. 2002; Zhang et al. 2004a; Card et al. 2004; Huai et al. 2004; Burgin et al. 2010; Wang et al. 2007a; Kranz et al. 2009; Fox et al. 2014; Gurney et al. 2011), and therefore act, at least in part, as competitive inhibitors of cAMP hydrolysis, the similarity among the catalytic sites of the isoforms has greatly complicated the development of inhibitors selective for any individual isoform, or even for all the isoforms encoded by one PDE4 gene. Although some newer compounds may be more selective (Bruno et al. 2011), most PDE4-selective inhibitors have less than a tenfold difference in potency (i.e., IC_{50}) for isoforms encoded by different PDE4 genes (Hatzelmann et al. 2010; Burgin et al. 2010; Wang et al. 2007a).

4.3 Dimerization of PDE4 Isoforms and Its Implication for Drug Discovery

Long PDE4 isoforms, such as PDE4B1 and PDE4D5, have been demonstrated by a variety of assays to form homodimers (Richter and Conti 2002; Richter and Conti 2004; Xie et al. 2014; Bolger et al. 2015). Recently, the dimerization of long PDE4 isoforms has been greatly illuminated by structural and enzymatic studies (Cedervall et al. 2015). The structural data built on prior interaction studies, including yeast 2-hybrid and co-immunoprecipitation, and extensive mutagenesis studies, that suggested an interaction between specific regions of UCR1 and UCR2, which appeared to form a module that in turn interacted with the catalytic domain (Bolger et al. 1993; Richter and Conti 2002; Richter and Conti 2004; Xie et al. 2014; Bolger et al. 2015; Lim et al. 1999; Beard et al. 2000). They have also demonstrated conclusively, consistent with previous data (Richter and Conti 2002; Richter and Conti 2004; Xie et al. 2014; Bolger et al. 2015), that long PDE4 isoforms can form dimers, with UCR1 and UCR2 being essential components of the dimeric structure (Cedervall et al. 2015). Collectively, these approaches have shown that dimerization is mediated by an interaction of α-helical regions in the C-terminus of UCR1 with the N-terminus of UCR2, forming a tight 4-helix bundle (Richter and Conti 2002; Cedervall et al. 2015; Beard et al. 2000). Also present in the dimer is an interaction between UCR2 of one member of the dimer and the catalytic region of the other, providing a mechanism by which UCR2 serves as an auto-inhibitory domain (Cedervall et al. 2015). Finally, there is a smaller, but nonetheless biochemically significant, interface between the two catalytic domains, mediated by electrostatic interactions between Asp463 and Arg499 (PDE4D5 co-ordinates; Asp 471 and Arg507 in PDE4B1; refs. (Bolger et al. 2015; Cedervall et al. 2015)).

Dimerization provides many new insights into the enzymology and pharmacology of long PDE4 isoforms. The enzymatic and pharmacologic characteristics of the dimeric form are markedly different from those of the corresponding monomer. The dimeric form appears to exist as a “closed” or less-active conformation of the enzyme, with a specific activity for cAMP hydrolysis of dimeric PDE4B1 being roughly 50-fold lower than the corresponding monomeric form (Cedervall et al. 2015). Dimerization also affects the ability of long PDE4 isoforms to be inhibited by many PDE4-selective inhibitors; the effect of dimerization has been best-studied with the prototypical PDE4 inhibitor rolipram (Cedervall et al. 2015). These differences are mediated by a specific α-helical domain in the C-terminal half of UCR2 that, in the dimer, associates *in trans* with the catalytic domain (Cedervall et al. 2015), to create a high-affinity rolipram binding site (HARBS). In contrast, in the monomer, inhibitor binding is mediated exclusively by the catalytic region, to form a low-affinity rolipram-binding site (LARBS). The presence of a HARBS therefore reflects a conformational state unique to long PDE4 isoforms; short PDE4 isoforms, which lack UCR1 and therefore cannot dimerize, do not have a HARBS (Richter and Conti 2004; Huston et al. 1996; Rocque et al. 1997a; Rocque et al. 1997b; Souness and Rao 1997). These insights expand and modify prior models of PDE4

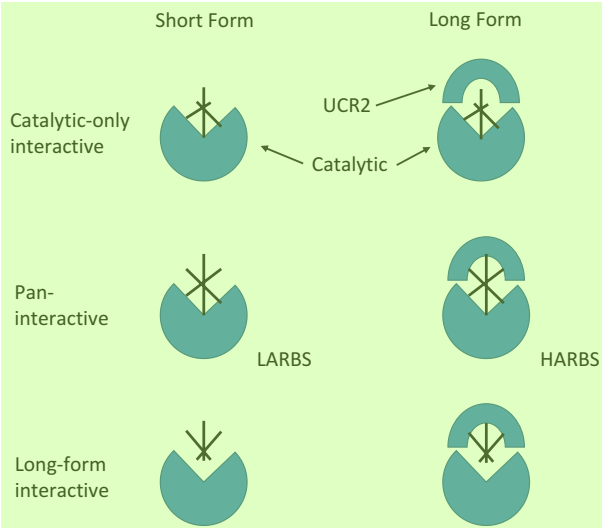

Fig. 4.2 Structure of the drug-binding site in short and long PDE4 isoforms and the effects of various classes of inhibitors. Schematic representations of the PDE4 short and long isoforms are shown in the *left and right columns*, respectively. Short isoforms form monomers with no UCR2-catalytic interaction; long isoforms form dimers with a specific UCR2-catalytic interaction. PDE4-selective inhibitors are represented by the *intersecting black bars*. Catalytic-only inhibitors (*top row*) interact primarily with the catalytic region and less avidly with UCR2; they would have activity against both long and short isoforms. Pan-interactive inhibitors (*middle row*) interact with both the catalytic regions and UCR2; when UCR2 is not present, the interaction site has the conformation of a LARBS; when UCR2 is present, the interaction site has the conformation of a HARBS. They would have activity against both long and short isoforms, but with different inhibitory characteristics. Long-form inhibitors (*lower row*) interact primarily with UCR2 and less avidly with the catalytic region and therefore would have activity against only long isoforms

active site conformation (Lee et al. 2002; Zhang et al. 2004a; Card et al. 2004; Burgin et al. 2010; Wang et al. 2007a; Kranz et al. 2009; Fox et al. 2014; Gurney et al. 2011; Huai et al. 2006) and are highly likely to stimulate the identification of inhibitors that interact primarily with UCR2, with relatively less interaction with the catalytic domain (Fig. 4.2). These "long-isoform interactive" PDE4 inhibitors might therefore have a safety and/or efficacy profile distinct from the current generation of PDE4 inhibitors (Cedervall et al. 2015; Zhang et al. 2006; Zhao et al. 2003a).

Given these new findings, it is of interest to review the action of currently-approved PDE4 inhibitors. Roflumilast clearly acts similarly (i.e., with an IC_{50} less than fivefold different) on the long and short forms encoded by any individual PDE4 gene (Hatzelmann et al. 2010). Similarly, the data on apremilast suggests that, like cilomilast (Giembycz 2001), it acts roughly equally on both long and short forms (Schafer et al. 2014). Another important characteristic of both roflumilast and apremilast is that their penetration into the CNS may be limited by the blood-brain barrier. There is little published pre-clinical data on crisaborole, which is designed for topical application. These characteristics of the currently-approved PDE4

inhibitors probably account for their improved tolerability in inflammatory and pulmonary disorders, compared to older agents, such as rolipram. However, it is clear that these clinically-useful characteristics of these three drugs actually reduces their potency in the CNS, indicating that further compound development work is essential to optimize the CNS-selectivity and effectiveness of PDE4-selective inhibitors. I present a potential pathway for these developmental activities below.

4.4 Dimerization and the Phosphorylation of PDE4s

The functions of PDE4 isoforms are dynamically regulated through phosphorylation by kinases such as PKA, ERK1/2, MK2, and AMPK, as well as modification by ubiquitination and sumoylation (Marchmont and Houslay 1980; Sette et al. 1994a; Sette et al. 1994b; Sette and Conti 1996; Hoffmann et al. 1998; MacKenzie et al. 2002; Collins et al. 2008; Baillie et al. 2001; Hoffmann et al. 1999; Baillie et al. 2000; MacKenzie et al. 2000; Mackenzie et al. 2011; Sheppard et al. 2014; Hill et al. 2006; Li et al. 2010). The activity of all long PDE4 isoforms is increased by two- to sixfold upon PKA phosphorylation, and PKA phosphorylation also changes the ability of the enzyme to be inhibited by PDE4-selective inhibitors, such as rolipram (Sette et al. 1994a; Sette et al. 1994b; Sette and Conti 1996; Hoffmann et al. 1998; MacKenzie et al. 2002). In contrast, ERK1/2 phosphorylation attenuates PDE activity (Hoffmann et al. 1999; Baillie et al. 2000; MacKenzie et al. 2000; Mackenzie et al. 2011). MK2 kinase serves to attenuate the degree of activation conferred by PKA phosphorylation and, in the case of PDE4D5, serves as a site for mono-ubiquitination by the β-arrestin-sequestered E3 ligase, Mdm3, which gates poly-ubiquitination of the PDE4D5 isoform-specific N-terminal region (Sheppard et al. 2014).

Recently, we have assessed the effects of phosphorylation on PDE4 dimerization. PKA phosphorylates a site (S54 in PDE4D3, S126 in PDE4D5 and S133 in PDE4B1; Fig. 4.1) in the motif QRRES located at the N-terminus of UCR2 (Sette et al. 1994a; Sette et al. 1994b; Sette and Conti 1996; Hoffmann et al. 1998; MacKenzie et al. 2002). ERK1/2 phosphorylates a site (S579 in PDE4D3, S651 in PDE4D5 and S659 in PDE4B1) located on the outer surface of the catalytic domain (Hoffmann et al. 1999; MacKenzie et al. 2000). MK2 phosphorylates a serine (S61 in PDE4D3, S133 in PDE4D5 and S140 in PDE4B1) close to the PKA site, within UCR1 (Sheppard et al. 2014).

Although all of these phosphorylation sites are located in highly flexible areas of the protein that are disordered in the crystal structure, suggesting that these regions are not essential for creation or maintenance of the dimer (Cedervall et al. 2015), we have shown recently that mutations of PKA, ERK1/2, MK2 and oxidative stress kinase phosphorylation sites can affect dimerization. Specifically, blocking phosphorylation at both the PKA and ERK1/2 phosphorylation sites diminished dimerization; mutations of each individual site had only modest effect (Bolger 2016). The precise mechanism of how PKA-ERK1/2 phosphorylation might

promote dimerization is uncertain; however, it is likely that phosphorylation at these sites would affect the conformation of the dimer and thereby push the equilibrium towards the dimeric form. In contrast, our analysis of phospho-mimetic mutations at the MK2 and stress oxidation kinase sites suggests that their action would be to promote the monomeric form.

4.5 Dimerization and Interaction of PDE Isoforms with Their Protein "Partners"

Given the extensive surfaces on PDE4 long forms that are necessary for dimerization (Cedervall et al. 2015), we felt that it was highly possible that their protein partners would restrict access to these surfaces and thereby inhibit dimerization. Recently, we demonstrated that the dimerization of PDE4D5 was blocked by two well-characterized protein partners, specifically RACK1 and β-arrestin2 (Bolger 2016). Given the high avidity and multiple sites of interaction between PDE4D5 and both of these proteins (Perry et al. 2002; Bolger et al. 2003; Baillie et al. 2003; Yarwood et al. 1999; Bolger et al. 2002; Steele et al. 2001; Li et al. 2009a; Bolger et al. 2006; Baillie et al. 2007; Smith et al. 2007), it is perhaps not surprising that they would have such an effect. However, since our prior studies have shown that both RACK1 and β-arrestin2 largely interact with the unique N-terminal and C-terminal regions of PDE4D5 (Bolger et al. 2003; Yarwood et al. 1999; Bolger et al. 2002; Bolger et al. 2006; Smith et al. 2007), which are unstructured in the dimer (Cedervall et al. 2015), it is unlikely that they act to directly restrict interaction at the UCR1/UCR2/catalytic or catalytic/catalytic interfaces that mediate dimerization. Instead, they presumably have indirect effects, possibly by sequestering the monomeric protein and thereby preventing it from forming a dimer, or by affecting its conformation in other ways. Inhibiting the dimerization of PDE4D5 could have multiple possible functional roles, such as increasing the enzymatic activity of PDE4D5 in certain cellular contexts, or targeting monomeric PDE4D5 to specific subcellular compartments.

RACK1 and β-arrestin2 have very different avidities for the "closed" or obligate-dimer conformation of PDE4D5. RACK1 interacts avidly with the "closed" conformation of PDE4D5, which is not entirely surprising, given its high avidity and selectivity for PDE4D5 and the extensive regions on PDE4D5 that can interact with RACK1 (Bolger 2016). However, in contrast, β-arrestin2 did not detectably interact with the "closed" conformation (Bolger 2016). This observation could provide novel insight into the physiological mechanism of the PDE4D5-β-arrestin2 interaction, in which β-arrestin2 serves to recruit PDE4D5 to the ligand-occupied, GRK2-phosphorylated state of the β_2-adrenergic receptor and thereby down-regulate cAMP signaling (Perry et al. 2002; Baillie et al. 2003). Since the major function of this recruitment is to move PDE4 enzymatic activity close to the β_2-adrenergic receptor, it would be logical that β-arrestin2 preferentially recruit the monomeric, or "open," form of PDE4D5, as this has much higher catalytic activity (50-fold greater, as

measured for PDE4B1; Cedervall et al. 2015). Therefore, the preferential interaction of β-arrestin2 with the monomeric form would maximize its physiologic function.

In summary, much has now been learned about the regulation of PDE4 isoforms by protein-protein interactions, including dimerization, and by phosphorylation. Since both of these processes require intimate contact between a PDE4 protein and its "partner" or kinase, these studies have also provided support for the concept that PDE4 regulation is highly spatially-dependent in cells, thereby providing a mechanism for the regulation of cAMP abundance in specific sub-cellular compartments (Francis et al. 2011; Conti and Beavo 2007; Houslay 2010; Bolger et al. 2007). This concept is particularly attractive in neurons, where PDE4 action could be targeted to specific synapses, axons, or dendrites, or other sub-cellular structures, rather than modulating cAMP levels globally throughout the cell. This compartmentalization of cAMP signaling, and PDE4 action in particular, is in turn compatible with PKA having different substrates in specific cellular compartments that are in turn regulated by different PDE4 isoforms. Selective targeting of these PDE4 isoforms could therefore produce highly specific pharmacologic effects, as discussed in the next section.

4.6 PKA Substrates as Mediators of PDE4 Action in the CNS

Key to understanding the cellular and organismal functions of the PDE4s is determining their downstream targets of action. Extensive research has demonstrated that cAMP binds to, and regulates the activity of, three effectors: (1) the regulatory subunit of cAMP-dependent protein kinase (kinase A; PKA); (2) the exchange protein directly activated by cAMP (Epac; refs. (de Rooij et al. 1998; Kawasaki et al. 1998; Gloerich and Bos 2010)) and (3) cAMP-gated ion channels. The cAMP-binding domains of each of these targets show significant structural similarity, reflecting their common function in binding cAMP (Rehmann et al. 2003; Kim et al. 2005; Zagotta et al. 2003). Epac acts as a cAMP-regulated guanine nucleotide exchange factor for Rap1 and has a range of physiologic functions (Gloerich and Bos 2010; Munoz-Llancao et al. 2015; Consonni et al. 2012; Gloerich et al. 2011). In contrast to the unique downstream effector of Epac, PKA has numerous substrates, the physiologic significance of which continues to evolve. In this section, we will focus on the following PKA substrates as being especially important in explaining PDE4 functions in the CNS:

4.6.1 CREB

The loop-helix loop transcription factor cAMP-response element binding protein (CREB) is phosphorylated by PKA, ERK1/2 and several other kinases at a single serine (S133). CREB and phospho-CREB are expressed widely in the brain and their abundance changes in response to numerous neurotransmitters, drugs, and

stimuli, including those necessary for learning/memory and other behavioral processes (Silva et al. 1998; Frank and Greenberg 1994). Knock-out and dominant-negative genetic approaches have demonstrated that CREB has an essential role in learning and memory in a wide range of organisms, from *Aplysia californica*, to *Drosophila melanogaster*, rodents, and humans (Bourtchuladze et al. 1994; Yin et al. 1995; Cho et al. 1998; Kida et al. 2002; Ahn et al. 1999; Bartsch et al. 1998; Pittenger et al. 2002; Barco et al. 2002; Pittenger et al. 2006; Han et al. 2009; Lonze et al. 2002). CREB has been implicated in a variety of CNS phenotypes, including those implicated in affect (depression), reward (drug-seeking behavior and addiction) and several others (Newton et al. 2002; Carlezon et al. 1998). Investigators using PDE4 mutant mice have implicated CREB as an important contributor to the phenotypes seen in these mice, as described in more detail below. 1

A number of gene-expression and proteomic studies have attempted to identify CREB-responsive genes. Whole-genome sequencing has identified cAMP-response elements (CREs) in the promoters of numerous genes, some of which have been determined experimentally to be of functional significance in the transcriptional regulation of those genes (Kim et al. 2010). mRNA expression studies have identified numerous genes that are differentially regulated upon phosphorylation of CREB in cells, many of which contribute to neuronal growth and differentiation and synaptic plasticity (Casadio et al. 1999; Barco et al. 2005; Crino et al. 1998). However, the precise role of CREB phosphorylation in the regulation of many of these genes is not known. Collectively, however, these studies suggest strongly that many of the biochemical and cellular effects of PDE4 modulation in the CNS might be mediated through CREB, a hypothesis that has been tested extensively in the cellular and animal experiments reviewed below. 2

4.6.2 Cytoplasmic PKA Targets: LKB1 and GSK-3β Kinases 3

PKA phosphorylates a number of kinases implicated in neuron growth and differentiation, especially in the hippocampus (Seino and Shibasaki 2005). Among the best-studied of these kinases are LKB1 and GSK-3β, both of which are essential for neuronal polarity during development and hippocampal neurogenesis (Song et al. 1997; Shelly et al. 2007; Ming et al. 1997; Huang et al. 2014; Barnes et al. 2007; Jiang et al. 2005; Yoshimura et al. 2005; Shelly et al. 2010). Treatment of cultured cortical neurons with rolipram, or transfection with siRNA directed against PDE4D isoforms, increases phosphorylation of LKB1 by PKA and impairs the development of neural polarity and reduces neural migration (Shelly et al. 2010). A number of extracellular or cell-surface components implicated in neuronal growth and differentiation, such as brain-derived neurotrophic factor (BNDF), NGF, netrin-1, laminin, or Wnt, could modulate cAMP levels in these cells. Although the physiological mechanism of cAMP elevation remains uncertain, these experiments implicate LKB1 and GSK-3β as likely PDE4-regulated PKA substrates in cortical neurons. It is highly possible that additional kinases, some of which may also be PKA substrates, contribute to these effects. 4

4.6.3 Cytoplasmic PKA Targets: DARPP32

The primarily cytoplasmic protein DARPP32 is an important PKA substrate in the CNS (Svenningsson et al. 2004). It is a 32 kDa protein that is phosphorylated at T34 by several kinases, including PKA, and at T75 by Cdk5. Phosphorylation of DARPP32 at T34 in turn depends on the phosphorylation state of S102 and S137, which are phosphorylated by CK2 and CK1, respectively (Svenningsson et al. 2004). Activation of the D1 dopamine receptor, a GPCR, by dopamine activates adenylyl cyclase and thereby PKA, increasing pT34-DARPP32 (Svenningsson et al. 2004; Stipanovich et al. 2008). Dopamine antagonists, such as haloperidol, and many drugs of abuse, such as cocaine, exert many of their effects through T34-DARPP32 phosphorylation (Bateup et al. 2008; Volkow and Morales 2015). As pT34-DARPP32 is in turn a potent inhibitor of PPT1 and pT75-DARPP32 is a potent inhibitor of PKA (Svenningsson et al. 2004), phosphorylation of DARPP32 produces profound changes in many cellular signaling pathways (Nishi et al. 2008; Svenningsson et al. 2004). pT34-DARPP32 can translocate to the nucleus, where it can inhibit nuclear PPT-1, enhance phosphorylation of histone H3, and regulate transcription (Stipanovich et al. 2008). Rolipram has been shown to enhance pT34-DARPP32 phosphorylation in striatopallidal neurons; this effect is accompanied by significant PKA-mediated phosphorylation of tyrosine hydroxylase (TH), essential for dopamine synthesis and turnover (Nishi et al. 2008). In contrast, PDE10 inhibition has no effect on TH phosphorylation, but substantially increases pT34-DARPP32 phosphorylation in striatal neurons (Nishi et al. 2008). The differential effects of these PDE4 inhibitors on dopamine signaling support investigation of PDE4-selective inhibitors as therapy in psychiatric and drug abuse disorders mediated, at least in part, by dopamine neurotransmission.

4.6.4 Ion Channels

There are two mechanisms by which cAMP can regulate ion channel activity. In the first mechanism, cAMP binds directly to a conserved intracellular cyclic nucleotide-binding domain (CNBD); this mechanism is important in several classes of cyclic nucleotide-gated ion channels (CNGs and HCNs; refs. (Zagotta et al. 2003; Craven and Zagotta 2006; Puljung et al. 2014)) whose functions in the mammalian CNS are an active area of research (DiFrancesco and DiFrancesco 2015; Nolan et al. 2004; Wang et al. 2007b; Kaupp and Seifert 2002). In the second mechanism, the ion channel is phosphorylated by PKA; a classical example of this mechanism is the cystic fibrosis transmembrane regulator (CFTR), which is a Cl^- ion channel that is mutated in the disease cystic fibrosis and which has multiple PKA phosphorylation sites (Lambert et al. 2014; Baker et al. 2007).

PKA modulates the activity of a number of CNS-expressed ion channels, largely through the property of PKA to be tethered close to these ion channels by its interaction

with specific A-kinase anchoring proteins (AKAPs). For example, the strong inwardly rectifying potassium channel Kir2.1 forms a complex with AKAP79/150 and the related channel Kir6.2 is PKA-phosphorylated in its regulatory region in response to GPCR activation (Dart and Leyland 2001; Light et al. 2002). AKAPs are likely to be involved in the PKA-mediated phosphorylation at S333 of the potassium ion channel TREK-1, which is expressed widely in the CNS (Maingret et al. 2000). PKA-mediated phosphorylation of the A-type potassium channel Kv4.2 subunit occurs at two sites and requires the participation of a multi-protein regulatory complex (Schrader et al. 2002). The role of PDEs in the regulation of these channels remains to be determined. 1

AKAP79/150 also recruited into complexes at the postsynaptic membrane of excitatory synapses with N-methyl-d-aspartic acid (NMDA) or alpha-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid (AMPA)-subtype glutamate GluA receptors, where it tethers PKA, protein kinase C, and protein phosphatase-2B (PP2B/calcineurin) into a dynamic regulatory complex (Westphal et al. 1999; Greengard et al. 1991; Banke et al. 2000; Tavalin et al. 2002). Recent studies have implicated PDE4, most notably ERK1/2-mediated phosphorylation of PDE4, in the regulation of membrane insertion of GluA1 (Song et al. 2013). GluA1 is also PKA-phosphorylated at a specific site (S845), but this phosphorylation is increased by PDE10, rather than PDE4, inhibition (Nishi et al. 2008; Greengard et al. 1991). 2

Extensive work in models of cardiac function has demonstrated that PDE4D3, and possibly other PDE4 isoforms, forms a complex with, and regulates PKA phosphorylation of, the cardiac ryanodine receptor (Beca et al. 2011; Lehnart et al. 2005) and other structures involved in the generation of cardiac calcium currents (Kerfant et al. 2007; Weninger et al. 2013; Leroy et al. 2011; Sin et al. 2011). Since calcium currents are also essential for many aspects of neuronal function, it would seem reasonable to search for PDE4-dependent activity of neuronal calcium flux; to date, however, such attempts have been unsuccessful. 3

4.6.5 Synaptic Vesicle Proteins 4

The synaptic protein Rim1α is an important PKA target, being phosphorylated at two separate sites (Seino and Shibasaki 2005; Lonart et al. 2003; Park et al. 2014). However, Rim1α has also been shown to interact with Epac2 (Seino and Shibasaki 2005). Mutant RIM1α lacking the N-terminal PKA phosphorylation site was unable to rescue LTP in *RIM1α* knockout neurons but selectively suppressed LTP in wild-type neurons, clearly implicating a role of PKA-mediated phosphorylation Rim1α on presynaptic LTP (Lonart et al. 2003). A number of other synaptic vesicle proteins also appear to be PKA substrates (Seino and Shibasaki 2005; Park et al. 2014), although the exact physiological consequences of their PKA phosphorylation are not clear. 5

4.6.6 Ubiquitin Ligases 1

The HECT domain E3 ubiquitin ligase UBE3A targets proteins to proteasome-mediated degradation (Yi et al. 2015). Duplication or truncation mutations in UBE3A have been linked to autism, while numerous different single amino acid mutations in UBE3A have been linked to Angelman syndrome (AS), a multi-component CNS disorder (Kishino et al. 1997; Jiang et al. 1998). Deletion of *Ube3a* in mice impairs synapse development and plasticity and produces a number of neurobiological phenotypes that mimic human AS (Yi et al. 2015). UBE3A is phosphorylated at T485 by PKA, and PKA-mediated phosphorylation of T485 inhibits UBE3A activity. Pharmacologic agents that elevate cAMP in dissociated mouse cortical neurons, including rolipram, augment phosphorylation of UBE3A by PKA. An AS-associated single amino acid mutation, T485A, blocks PKA action (Yi et al. 2015), thereby elevating UBE3A activity in cells, with enhanced substrate turnover and excessive dendritic spine development (Yi et al. 2015). These findings implicate a role for PDE4-mediated regulation of PKA activity in CNS development, with potential implications in several genetic disorders, including acrodysostosis, as discussed in greater detail in a section below. 2

4.7 Cellular Functions of PDE4 Action in the CNS 3

Given the diversity of PDE4 isoforms, and the large number of PKA substrates both in and outside of the CNS, it should not be surprising that numerous cellular functions are influenced in some way by the actions of PDE4 isoforms (Conti and Beavo 2007; Houslay 2010; Maurice et al. 2014; Bolger et al. 2007). Many of these functions are specific to organs or tissues outside the CNS (e.g., cardiac function, refs. (Maurice et al. 2014; Zaccolo 2009; Nikolaev et al. 2010; Richter et al. 2011; Eschenhagen 2013)) and are not discussed here. For this review, we will focus on two CNS-specific cellular functions: neurogenesis and synaptic plasticity. 4

4.7.1 PDE4s and Neurogenesis 5

Appropriate levels of hippocampal neurogenesis are essential for normal learning and memory, pattern and spatial recognition, and potentially other functions (Gage 2000; Lie et al. 2004; Sahay et al. 2011; Zhao et al. 2008; Kitamura et al. 2009). Hippocampal neurogenesis occurs throughout human life, with a modest decline accompanying aging (Spalding et al. 2013). Neurogenesis appears to be essential for the anti-depressant effects of fluoxetine, a serotonin-selective re-uptake inhibitor (SSRI), in murine models of depression (Malberg et al. 2000; David et al. 2009; Santarelli et al. 2003). One study has shown that chronic fluoxetine can increased 6

dendritic arborization of newly-generated immature neurons (Wang et al. 2008). This study also showed that chronic fluoxetine accelerated the maturation of immature neurons. The effects of fluoxetine on neurogenesis are generalizable to other anti-depressants, such as rolipram and other PDE4-selective inhibitors (Li et al. 2009b; Xiao et al. 2011). They are also consistent with the results from the study of genetically-altered PDE4 mice, as described in more detail below.

4.7.2 PDE4s, Neuronal Polarity and the Formation of Axons and Dendrites

As described above, the phosphorylation of LKB1 is dependent on a reciprocal interaction between cAMP and cGMP (Shelly et al. 2010). This reciprocal interaction also has an important role in neuronal development. High local concentrations of cAMP stimulate the differentiation of neurites from embryonic hippocampal neurons into axons, while cGMP stimulates the development of dendrites (Shelly et al. 2010). As predicted, PDE4D siRNA impaired the migration of neural precursor cells to the cortical plate and suppressed neuronal polarity during embryogenesis (Shelly et al. 2010). Although the functional implications of these processes in the intact brain remain uncertain, they may have important implications in a number of neurobiological processes, including cognition, learning and memory, and affect.

4.7.3 PDE4s and Synaptic Function

Modulation of synaptic plasticity underlies, or is influenced by, numerous CNS functions, including learning and memory (Kandel et al. 2014), addiction (Volkow and Morales 2015), and sleep (Yang et al. 2014; Attardo et al. 2015). Emerging evidence from several systems has suggested that select PDE4 isoforms are targeted to synapses, where they can regulate cAMP levels in the local synaptic environment, affect PKA activity, and modulate plasticity. Among the best-understood of these mechanisms is the interaction of PDE4B1 with DISC1 (Millar et al. 2005; Murdoch et al. 2007; Bradshaw et al. 2011; Hayashi-Takagi et al. 2010; Bradshaw and Porteous 2012; Brandon and Sawa 2011), in which PDE4B1 and DISC1 form a complex with several other proteins, including dynein, LIS1, NDE1, and NDEL1 (Collins et al. 2008; Bradshaw et al. 2008). According to some models, DISC1 is felt to act as a scaffold for this complex and to recruit PDE4B1 to the synapse (Hayashi-Takagi et al. 2010; Bradshaw et al. 2008; Wang et al. 2011); other models have suggested that a major location of this complex is in the centrosome or nucleus, where it regulates gene expression (Bradshaw et al. 2011; Sheppard et al. 2014; Bradshaw and Porteous 2012; Soda et al. 2013; Ishizuka et al. 2011) and is active in early brain development (Greenhill et al. 2015; Mao et al. 2009; Niwa et al. 2010).

In both of these models, PDE4B1 is felt to regulate PKA's ability to phosphorylate T131 of NDE1 (Bradshaw et al. 2011) and S58 of DISC1 (Soda et al. 2013). DISC1 has been shown to interact with numerous proteins (Bradshaw and Porteous 2012), not all of which appear to be present in the complex under all physiologic circumstances, and the precise protein components of the PDE4B1-DISC1 complex, and its precise physiologic function(s), remain objects of intense investigation. 1

A number of PDE4 isoforms other than PDE4B1 have been implicated in synaptic function and, specifically, in hippocampal functions essential to learning and memory (see Sanderson and Sher 2013 for a review). For example, the PDE4B3 isoform has been implicated in LTP, especially late-phase LTP, in rat hippocampal neurons, where it has been localized to cell bodies and dendrites of neurons in hippocampal CA1 (Ahmed and Frey 2003). Another group has demonstrated that the anchoring protein gravin recruits a signaling complex containing PKA, PKC, calmodulin, and PDE4D isoforms to the β_2-adrenergic receptor (Havekes et al. 2012). Mice lacking the alpha-isoform of gravin have deficits in PKA-dependent long-lasting forms of hippocampal synaptic plasticity, including β_2-adrenergic receptor-mediated plasticity, and selective impairments of long-term memory storage (Havekes et al. 2012). These studies have collectively implicated a number of different PDE4 isoforms in synaptic plasticity, and particularly in learning and memory, and provide an essential background to interpretation of studies on genetically-modified PDE4 mice, which will be described in detail below. 2

4.8 Regional Expression of PDE4 Isoforms in the CNS and Potential Functional Implications 3

Each of the PDE4 isoforms has a distinct pattern of expression in cells and tissues and the vast majority of them has been demonstrated to have an isoform-specific pattern of expression in the CNS (Bolger et al. 1994; Cherry and Davis 1999; Miro et al. 2002; D'Sa et al. 2005; D'Sa et al. 2002; Reyes-Irisarri et al. 2008; Nishi et al. 2008; Mori et al. 2010; Kuroiwa et al. 2012; Ahmed and Frey 2003; Shakur et al. 1995; Suda et al. 1998; Farooqui et al. 2000; Zhang et al. 1999a; McPhee et al. 2001; Mackenzie et al. 2008; Perez-Torres et al. 2000; Johansson et al. 2012; Johansson et al. 2011; Braun et al. 2007). The regional expression of many isoforms, especially those identified recently, has yet to be determined. Unfortunately, there is little or no isoform-specific data in commonly-used CNS gene expression databases, such as the Allen Brain Atlas. Some isoforms, such as PDE4D5, are broadly-expressed in multiple CNS and non-CNS tissues (Miro et al. 2002; Bolger et al. 1997), while others, such as PDE4A1, are expressed strongly in a few tissues (e.g., cerebellum for PDE4A1) and expressed at much lower levels elsewhere (Shakur et al. 1995). These pronounced differences in regional expression in the CNS suggest strongly that each isoform has a distinct function; however, in most cases, the precise neurobiological function(s) of each isoform have only begun to be 4

appreciated. Better knowledge of the regional expression of PDE4 isoforms would in turn provide improved understanding of the phenotypes of genetically-altered PDE4 mice, as described in detail in a subsequent section.

4.8.1 Regional Distribution of PDE4 Isoforms in Brain Regions Involved in Dopaminergic Signaling: Addictive Behaviors, Depression and Schizophrenia

A major objective of PDE4 CNS research has been to identify the functional role(s) of PDE4s in additive behavior. Experimental studies of addiction in a variety of model systems have identified many of the neuronal circuit, behavioral, and synaptic mechanisms involving this process (Volkow and Morales 2015). These studies have identified and characterized a drug-reward neuronal pathway in the CNS, extending from dopaminergic neurons in the ventral tegmental area (VTA) to the nucleus accumbens (NAc). Many drugs of abuse, including opioids and cocaine, increase dopamine release in the shell subregion of the NAc (Di 2002) and elsewhere. Dopaminergic D1 and D2 receptors increase cAMP levels and the phosphorylation of CREB (Bibb 2005; Dudman et al. 2003; Antoine et al. 2013). Rolipram administration given prior to drug administration substantially reduced morphine-, cocaine- and cannabinoid-induced conditioned place preference in mice (Thompson et al. 2004; Zhong et al. 2012; Janes et al. 2009). Additionally, rolipram and other PDE4-selective inhibitors blocked inhibitory LTD and acute depression of inhibitory postsynaptic currents induced by D2 receptor and cannabinoid receptor agonists in VTA dopamine neurons (Zhong et al. 2012).

A number of studies have also implicated PDE4 isoforms in the NAc shell in the pathogenesis of depression. PDE4B and PDE4D isoforms are present in the NAc shell and that their expression is increased upon chronic administration of antidepressants (Cherry and Davis 1999; Takahashi et al. 1999). These effects are likely to be mediated by CREB, as over-expression of dominant-negative CREB in the NAc had an antidepressant effect in the learned-helpless model, while over-expression of wild-type CREB had an opposite effect (Newton et al. 2002). The specificity of these studies to depression is not clear, especially as chronic treatment with a number of antidepressants having different mechanisms of action (including tricyclics, SSRIs and PDE4 inhibitors) all increase levels of various PDE4 isoforms in a number of different areas of the brain (D'Sa et al. 2005; D'Sa et al. 2002; Ye et al. 1997; Ye et al. 2000; Zhao et al. 2003b; Dlaboga et al. 2006). In contrast, diminished stimulation of beta-adrenergic receptors, either by loss of noradrenergic innervation or by receptor blockade, reduces PDE4 activity (Farooqui et al. 2000; Ye and O'Donnell 1996; Zhang et al. 1999b).

Finally, immunohistochemical studies have demonstrated expression of PDE4A, PDE4B and PDE4D isoforms in frontal cortex, probably in D1-receptor-positive

neurons (Kuroiwa et al. 2012). Its location in these areas may contribute to the anti-schizophrenic effect of D1-receptor agonists.

Related to the role of PDE4 isoforms in depression and learning is the important influence of sleep and sleep disorders in these processes (Yang et al. 2014; Vecsey et al. 2009; Havekes et al. 2014); see refs. (Havekes et al. 2015; Meerlo et al. 2015) for a review. Normally, sleep promotes the development of dendritic spines after learning, implicating a beneficial role of sleep in memory consolidation (Yang et al. 2014). In contrast, sleep deprivation has been shown to produce memory loss in a number of rodent models of learning and memory, which is associated with impairment of cAMP- and PKA-dependent forms of hippocampal synaptic plasticity (Vecsey et al. 2009). Sleep deprivation increases PDE4 activity, possibly as a compensatory process (Vecsey et al. 2009). Transiently elevating cAMP levels in hippocampal excitatory neurons during sleep deprivation prevents memory consolidation deficits associated with sleep loss. These observations provide further evidence for the benefit of PDE4 inhibition on cognition and memory. The specificity of the benefit of PDE4 inhibition to sleep-disordered memory loss is uncertain, however, as rolipram and other PDE4 inhibitors improve cognitive function generally in mice, as described in greater detail in the next section.

Chronic stress (modeled in mice by an acute and unpredictable tail-shock), like sleep deprivation, increases PDE4 activity in hippocampal CA3 neurons and is associated with a marked impairment of hippocampal LTP (Chen et al. 2010).

4.9 CNS Effects of PDE4 Inhibitors

The molecular, cellular and regional studies described in the preceding sections provide a perspective essential to studying the phenotypes of PDE4 inhibition or ablation in the intact organism. Therefore, we will now discuss the whole-organism pharmacology of PDE4 inhibitors and then move to genetic models.

The prototypical PDE4 inhibitor rolipram was first identified by virtue of its antidepressant-like activity in humans and rodents (Fleischhacker et al. 1992; Scott et al. 1991; Hebenstreit et al. 1989; Eckmann et al. 1988; Zeller et al. 1984; Bobon et al. 1988; Kehr et al. 1985; Wachtel 1983). Its activity as a highly-selective PDE4 inhibitor was determined only after the publication of these early behavioral studies (Nemoz et al. 1985). Extensive testing of rolipram and numerous other PDE4-selective inhibitors in behavioral assays in rodents has demonstrated that they have activity that is broadly similar to other antidepressant agents, such as tricyclic antidepressants, SSRIs and SNRIs. Specifically, PDE4-selective inhibitors have antidepressant-like activity in hypothermia assays and in the forced-swim and tail-suspension tests (Barad et al. 1998; Bach et al. 1999; Titus et al. 2013; Mueller et al. 2010; Nibuya et al. 1996; O'Donnell and Zhang 2004; Zhang et al. 2006; Xiao et al. 2011; Jindal et al. 2012) and other assays (Wachtel 1983; O'Donnell 1993; O'Donnell and Frith 1999; Wachtel and Schneider 1986) used in the pre-clinical

testing of antidepressants. Numerous studies have also demonstrated that most classes of antidepressant drugs, although having disparate immediate targets, ultimately have overlapping effects on cAMP signaling pathways (Zhang et al. 2005b). For example, in rodents, several different classes of antidepressants elevate PDE4 levels, especially levels of PDE4D (Takahashi et al. 1999; Ye et al. 1997; Ye et al. 2000; Zhao et al. 2003b; Dlaboga et al. 2006) and increases levels of CREB (Nibuya et al. 1996) and phospho-CREB (Li et al. 2009b). 1

In addition to their antidepressant effects, PDE4 inhibitors have cognitive and memory-enhancing effects in rodents and possibly in humans. The potential memory-enhancing effects of PDE inhibition have been investigated for decades (Villiger and Dunn 1981) and the effects of rolipram studied soon after it was first synthesized (Randt et al. 1982) and subsequently (Egawa et al. 1997; Imanishi et al. 1997). The potential value of PDE4 inhibition in disorders of cognition and memory received support from two studies from the Kandel laboratory in 1999 that suggested that PDE4-selective inhibitors have cognitive- and memory-enhancing activity in mice (Barad et al. 1998; Bach et al. 1999). These results have been confirmed by other groups, using a range of experimental conditions (Zhang et al. 2005a; Titus et al. 2013; Mueller et al. 2010; Kuroiwa et al. 2012; Ahmed and Frey 2003; Xiao et al. 2011; Zhang et al. 2000; Zhang et al. 2004b; Hajjhussein et al. 2007; Rutten et al. 2009; Rutten et al. 2007a; Rutten et al. 2007b; Cheng et al. 2010; Li et al. 2011a; Rutten et al. 2008a; Rutten et al. 2006; Navakkode et al. 2005; Wang et al. 2012; Wang et al. 2013; Guan et al. 2011; Werenicz et al. 2012; Hotte et al. 2012; Giralt et al. 2011; Li et al. 2011b). One distinct experimental approach has been the use of NMDA inhibitors as pre-treatment prior to PDE4 inhibition; PDE4 inhibition clearly can reverse, at least in part, memory loss produced by these inhibitors (Zhang et al. 2005a; Zhang et al. 2000; Hajjhussein et al. 2007; Suvarna and O'Donnell 2002; Kato et al. 1997; Wieschollеck and Manahan-Vaughan 2012). These cognition/memory-enhancing effects have also been demonstrated in other rodent models, including the rat (Rutten et al. 2007a; Wieschollеck and Manahan-Vaughan 2012; Schaefer et al. 2012; Zhang and O'Donnell 2000). The effects of rolipram and other PDE4-selective inhibitors on cognition, learning and memory appear to be distinct from their antidepressant effects, as antidepressants of other classes do not seem to have these effects (Makhay et al. 2001). The results of all these studies have stimulated the development of PDE4 inhibitors specifically targeted at cognition and memory enhancement (Zhang et al. 2005a; Zhang et al. 2006); however, clinical trials of these compounds to date have proved to be disappointing. 2

Pre-clinical testing of PDE4-selective inhibitors in rodent models of emesis, such as in the ferret, have shown consistently that they have pro-emetic properties; this effect is mediated, at least in part, by central mechanisms (i.e., via the area postrema; refs. (Mori et al. 2010; Robichaud et al. 1999; Robichaud et al. 2002; Duplantier et al. 1996)). PDE4-selective inhibitors also have significant class-specific effects on the GI tract, in that they increase gastric production and bowel chloride secretion, leading to emesis and diarrhea (Fabbri et al. 2009; Calverley 3

et al. 2009; Schafer et al. 2014; Kavanaugh et al. 2015; Papp et al. 2015). These side effects of PDE4-selective inhibitors appear to be related to their pharmacologic mechanism of action, in that gastric acid production and secretory diarrhea are both caused by elevation of cAMP levels in GI epithelium (Hatzelmann et al. 2010; Lambert et al. 2014; Barnette et al. 1995; Okuda et al. 2009). Studies of both the CNS and non-CNS side effects of PDE4 inhibitors have been complicated by the lack of selectivity of PDE4 inhibitors for any individual PDE4 isoform, or subset of PDE4 isoforms, thereby rendering it uncertain which PDE4 isoform(s) are responsible for any specific side effect. However, experimental studies of emesis in *Pde4d* knockout mice have implicated the isoforms encoded by this gene as being most likely to be contributing to this effect (Robichaud et al. 2002).

4.10 Studies of PDE4 Function in the CNS Using Genetically-Modified Mice

Essential to the understanding of the functions of PDE4 isoforms in the CNS has been the development of mice with mutations or knockdowns in specific PDE4 isoform(s). Three approaches have been employed: gene knockouts, lentiviral siRNA, and dominant-negative approaches, respectively.

4.10.1 PDE4 Gene Knockouts

The phenotypes of mice with knockouts in each of the *Pde4a, Pde4b* and *Pde4d* genes have been generated and studied extensively.

Pde4a−/− mice have been studied to date by a single group (Hansen et al. 2014). The knockout seems to have a beneficial effect on cognition and/or memory, based on one assay (the step-through-passive-avoidance test), but not in other assays, such as the Morris water maze. The mice also seem to have increased anxiety-like behavior, based on the elevated-plus maze, holeboard, light-dark transition, and novelty suppressed feeding tests. Consistent with the anxiety profile, *Pde4a−/−* mice had elevated corticosterone levels. The knockout did not seem to produce any change on tests of depression, such as the forced swim or tail suspension tests. Therefore, *Pde4a* may be important in the regulation of emotional memory and anxiety-like behavior.

Pde4b−/− mice have been studied by a number of groups, with disparate results (Zhang et al. 2008; Siuciak et al. 2008; Siuciak et al. 2007; Rutten et al. 2011). Some studies of *Pde4b−/−* mice have shown them to have behavioral characteristics that mimic the actions of antidepressants (Zhang et al. 2008; Siuciak et al. 2008; Zhang et al. 2002); for example, decreased immobility in tail-suspension and

forced-swim tests. However, other studies of the same genotype show only weak or modest effects in what appear to be similar assays (Siuciak et al. 2007; Rutten et al. 2011). Increased activity was also noted by some groups. There was no consistent effect on cognition or memory among the studies. These disparate findings are difficult to reconcile, although differences in genetic background, age at the time of study, or assay conditions could be responsible. 1

Pde4d−/− mice have also been studied by several groups. Some studies of *Pde4d−/−* mice have shown them to have augmented activity in tests of learning and memory (Li et al. 2011a; Zhang et al. 2002), while studies of the identical genotype by other groups do not show this effect (Rutten et al. 2008b). Almost all studies have shown increased levels of pCREB and increased hippocampal neurogenesis in these mice. Some groups also have shown that this knockout has an anti-depressant phenotype, consistent with the concept that PDE4D mediates antidepressant effects (Zhang 2009). 2

PDE4D−/− rats have also been generated recently (Kaname et al. 2014), although detailed characterization of their CNS phenotype awaits further publication. Of interest, however, is that they have skeletal abnormalities reminiscent of those seen in the human *PDE4D*-mutant disorder, acrodysostosis (see below). 3

Study of all PDE4 mouse knockouts have been complicated by non-CNS effects (Jin et al. 1999; Jin and Conti 2002), such as slow growth, small adult size and impaired fertility. In addition, assessment of the CNS phenotype of these knockouts has also been complicated by the fact that all of them have knocked out their respective gene in the entire organism, which, given the given the expression of isoforms from their respective genes in a number of brain areas (see section above), complicates assessment of their phenotype in any one area of the brain, such as the striatum or forebrain/hippocampus. Region-specific knockouts would allow exploration of these phenotypes. 4

4.10.2 Lentiviral siRNA 5

Several groups have employed lentiviruses expressing siRNA to knock down a specific PDE4 isoform in the murine or rat CNS (Li et al. 2011a; Wang et al. 2013; Schaefer et al. 2012; Wang et al. 2015). The lentiviruses were injected into specific areas of the brain, typically the hippocampus, of wild-type or knockout mice. These experiments have the advantage of targeting both a specific PDE4 isoform and a specific region of the CNS. However, potential off-target effects of the siRNA and trauma related to the injection process remain legitimate concerns. These studies have confirmed and expanded the concept the *Pde4d* is essential to memory, hippocampal neurogenesis and the regulation of pCREB. *Pde4d* siRNA also has a profound effect on neuronal polarization, with potential implications for neural development and learning (Shelly et al. 2010). 6

4.10.3 Dominant-Negative PDE4 Mutants

Two groups have now reported studies in which they used the over-expression of a dominant-negative PDE4B1 mutant as a transgene in the murine CNS (McGirr et al. 2016). As a precedent for this approach, we and our collaborators have used dominant-negative PDE4 mutants successfully in cell-based studies (Perry et al. 2002; Baillie et al. 2003; Bolger et al. 2006). In these cell-based studies, the dominant-negative mutant protein has been shown to displace the corresponding endogenous PDE4 isoform from its protein partner(s) and therefore disrupt its cellular function(s). The use of a dominant-negative mutant has the potential to be more isoform-selective than a gene knockout: The murine *Pde4b* and human *PDE4B* gene both encode five isoforms (Bolger et al. 1993; Bolger et al. 1994; Swinnen et al. 1991; Huston et al. 1997; Shepherd et al. 2003; Cheung et al. 2007; Johnson et al. 2010), each with a distinct protein structure and pattern of expression in tissues. Therefore, the *Pde4b−/−* mice described above have a phenotype that reflects the combined deficiency of all five PDE4B isoforms, which greatly complicates analysis of the effect(s) of any individual isoform, such as PDE4B1. The generation of dominant-negative mutants as transgenes also follows a strategy used by other groups who have expressed a dominant-negative PKA RIα subunit (Abel et al. 1997), or a dominant-negative CREB mutant (Silva et al. 1998; Kida et al. 2002; Ahn et al. 1999; Pittenger et al. 2002; Barco et al. 2002; Pittenger et al. 2006; Lonze et al. 2002; Vecsey et al. 2009) in the CNS. In the vast majority of these studies, the dominant-negative transgene was expressed off the CaMKIIα promoter (Mayford et al. 1996a; Mayford et al. 1996b; Tsien et al. 1996). This promoter is active preferentially in excitatory neurons of forebrain areas, including the hippocampus, amygdala, cortex and striatum (Mayford et al. 1996a; Mayford et al. 1996b). It is also silent until several days after birth (Burgin et al. 1990), when most neural circuits are already formed, thereby possibly minimizing any adverse effects of the transgene on the normal development of the brain (Tsien et al. 1996). The PDE4B1 dominant-negative approach is designed to target just the PDE4B1 isoform and therefore has greater specificity than a *Pde4b* knockout. This specificity is the likely explanation for the differences in phenotype in PDE4B1 dominant-negative mice, compared to *Pde4b−/−* mice. The PDE4B1 dominant-negative transgene clearly produces increased activity, levels of pCREB and neurogenesis, and may produce antidepressant effects in several assays (McGirr et al. 2016). One potential drawback of this approach is that the PDE4B1 dominant-negative transgene might not fully block PDE4B1 function, or, alternatively, might have some action against other PDE4 isoforms, including those encoded by the *Pde4a* and *Pde4d* genes. Despite these potential issues, the dominant-negative approach has merit and indeed appears to be best available way to study the relationship of a PDE4 isoform with its specific interacting partners, such as the interaction of PDE4B1 and DISC1.

4.10.4 What Have We Learned from the Mouse Models?

The mouse genetic models collectively appear to have phenotypes that are broadly similar to those that would be predicted on the basis of the known CNS actions of PDE4-selective inhibitors: there is activation of PKA and phosphorylation of CREB, with antidepressant-like activity being detected in most although certainly not all, of the models. There also appears to be some effect on learning and memory in many of the models. Augmented neurogenesis has been detected in almost all the models that have been assayed and provides a likely cellular mechanism for both the antidepressant and memory-augmentation phenotypes that have been observed. The antidepressant effects seem to be mediated more by *pde4b* isoforms, whereas the memory effects are mediated more by *pde4d* isoforms, although the relative contributions of these two genes are likely to overlap substantially. These results are generally reassuring for drug development: they provide essential confirmation that the CNS effects of PDE4-selective inhibitors are indeed produced by their ability to inhibit PDE4 enzymatic activity, and not by some as-yet-unappreciated off-target effect. They are also compatible with generally-accepted theories of learning and memory (Silva et al. 1998; Volkow and Morales 2015; Kandel et al. 2014), and depression (Gage 2000; Lie et al. 2004; Zhao et al. 2008; Spalding et al. 2013; Nestler and Hyman 2010), and thereby provide continued impetus for the development of PDE4-selective inhibitors that can produce such effects therapeutically in humans.

A number of questions remain. One important question is determining the specific region(s) of the brain that are essential for PDE4-mediated phenotypes. The dominant-negative models that use the CamIIα promoter tend to confirm numerous prior observations that the hippocampus and forebrain are essential for the PDE4-related learning and memory phenotype; however, this conclusion is obviously dependent on the accuracy of prior observations on the tissue specificity of this widely-used promoter (Mayford et al. 1996a; Mayford et al. 1996b; Tsien et al. 1996; Mayford et al. 1995); see also (Hitti and Siegelbaum 2014). The models provide fewer insights into the regions essential for the antidepressant actions of PDE4-selective inhibitors. Further studies that employ tissue-specific or region-specific methods, such as cre/lox knock-out/knock-in methods, or optogenetic approaches, should provide additional insights.

4.11 Human PDE4D Mutations: Acrodysostosis Syndromes

The phenotypes of mice with PDE4 mutations contrast sharply with those identified to date in humans. Mutations in the gene encoding the PKA regulatory subunit Type 1A (PRKAR1A) have been identified as the cause of Carney Complex, a multi-spectrum disorder with cutaneous, cardiac and endocrine features and a

predisposition to several cancers (Carney et al. 1985; Kirschner et al. 2000; Salpea and Stratakis 2014). Intriguingly, a different set of PRKAR1A mutations have been detected in patients with acrodysostosis, a complex disorder affecting bone formation, growth and the CNS (Linglart et al. 2011; Lee et al. 2012; Linglart et al. 2012; Michot et al. 2012; Nagasaki et al. 2012; Muhn et al. 2013; Lindstrand et al. 2014). More recently, PDE4D mutations have been identified in patients with acrodysostosis that lack PRKAR1A mutations (Lee et al. 2012; Linglart et al. 2012; Michot et al. 2012; Lindstrand et al. 2014; Lynch et al. 2013). The skeletal dysplasia in patients with acrodysostosis with PRKAR1A mutations resembles the osteodystrophy seen in patients with pseudohypoparathyroidism Type 1a, in that they are resistant to the action of the hormones PTH and TSH (Linglart et al. 2011; Linglart et al. 2012), two hormones that activate adenylyl cyclase through GPCRs. However, patients with PDE4D mutations do not demonstrate resistance to these hormones (Linglart et al. 2011; Linglart et al. 2012), consistent with the gene defect being in a different portion (PDE4D v PKA) of the cAMP signaling pathway. 1

Of considerable interest to neurobiologists is that most patients with acrodysostosis and PDE4D mutations have significant mental retardation (Lee et al. 2012; Linglart et al. 2012; Michot et al. 2012; Lindstrand et al. 2014; Lynch et al. 2013); this is not typically seen in patients with acrodysostosis and PRKAR1A mutations, although some of those individuals have behavioral disorders. The presence of intellectual disorders in PDE4D acrodysostosis patients has led a number of investigators to test for PDE4D mutations in a broader population of patients with mental retardation and skeletal abnormalities. These efforts have led to the recent study of a mirror phenotype, involving intellectual disability and skeletal abnormalities different from acrodysostosis; genetic testing revealed PDE4D haploinsufficiency in these patients (Lindstrand et al. 2014). It seems quite likely that additional PDE4D-mutant syndromes affecting the CNS will be identified in the near future. 2

It is of considerable interest to compare the CNS phenotypes in the acrodysostosis patients to those seen in the PDE4D knockout mice. It is clear that the human phenotype is considerably more severe and affects multiple aspects of cognition and memory. Whether this reflects a purely species difference, or a different mutation mechanism (the acrodysostosis mutants may have a dominant-negative effect, as discussed below) is uncertain. There are no murine models of the acrodysostosis mutations; the CNS phenotype of the rat PDE4D−/− rat, which has skeletal abnormalities reminiscent of acrodysostosis, has yet to be published (Kaname et al. 2014). 3

4.12 Dimerization and the PDE4D Acrodysostosis Mutations 4

The structural data on the PDE4 dimer also provide great insight into the possible functional effects of PDE4D mutations that have been implicated in acrodysostosis. Of the 16 different single amino acid acrodysostosis mutations that have been identified to date, 15 map to the interface between UCR1/2 and the catalytic domain, or 5

to the "hinge" region connecting the dimerization domain to UCR1/2 and the catalytic domains (Cedervall et al. 2015). The 16th acrodysostosis mutation is at S133, the PKA catalytic site (Lindstrand et al. 2014); note that this and other genetic references use GenBank NM_001104631.1 for the mutation co-ordinates, with S133 in PDE4D5 being S190 in the GenBank entry. 1

The structural model may provide insight into the profound disability seen in patients with acrodysostosis mutations. Given that one of the acrodysostosis mutations is at the PKA phosphorylation site and completely blocks PKA phosphorylation of long PDE4D isoforms, and that all the PDE4D acrodysostosis mutations have a similar phenotype, it is quite possible that all PDE4D acrodysostosis mutations serve to inhibit PKA-mediated activation of PDE4D enzymatic activity, or lower PDE4D enzymatic activity in other ways (Kaname et al. 2014). Therefore, cAMP levels would be elevated in PDE4D acrodysostosis cells, activating PKA activity at its substrates and producing a potentially broad range of phenotypes. Consistent with this model is the observation of compensatory activation of other PDE4 isoforms (e.g., PDE4A and PDE4B) in acrodysostosis cells (Kaname et al. 2014). 2

Since acrodysostosis mutations lower PDE4D enzymatic activity, which is also the pharmacologic effect of rolipram and other PDE4-selective inhibitors, the severe bone and CNS manifestations of acrodysostosis provide a rationale for caution in the human use of PDE4-selective inhibitors. It is possible that disorders of bone (or of the CNS) might be unanticipated side effects of PDE4 inhibition. To date, bone abnormalities (e.g., osteoporosis) have not been reported as a side effect of adult use of PDE4 inhibitors, however, exposure earlier in development (e.g., in utero) might produce profound skeletal development effects and remains a legitimate concern. 3

4.13 Conclusion: Implications for Future PDE4 CNS Drug Development 4

There are now grounds for reasonable optimism for the development of PDE4-selective inhibitors that would target the CNS and thereby be potentially useful in the treatment of depression and other affective disorders, psychosis, cognitive and memory dysfunction, addictive disorders, and possibly other conditions. I propose that such agents should target the long PDE4 isoforms preferentially. Expression studies show that long forms are preferentially expressed in the CNS. Furthermore, only the long isoforms are capable of dimerization, with the corresponding change in enzymatic properties and the formation of a HARBS. Indeed, it is generally agreed that CNS tissues are enriched in HARBS (Rocque et al. 1997a; Rocque et al. 1997b; Souness and Rao 1997; Zhang et al. 2006; Zhao et al. 2003a; Zhao et al. 2003b). To be effective therapeutically, these new inhibitors would also need to have low emetic potential; given our lack of knowledge of the exact targets for PDE4 inhibitors in the area postrema, this could remain a significant problem. 5

Finally, these PDE4 inhibitors would need to permeate the blood-brain barrier and have appropriate bioavailability. Despite these obstacles, there are grounds for optimism.

Conflict of Interest The author declares that he has no conflicts of interest.

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