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Clinical Relevance and Role of Neuronal AT_1 Receptors in ADAM17-Mediated ACE2 Shedding in Neurogenic Hypertension

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Abstract

Rationale—Neurogenic hypertension is characterized by an increase in sympathetic activity and often resistance to drug treatments. We previously reported that it is also associated with a reduction of Angiotensin Converting Enzyme 2 (ACE2) and an increase in A Disintegrin And Metalloprotease 17 (ADAM17) activity in experimental hypertension. In addition, while multiple cells within the central nervous system have been involved in the development of neurogenic hypertension, the contribution of ADAM17 has not been investigated.

Objective—To assess the clinical relevance of this ADAM17-mediated ACE2 shedding in hypertensive patients and further identify the cell types and signaling pathways involved in this process.

Methods and Results—Using a mass spectrometry-based assay, we identified ACE2 as the main enzyme converting Ang II into Ang-(1–7) in human cerebrospinal fluid (CSF). We also observed an increase in ACE2 activity in the CSF of hypertensive patients, which was correlated

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DISCLOSURES

None.

AUTHOR CONTRIBUTIONS

JX, SS, HX, MP and EL designed experiments. JX, SS, HX, and OD performed experiments. LMW and FC contributed patient samples. JX, MP and EL wrote the manuscript. All authors contributed to manuscript revision.

with systolic blood pressure. Moreover, the increased level of tumor necrosis factor (TNF)-α in those CSF samples confirmed that ADAM17 was up-regulated in the hypertensive patients' brain. To further assess the interaction between brain renin-angiotensin system and ADAM17, we generated mice lacking Angiotensin II type 1 receptors (AT_1R) specifically on neurons. Our data reveal that despite expression on astrocytes and other cells types in the brain, ADAM17 up-regulation during DOCA-salt hypertension occurs selectively on neurons and neuronal AT_1R are indispensable to this process. Mechanistically, reactive oxygen species (ROS) and extracellular signal-regulated kinase (ERK) were found to mediate ADAM17 activation.

Conclusions—Our data demonstrate that AT_1R promote ADAM17-mediated ACE2 shedding in the brain of hypertensive patients, leading to a loss in compensatory activity during neurogenic hypertension.

Keywords

Renin-angiotensin system; hypertension; ACE2; TNF-alpha convertase enzyme; central nervous system

Subject Terms

ACE/Angiotensin Receptors/Renin Angiotensin System; Autonomic Nervous System; Hypertension; Cell Signaling/Signal Transduction

INTRODUCTION

The brain renin-angiotensin system (RAS) plays a critical role in the development of neurogenic hypertension. Angiotensin (Ang) II, the main peptide in this system, increases sympathetic outflow and blood pressure (BP), while decreasing baroreflex gain and vagal tone, by acting on brain Ang II type 1 receptors (AT_1R). In addition to this classic axis, compensatory mechanisms exist within the RAS. Angiotensin-converting enzyme type 2 (ACE2) was discovered almost 2 decades ago and has been established as a major component of the rescue mechanisms. ACE2 transforms Ang II into Ang-(1–7), thus turning the vasoconstrictor into a vasodilator peptide, promoting nitric oxide release, reducing sympathetic outflow, increasing baroreflex sensitivity and ultimately reducing high BP. Our group previously reported that ACE2 is expressed throughout the brain, including in regions involved in central regulation of BP, such as the subfornical organ (SFO), paraventricular nucleus of hypothalamus (PVN), nucleus of the solitary tract (NTS) and rostral ventrolateral medulla (RVLM).[1, 2] Regardless of the enzyme's compensatory effects, during neurogenic hypertension, brain expression and activity of ACE2 are found to be compromised.[3, 4] Previous work has shown that central ACE2 is down-regulated in several experimental hypertension models[5, 6] and ACE2 gene therapy can lead to reduced sympathetic drive and improved baroreflex sensitivity, contributing to a reduction of BP in those models. Despite these observations, the mechanisms leading to ACE2 down-regulation in hypertension remain unknown.

ADAM17, a member of the 'A Disintegrin And Metalloproteases' (ADAM) family, is known to cleave a variety of membrane-anchored proteins.[7] We previously demonstrated an

ADAM17-mediated mechanism responsible for the impairment of ACE2 compensatory function in the mouse hypothalamus during neurogenic hypertension.[5] Accordingly, upon brain RAS over-activation, ADAM17 is up-regulated, leading to an increased ectodomain shedding of ACE2. As a result, the balance between classic and compensatory axes of the RAS is compromised, favoring the development of neurogenic hypertension. In our previous study, we firstly associated ADAM17-mediated shedding to the loss of membrane-bound ACE2 and increase in the shed form of ACE2 (sACE2).[5] However, there has been no study investigating the existence of this potentially deleterious mechanism in the central nervous system (CNS) of hypertensive patients, or how it is regulated during hypertension development.

In this study, we investigated the correlation between ACE2 shedding and hypertension using cerebrospinal fluid (CSF) collected from normotensive and hypertensive patients. Since intracerebroventricular (icv) losartan blocked ADAM17 up-regulation,[5] central AT_1R were identified as critical players in ADAM17-mediated ACE2 shedding. However, AT_1R are expressed on various cell types in the brain, including neurons, microglia and astrocytes, which have been shown to contribute to neurogenic hypertension.[8–10] To further investigate the relationship between brain RAS and ADAM17 activation, we used the deoxycorticosterone acetate (DOCA)-salt model, which promotes RAS activation specifically in the brain, to test the hypothesis that neuronal $AT_{1a}R$ are essential for ADAM17-mediated ACE2 shedding in neurogenic hypertension.[5, 11]

In patients, we observed an increase in sACE2, indicating increased ACE2 ectodomain shedding in hypertensive individuals. This rise in sACE2 was significantly correlated with elevated systolic BP and normalized by BP medications. Using transgenic mice with neuron-specific $AT_{1a}R$ deficiency, our data provide strong evidence that neuronal $AT_{1a}R$ are required for central ADAM17 up-regulation during the development of DOCA-salt hypertension, in addition to its important roles in the regulation of sympathetic outflow and BP. Furthermore, we showed that signaling molecules downstream of $AT_{1a}R$, such as reactive oxygen species (ROS) and extracellular signal-regulated kinase (ERK), play a critical part in mediating the $AT_{1a}R$-driven increase in ADAM17 activity in neurons.

METHODS

A detailed Methods section is available in the online supplemental material.

Patients' cerebrospinal fluid (CSF) samples

Excess of CSF collected for diagnostic purposes was obtained from 27 patients undergoing neurosurgery or seeking neurological treatment at the LSU Emergency department. All procedures were approved by the LSU Health Sciences Center-NO Institutional Review Board committee (#7832) and the Institutional Biosafety Committee (#12086).

Transgenic mice and animal husbandry

Experiments were performed in adult (14–16 weeks old, 25–30 g) male neuronal $AT_{1a}R$ knock-down mice (AT1N) and their control littermates. All procedures were approved by the LSU Health Sciences Center-NO Animal Care and Use Committee (#3112) and are in

agreement with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Statistics

Data are presented as means ±SEM. Data were analyzed by repeated measures ANOVA, or two-way ANOVA, followed by Bonferroni post-hoc test for multiple comparisons between means, as appropriate. Correlations were analyzed using a standard (Pearson) correlation test. Statistical analyses were performed using Prism5 (GraphPad Software). Differences were considered statistically significant at $P<0.05$.

RESULTS

ACE2 shedding is elevated in the brain of hypertensive patients

We previously reported that neurogenic hypertension is associated with a reduction of ACE2 activity and an increase in ADAM17 activity in the mouse hypothalamus.[5] To address the relevance of ADAM17-mediated ACE2 shedding in humans and its potential link with neurogenic hypertension, sACE2 levels were assessed in the CSF of 27 patients (Online Table II), including 10 males (37 ±4 years) and 17 females (39 ±2 years). To first determine the assay's specificity, CSF samples from different patients were pooled together and incubated in the presence of increasing doses of recombinant human ACE2 and the ACE2 inhibitor MLN-4760. The data show a dose-dependent increase in Ang-(1–7) formation in this standard addition experiment, confirming assay specificity, as well as the efficiency of the antagonist to block the reaction (Figure 1A). To further validate the assay, random CSF samples were selected and pre-treated with MLN-4760. Results show a dramatic reduction in their ability to form Ang-(1–7) from the spiked Ang II (Figure 1B), establishing sACE2 as the main enzyme converting Ang II into Ang-(1–7) in human CSF. When comparing the different patient groups, our data reveal that sACE2 activity was more than doubled in the CSF of individuals with uncontrolled hypertension (systolic BP: 159 ±7 mmHg) compared to normotensive patients (systolic BP: 113 ±3 mmHg; $P<0.05$; Figure 1C). Patients with controlled BP (systolic BP: 125 ±4 mmHg) exhibited a normalization of sACE2 activity ($P<0.05$; Figure 1C). Together, these data suggest that ADAM17-mediated ACE2 shedding is taking place in the CNS during human hypertension. To further confirm that the activity of ADAM17 is indeed increased in these patients, TNFα levels were assessed. This cytokine showed exactly the same profile as sACE2, with a 2-fold increase in hypertension and a return to baseline in patients with controlled BP (Figure 1D). A significant positive correlation (P=0.012) was also established between sACE2 and systolic BP levels in these patients (Figure 1E).

Neuron-targeted deletion of $AT_{1a}R$ reduces DOCA-salt-induced hypertension and dysautonomia

In our previous study, icv losartan infusion in DOCA-salt-treated mice significantly attenuated both BP rise and increased ADAM17 expression,[5] indicating the pivotal role of $AT_{1a}R$ in ADAM17 up-regulation during RAS overactivity-related neurogenic hypertension. However, in the CNS, $AT_{1a}R$ and ADAM17 are co-localized in various cell types that have all been shown to be involved in BP regulation. Therefore, we hypothesized that neuronal

$AT_{1a}R$ play a major role in the regulation of ADAM17 activity during DOCA-salt treatment and the ensuing development of neurogenic hypertension. To assess the contribution of neuronal $AT_{1a}R$, a new neuronal knockout mouse (AT1N) was generated, in which cre-recombinase expression is driven by the Nefh promoter (Figure 2A–B).[12] Using primary neurons cultures, specific cre expression to neurons was verified by double-labeling with MAP2 (Figure 2C). Quantitative RT-PCR performed with these cells confirmed a significant reduction of $AT_{1a}R$ mRNA in AT1N mice compared to controls (Figure 2D), thus validating the knockdown of $AT_{1a}R$ in these mice neurons.

DOCA-salt, a well-accepted neurogenic hypertension model,[11] was previously reported by us to up-regulate ADAM17 through RAS over-activation.[5] Before the DOCA-salt paradigm, BP and HR were not significantly different between AT1N mice and their control littermates, suggesting that neuronal $AT_{1a}R$ are not involved in the maintenance of baseline BP and HR (Figure 3A–C). However, daily BP recording (Figure 3A), uncovered major differences between these 2 genotypes upon DOCA-salt treatment. Indeed, while BP progressively increased in control littermates and rose by ~30 mmHg, it reached a plateau at a lower level in AT1N mice (Figure 3A). The BP traces difference started as early as the first day post DOCA-salt and remained until the last day of treatment, where BP in AT1N mice was significantly lower than in controls (21 ±3 *vs.* 34 ±4 mmHg; $P<0.001$; Figure 3B). Interestingly, despite a smaller rise of BP in AT1N, the bradycardic response was similar between the 2 DOCA-salt-treated groups (Figure 3C), suggesting that baroreflex and/or autonomic function might be altered following $AT_{1a}R$ knockdown. Indeed, the impaired baroreflex sensitivity induced by DOCA-salt was significantly attenuated in AT1N mice (Figure 3D). Analysis of autonomic function in control mice showed a typical increase in both cardiac (Figure 3E) and vascular (Figure 3F) sympathetic activities as well as a reduction of vagal tone (Figure 3G), which contribute to the maintenance of hypertension in DOCA-salt-treated animals ($*P<0.05$ and $**P<0.01$ *vs.* control). The similarity of intrinsic HR between groups, following ganglionic blockade (Figure 3H), suggests that these changes are related to autonomic function and not cardiac function. Deletion of $AT_{1a}R$ was not associated with baseline changes in autonomic function. However, the dysautonomia observed in the control+DOCA group, was prevented in AT1N mice as neither sympathetic drive nor vagal tone were altered following DOCA-salt treatment in these animals.

Control+DOCA mice exhibited a ~50% left ventricular (LV) mass increase (Figure 3I), associated with early signs of heart failure, as evidenced by reduced ejection fraction and fractional shortening (Online Figure I: A–C) and NE release, an index for sympathetic activity, was dramatically increased in these animals (Figure 3J). Interestingly, AT1N exhibited no sign of altered cardiac function while subjected to the DOCA-salt paradigm. Notably, LV mass was not increased (Figure 3I) despite identically-increased fluid intake and urine output (Online Figure I: D–E), suggesting similar blood volumes between control and AT1N mice under DOCA-salt treatment. On the other hand, despite remaining elevated, NE excretion was reduced by more than 50% in AT1N treated with DOCA-salt (Figure 3J). Taken together, these data highlight the critical role of brain $AT_{1a}R$ in promoting cardiac dysfunction through enhanced NE release in DOCA-salt hypertension.

Neuronal $AT_{1a}R$ are required for ADAM17 activation during DOCA-salt hypertension

Following DOCA-salt or sham treatment, the hypothalamus was collected to assess ADAM17 and ACE2 enzymatic activities. In control mice, ADAM17 activity was enhanced by ~2-fold in the hypothalamus after DOCA-salt treatment ($P<0.01$ *vs.* control sham; Figure 4A). As a result, both ACE2 activity and expression were attenuated by ~25% ($P<0.05$ *vs.* control sham; Figure 4B–C), confirming the opposite relationship between ADAM17 and ACE2. AT_2R gene expression was reduced as well in hypertensive animals (Figure 4D). Interestingly, $AT_{1a}R$ deletion on neurons did not affect baseline ADAM17 activity and had no impact on ACE2 expression or activity in the brain, suggesting that $AT_{1a}R$ do not regulate these enzymes in normal conditions. Surprisingly, in AT1N+DOCA mice, the enhanced ADAM17 activity, observed in control+DOCA animals, was totally blunted (Figure 4A), leading to the preservation of ACE2 expression and activity, as well as AT_2R gene expression (Figure 4B–D), in the hypothalamus of these mice. These data suggest that while constitutive ADAM17 enzymatic activity does not rely on the presence of neuronal $AT_{1a}R$, the DOCA-salt hypertension-mediated rise in its shedding activity requires $AT_{1a}R$ expression in neurons. To determine the specificity of this mechanism, ADAM17 activity was also assessed in the heart and kidney. Interestingly, DOCA-salt treatment failed to alter total ADAM17 activity in the kidney (Figure 4F) but produced a significant increase in the cardiac LV which was also prevented by $AT_{1a}R$ knockdown in neurons (Figure 4E).

Hypertension is not sufficient for ADAM17 activation in the brain

To further determine whether the lack of ADAM17 activation in AT1N mice is related to the absence of $AT_{1a}R$ on neurons or the reduction of BP in these animals, we tested whether brain ADAM17 activation could result from an elevated BP, independently of RAS activation. Wild type (WT) mice were infused with NE, a peripherally acting vasoconstrictor, at a dose previously reported to produce a sustained hypertension.[13] The 14-day infusion of NE drove significant cardiac and renal hypertrophy (Figure 5A–B), supporting a BP increase after NE treatment in these mice. Unlike DOCA-salt treatment, compared to the saline-infused normotensive mice, there was no significant increase in ADAM17 activity in the hypothalamus of the NE-treated mice (Figure 5C). As a result, hypothalamic ACE2 activity in those mice remained intact following NE infusion (Figure 5D). These observations demonstrate that ADAM17 activation during DOCA-salt hypertension, is mediated by neuronal $AT_{1a}R$ rather than increased BP. Interestingly, ADAM17 activation was increased in the LV of NE-infused mice (Figure 5E), suggesting that NE leads to enhanced ADAM17 activity as a result of cardiac hypertrophy, as reported previously.[14] Together these data indicate that neuronal $AT_{1a}R$ play an essential role in the ADAM17-mediated ACE2 ectodomain shedding following brain RAS over-activation, ultimately contributing to the development of neurogenic hypertension.

Neuronal ADAM17 is selectively up-regulated by $AT_{1a}R$ in DOCA-salt hypertension

To better understand the relationship between neuronal $AT_{1a}R$ and ADAM17, a flow cytometry protocol was developed to sort different types of hypothalamic cells from DOCA-salt-treated mice. Following labeling with NeuN-conjugated Alexa Fluor 488 and GFAP-conjugated Phycoerythrin (PE) antibodies, hypothalamic cells were sorted into neuronal,

astrocyte and non-neuronal/non-astrocyte (NNNA) populations (Figure 6A). ADAM17 gene expression was easily detected among the various cell populations but only neuronal ADAM17 was up-regulated after DOCA-salt treatment (Figure 6B, $P<0.05$). A trend to increased ADAM17 expression in NNNA population, however did not reach statistical significance ($P=0.19$). At baseline, there was no difference in ADAM17 gene expression between control and AT1N mice, indicating again that ADAM17 expression in the CNS is not dependent on the presence of neuronal $AT_{1a}R$. Consistent with previous results showing a lack of increase in ADAM17 activity in AT1N+DOCA mice, the increased neuronal ADAM17 gene expression was prevented in these animals and none of the other cell populations showed an increase in ADAM17 mRNA levels (Figure 6B). To confirm the FACS data, primary neurons cultured from control and AT1N mice were exposed to Ang II (100 nM). After 2 h, ADAM17 gene expression was significantly elevated by Ang II treatment in control mice (**$P<0.01$ *vs.* control+vehicle) but not in AT1N ($P=0.16$; Figure 6C). Interestingly, we observed that while neuronal TNFα mRNA was unaffected, it increased in control+DOCA mice in NNNA cells and this was prevented in animals lacking neuronal $AT_{1a}R$ (Figure 6D). These data suggest that while neurons are not the main source for TNFα, they might have the ability to regulate this cytokine expression on other cell types.

Oxidative stress and ERK/MAPK are involved in $AT_{1a}R$-mediated ADAM17 activation

Since basal ADAM17 levels were not affected by neuron-targeted $AT_{1a}R$ knockdown, signals that up-regulate ADAM17 during DOCA-salt treatment are thought to be downstream of $AT_{1a}R$ activation. Among those, ERK/MAPK activation[15–17] and oxidative stress[18] have been shown to contribute to ADAM17 activation. In control mice, DOCA-salt treatment led to a 2-fold increase in phosphorylated ERK in the hypothalamus, that was totally blunted in mice lacking neuronal $AT_{1a}R$ (Figure 7A–B). A rise in oxidative stress was also observed in control+DOCA mice, as evidenced by enhanced Nox4 protein expression in the hypothalamus (Figure 7C) and a dramatic increase in DHE staining, an index of ROS production, in the PVN (Figure 7D–E). Importantly, neither Nox4 expression nor ROS production were affected by DOCA-salt treatment in AT1N mice (Figure 7C–E), confirming that these signaling molecules are regulated by $AT_{1a}R$ in DOCA-salt hypertension. To confirm the involvement of ERK/MAPK and oxidative stress in neuronal ADAM17 activation, primary hypothalamic neurons were exposed to Ang II in the presence or absence of U0126, a MEK inhibitor (used to prevent ERK phosphorylation), SB203580 (a p38-MAPK inhibitor) and diphenyleneiodonium (DPI), a NADPH oxidase inhibitor. As shown by us previously in neuronal cell lines,[18] ADAM17 activity was significantly up-regulated by Ang II treatment (Figure 7F), and this effect was completely blocked by pre-treatment with U0126, SB203580, or DPI. Taken together, our data provide strong evidence that neuronal $AT_{1a}R$ mediate ADAM17 activation through its ERK/MAPK and oxidative stress downstream signals, which are also known to be up-regulated during RAS over-activation.

DISCUSSION

Although ADAM17 (*aka* TNFα convertase) was originally discovered as a key sheddase in the formation of cytokines, such as TNFα, its wide spectrum of substrates [19] also highlights

its important role, beyond inflammation, in the CNS and cardiovascular system.[7] An opposite relationship between ADAM17 and ACE2, a pivotal member of the compensatory RAS, was discovered by the observation of increased sACE2 levels in the culture medium of cells transfected with ADAM17.[20] ACE2 is a cell-surface-bound enzyme, with its catalytic site exposed to the extracellular surface,[21] therefore, ADAM17-mediated ectodomain shedding might compromise the RAS compensatory axis by impairing ACE2 enzymatic activity, or its ability to process Ang II on the cell surface. By demonstrating this opposite relationship in the hypothalamus, our previous study was first to point out the contributory role of ADAM17 in the development of neurogenic hypertension.[5] However, in the absence of data in humans, the relevance and potential therapeutic implications of this process in the CNS remained unknown.

Despite the numerous peptidases involved in the formation of Ang-(1–7),[22] our data suggest that ACE2 appears to be the main enzyme hydrolyzing Ang II into Ang-(1–7) in human CSF (Figure 1B), therefore preservation of ACE2 enzymatic activity is critical to the compensatory RAS in the CNS. Among the mechanisms leading to impaired ACE2 activity in hypertension, we previously reported ADAM17-mediated shedding and AT_1R-dependent internalization.[5, 6] While there are no data available for the latter, in humans, elevated sACE2 has been reported in the periphery in heart failure,[23, 24] hypertension,[24] severe acute respiratory syndrome[25] and Type 1 diabetes.[26] Here, we provide evidence of increased sACE2 in CSF, correlated with elevated systolic BP, in hypertensive patients. Despite a lack of direct evidence, the similarity between sACE2 and TNFα profiles (Figure 1C, D) in the CSF of these patients, points to ADAM17-mediated shedding. In addition, the normalization of sACE2 levels in patients with controlled BP also supports the link between overactive RAS, enhanced ADAM17 activity and elevated sympathetic drive since these individuals were taking a RAS blocker or a sympatholytic (Online Table II) in combination with a diuretic and/or calcium channel blocker. Therefore, it is conceivable that these drugs might have directly or indirectly reduced brain RAS activity, thwarting ADAM17 activation and ultimately contributed to the preservation of ACE2 on the cell surface. This hypothesis is based on the assumption that sACE2 is less efficient at converting Ang II into Ang-(1–7), than the membrane-bound enzyme. This reduction in enzymatic efficiency might due to decreased local metabolism or changed structure. Whether sACE2 has therapeutic benefits or is merely a degradation product resulting from RAS over-activation, remains controversial.

To further clarify the mechanisms involved in ADAM17-mediated ACE2 shedding, we took advantage of the DOCA-salt hypertension model, which was reported to have a strong neurogenic component and typical features including salt-sensitivity, low plasma renin levels and an overactive brain RAS.[11] As the main mediator of brain RAS over-activity, the $AT_{1a}R$ was previously demonstrated to prompt ADAM17 up-regulation both in vitro [18] and in vivo.[5] Moreover, in recent years cell-specific expression of $AT_{1a}R$, in the CNS, has been shown to contribute to neurogenic hypertension[10, 27, 28] and other ailments.[29] Since $AT_{1a}R$ and ADAM17 co-exist on various cell types, including endothelial cells,[30] vascular smooth muscle cells,[31] glial cells and neurons,[32] the contribution of cell-specific ADAM17 activation to the development of hypertension must be determined to insure selective targeting. To address the contribution of neuronal $AT_{1a}R$ to ADAM17 activation, we

generated a cell-specific knockout mouse by taking advantage of the neuron-specific Nefh promoter. Nefh is expressed throughout the brain, including in the hypothalamus, pons and medulla, which contain the majority of nuclei involved in BP regulation.[12] Accordingly, most of $AT_{1a}R$ neuronal expression is thought to be knocked-down in AT1N mice.

To the best of our knowledge, this study provides the first evidence that ADAM17 activation in the CNS, as a result of DOCA-salt treatment, is entirely dependent on neuronal $AT_{1a}R$ (Figure 5A). In addition, it is not secondary to hypertension since NE infusion, by which BP can be increased to ~160 mmHg,[13] failed to increase the sheddase activity in the brain (Figure 5C), while it did promote activation in the heart, as reported previously.[14] Importantly, this new mechanism is dependent upon brain RAS over-activation, as $AT_{1a}R$ deletion did not alter baseline ADAM17 activity, and it is restricted to neurons. In vitro studies have shown that caveolin-1, a membrane lipid raft marker, co-immunoprecipitates with AT_1R upon agonist stimulation and these rafts play a critical role for AT_1R-mediated signal transduction.[33, 34] Altogether, it is conceivable that upon RAS over-activation, $AT_{1a}R$ could move to a closer location, from which its downstream signals can affect ADAM17 in a more efficient way.

Upon acute stimulation, intracellular signals resulting from GPCR activation or oxidative stress can activate ADAM17 within a few minutes via phosphorylation of its cytoplasmic domain[17] or up-regulate ADAM17 availability through increasing its translocation and expression. In addition to its critical role in the migration of $AT_{1a}R$ into membrane micro-domains,[35] ROS are actually important to Golgi apparatus-mediated ADAM17 transport, which requires $p47^{phox}$.[36] In chronic states, such as DOCA-salt treatment, the expression of ADAM17 could be up-regulated through the activation of transcription factors, such as CREB and NFκB, which are downstream targets of ROS/ERK/MAPK-mediated signaling pathways, as confirmed by our data (Figure 7F).

Besides the brain, ADAM17 is also expressed in end organs of neurogenic hypertension, including heart and kidneys. In the heart, systemic Ang II infusion increases ADAM17 activity and ADAM17-mediated ACE2 shedding in mouse left ventricle, accompanied with up-regulated cardiac hypertrophy, which can be blocked by deletion of ADAM17 independently of decreasing BP.[14, 36] In the kidney, ADAM17-mediated ACE2 down-regulation has also been found to be altered in mouse and human renal diseases.[37, 38] In this study, we observed an up-regulation of ADAM17 activity in the mouse heart after DOCA-salt treatment presumably driven by increased NE level during hypertension (Figure 4 and 5). Although we did not see any significant change in overall renal ADAM17 activity, ACE2 shedding from tubular and glomerular epithelial cells, may still play a role in the alteration of renal function during DOCA-salt treatment.

While our data certainly highlight an essential role for neurons and position them upstream among the various contributors to ADAM17-mediated neurogenic hypertension, consideration should be given to other CNS cells, as all showed robust levels of ADAM17 mRNA. With regards to astrocytes, despite recent data suggesting a contribution to Ang II-mediated sympathetic outflow [28], the lack of detectable $AT_{1a}R$ mRNA (Figure 6E) and failure of DOCA-salt to increase ADAM17 gene expression in these cells suggest that their

role might be merely limited to the synthesis of angiotensinogen (Figure 6B). The situation is less clear for other cell types due to FACS resolution. The NNNA population which includes mostly microglia and vascular cells also showed a significant level of ADAM17 mRNA and a trend to up-regulation upon DOCA-salt treatment. Although $AT_{1a}R$ gene expression was undetectable in these cells, likely due to its over-abundance in neurons, the rise of TNFα, and maybe ADAM17, mRNA following DOCA-salt treatment highlights a partnership between neuronal $AT_{1a}R$ and these cells. The contribution of ADAM17 in the CNS to hypertension is not necessarily identical between cells and likely depends on its substrates and their location. In VSMC, ADAM17-mediated HB-EGF up-regulation is one of the mechanism of vascular remodeling,[39] which may contribute to the pathological process in late stage hypertension and increase the possibility of cerebrovascular accidents. ADAM17 is also the major source of soluble (*i.e.* active) TNFα in vascular and glial cells, therefore in DOCA-salt hypertension, the sheddase is poised to play a key role in the process of neuro-inflammation, most likely from microglia,[40] which can be activated by Ang II and infiltrating immune cells.[41] The cell-specificity of ADAM17 is further confirmed by the lack of TNFα mRNA up-regulation in neurons following DOCA-salt treatment while there was almost a 2-fold increase in NNNA cells. Interestingly, the rise of TNFα mRNA in NNNA cells was blunted in mice lacking neuronal $AT_{1a}R$ and treated with DOCA-salt (Figure 6D). These data clearly suggest a cross-talk between neurons and these other cells. Microparticles, capable of carrying mRNA as well as receptors, can be formed by Ang II and induce membrane trafficking in hypothalamic cells.[42, 43] While not the focus of this study, their involvement could be an attractive hypothesis to explain $AT_{1a}R$-ADAM17 cooperation between neurons and TNFα-producing cells in hypertension.

Limitations

Though we provide strong evidence that AT_1R promote ADAM17-mediated ACE2 shedding in the development of neurogenic hypertension, there are some limitations in our study regarding to the experimental methods we chose. 1) While DPI is wildly used as an inhibitor of flavoenzymes and often accepted as evidence of NADPH oxidase inhibition, it is a non-specific inhibitor that binds strongly to flavoproteins, including nitric oxide synthase, NADPH-ubiquinone oxidoreductase, NADPH oxidases and NADPH cytochrome P450 oxido-reductase.[44, 45] It is well documented that Ang II-associated ROS production is not limited to NOX,[46] therefore using DPI is an effective choice to block more sources of ROS, but on the other hand, the lack of specificity prevented from a clear identification of the signaling molecules involved in the regulation of ADAM17 activity, during Ang II treatment. 2) In this study we used DHE staining to detect DOCA-salt-induced ROS production in the PVN. Because of its ability to passively diffuse into cells and its high reactivity, DHE has been used to detect cytosolic superoxide. However, there are other products generated by DHE. Apart from superoxide-mediated oxidation to 2-hydroxyethidium, DHE can also undergo unspecific oxidation via $ONOO^-$ or $^{\bullet}OH$ into ethidium.[47] Since the difference in their fluorescence spectra is small, analytical methods, like HPLC and mass spectrometry, should be applied to verify the specific superoxide signals.[48] EPR (electron paramagnetic resonance) spectroscopy can also be used for ROS detection.[49] 3) The present data highlight significant variability within our ADAM17 activity assay, so it is not suitable for comparisons between experiments. When using this method, it

is imperative to include all experimental groups on the same assay and apply positive (ADAM17 overexpressing cell lysate) and negative (lysis buffer) controls to validate its efficiency. 4) In the DOCA-salt hypertensive mice, we saw significant hypertrophy in heart and kidney, both of which are important organs in the development of hypertension, and we assessed ADAM17 in these tissues. Although Salem *et al.*,[50] previously reported that renal ADAM17 expression was increased in whole kidney lysates, highlighting that total renal ADAM17 could be changed during the pathological process, it is more informative to perform such assays in dissected tissues or regions (*e.g.* cortex *vs.* medulla) as these tissues/regions contain multiple cell types with often opposite roles.

In summary, the present study describes a novel mechanism taking place in the CNS of hypertensive patients, by which ADAM17 cleaves ACE2 from the cell surface, potentially leading to the loss of compensatory activity and contributing to neurogenic hypertension. Neuronal $AT_{1a}R$ were further shown to be required for this process and for the formation of TNFα from other cells. We postulate that an important role for neuronal ADAM17-mediated ectodomain shedding is to unbalance the relationship between the classic RAS and its compensatory axis, thus increasing sympathetic activity in neurogenic hypertension.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Nonstandard Abbreviations and Acronyms

ACE2	angiotensin-converting enzyme 2
sACE2	shed form of ACE2
ADAM17	a disintegrin and metalloproteinase 17
AT_1R	angiotensin II type 1 receptor
Ang II	angiotensin II
Ang-(1–7)	angiotensin-(1–7)
BP	blood pressure
CNS	central nervous system

CSF	cerebrospinal fluid
DHE	dihydroethidium
DPI	diphenyleneiodonium
DOCA	deoxycorticosterone acetate
ERK	extracellular signal-regulated kinase
FACS	fluorescence-activated cell sorting
LV	left ventricle
MAPK	a mitogen-activated protein kinase
NE	norepinephrine
NNNA	non-neuronal/non-astrocyte
NOX	NADPH oxidase
RAS	renin-angiotensin system
ROS	reactive oxygen species
TNFα	tumor necrosis factor α
VSMC	vascular smooth muscle cell

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NOVELTY AND SIGNIFICANCE

What Is Known?

- Overactive brain renin-angiotensin system (RAS) is associated with decreased angiotensin-converting enzyme 2 (ACE2) level and activity in the brain during the development of neurogenic hypertension.
- ADAM17 is a member of the disintegrin and metalloproteinase family that has been shown to be up-regulated in the brain in deoxycorticosterone acetate (DOCA)-salt-induced hypertensive mice.

What New Information Does This Article Contribute?

- Ectodomain shedding of ACE2 takes place in the human brain and is up-regulated during hypertension, as indicated by increased ACE2 activity in cerebrospinal fluid (CSF) samples from human patients.
- The increased CSF TNFα (tumor necrosis factor α) level in those hypertensive patients further supports that central ADAM17 levels are up-regulated and highly likely mediate ACE2 shedding.
- Using a novel transgenic mouse model, which has Angiotensin II type 1 receptor (AT_1R) knocked-down in brain neurons, we demonstrate that AT_1R promote ADAM17 up-regulation selectively on neurons through increased expression and activity.

ACE2, an important link between classic RAS and its compensatory axis, is down-regulated in various animal models of cardiovascular disease. In the DOCA-salt mouse neurogenic hypertension model, reduced ACE2 activity in the central nervous system is mediated through ADAM17. In this study, we provide evidence that ADAM17-mediated ACE2 shedding is also present in the human brain and is up-regulated during the development of hypertension. Using a new transgenic mouse model, we show that neuronal cells play an important role in the development of neurogenic hypertension. AT_1R promote ADAM17 up-regulation selectively in neurons, through ROS (reactive oxygen species) and the ERK/MAPK signaling pathway. ADAM17-mediated ACE2 shedding is exacerbated in the brain of hypertensive patients and compromises the compensatory effect of Angiotensin-(1–7).

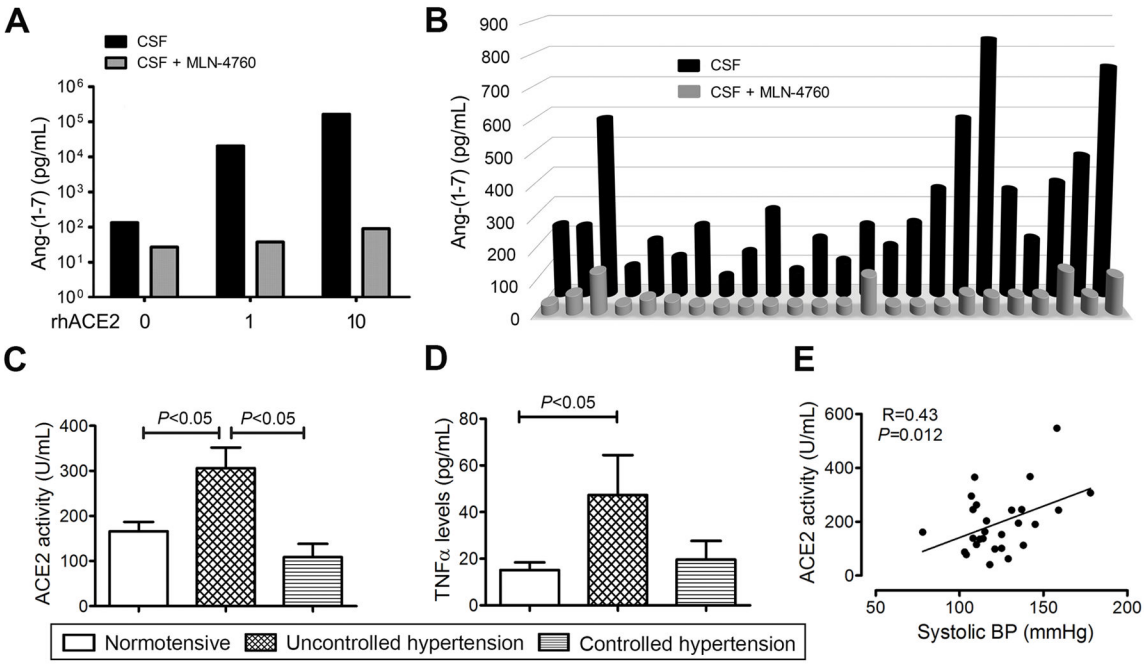

Figure 1. ACE2 shedding is increased in the brain of hypertensive patients

Cerebrospinal fluid (CSF) was obtained from 27 patients and processed for ACE2 activity. Specificity of the assay was validated using an ACE2 inhibitor (MLN-4760) in pooled CSF samples (**A**) while randomly selected samples (**B**) reveal the predominance of ACE2 as the main enzyme converting Ang II into Ang-(1–7) in humans CSF. Measurements of soluble ACE2 (sACE2) activity (**C**) and TNFα levels (**D**) between normotensive patients (n=17), hypertensive with uncontrolled blood pressure (BP) (n=7) and hypertensive with controlled BP (n=3) uncovered a 2- to 3-fold increase in both sACE2 activity and TNFα levels in uncontrolled hypertensive patients. BP medications significantly blunted sACE2 activity while only a trend was noted for TNFα levels (One-way ANOVA; $*P<0.05$). Data are shown as mean ±SEM. (**E**) A positive correlation (Pearson test) was detected between sACE2 activity and systolic BP in these patients.

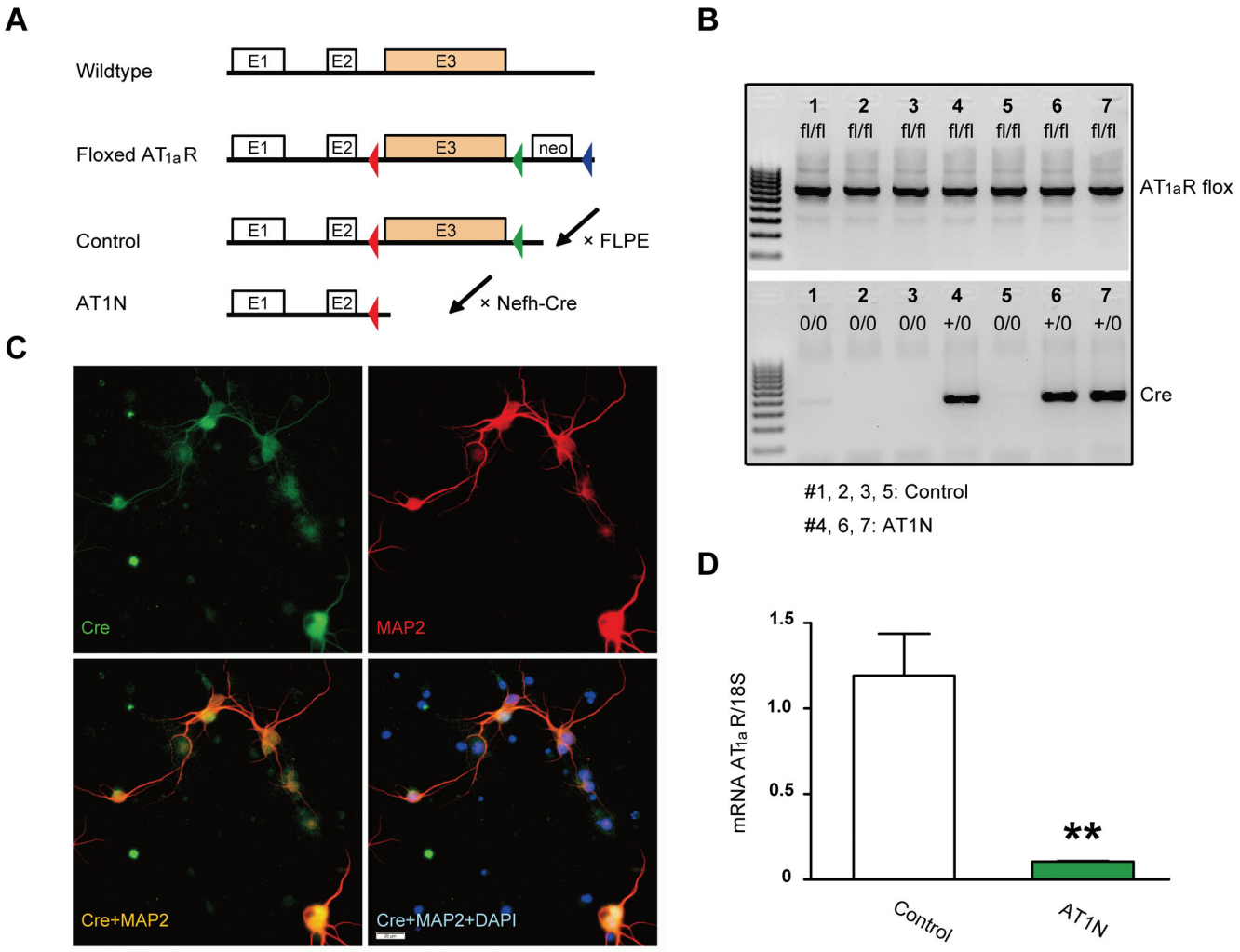

Figure 2. Genotyping and validation of $AT_{1a}R$ conditional knockout mice

(A) Schematic diagram showing the breeding strategy. Upon introduction of cre-recombinase (Nefh-cre), exon 3 of the $AT_{1a}R$ gene is excised specifically in neurons, allowing for the generation of the selective $AT_{1a}R$ knockout (AT1N) mice. **(B)** Representative genotyping results for AT1N and control littermates. Accordingly, pups #4, 6, and 7 were identified as AT1N: $AT_{1a}R^{fl/fl}$ with cre recombinase ($Cre^{+/0}$) while pups #1, 2, 3, and 5 were the control littermates: $AT_{1a}R^{fl/fl}$ without cre recombinase ($Cre^{0/0}$). **(C)** Immunofluorescence triple-labeling of primary cultured neurons from AT1N mice for visualization of Cre (green), MAP2 (red) and nuclei (DAPI, blue). The scale bar represents 20 μm. **(D)** Quantitative reverse transcription polymerase chain reaction (qRT-PCR) results showing that $AT_{1a}R$ expression is knocked-down in the cultured neurons from AT1N mice (Student's t-test: $^{**}P<0.01$ *vs.* control).

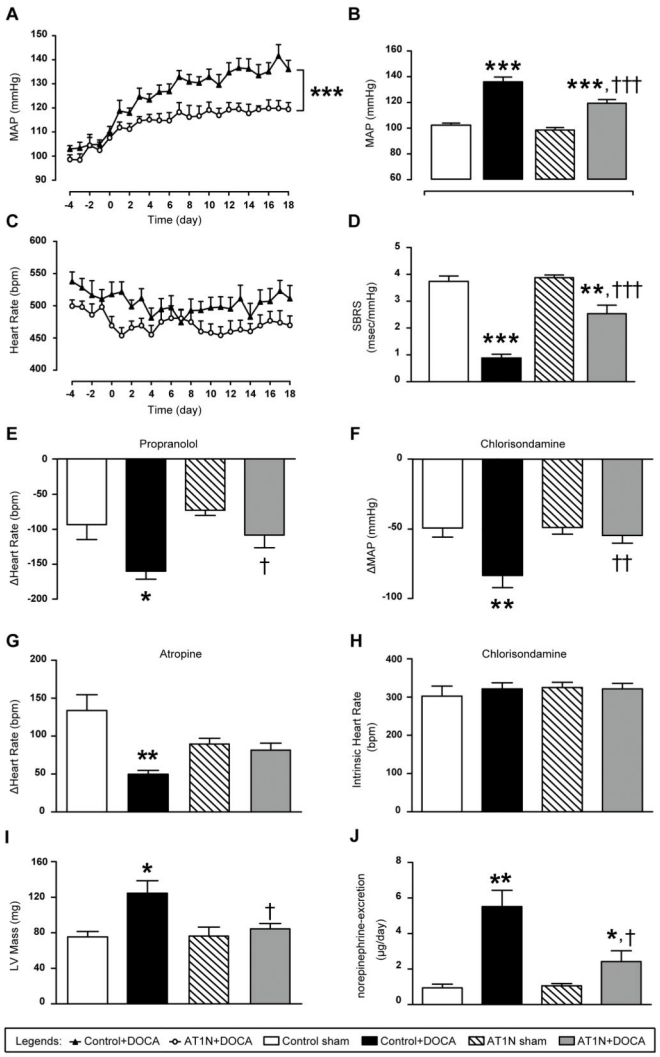

Figure 3. DOCA-salt-induced hypertension and dysautonomia are significantly attenuated in mice with neuronal $AT_{1a}R$ deficiency

(A) DOCA implanted subcutaneously (1 mg/g) and combined with 1% saline in the drinking water, induced a progressive increase of mean arterial pressure (MAP) in uninephrectomized control mice. This DOCA-salt-induced hypertension was blunted in AT1N mice (n=10/group). **(B)** Summary data for the MAP values before and after 18 days of DOCA-salt treatment. **(C)** A plot for daily heart rate, in which both control and AT1N mice were showing similar bradycardic response during DOCA-salt treatment. **(D)** After 18 days of DOCA-salt treatment, spontaneous baroreceptor reflex sensitivity (SBRS) was calculated using the sequence method. **(E–H)** Autonomic function was assessed pharmacologically by determining the changes in MAP (ΔMAP) and heart rate (ΔHeart Rate) after intraperitoneal injections of a β-blocker (**E**, propranolol, 4 mg/kg), a ganglionic blocker (**F** and **H**, chlorisondamine, 5 mg/kg), and a muscarinic antagonist (**G**, atropine, 1 mg/kg), n=6/group. (**I**) Left ventricle (LV) mass in DOCA-salt or sham-treated control and AT1N mice (n=6/sham group and n=9/DOCA-salt-treated group), measured via echocardiography. (**J**) Daily urinary norepinephrine excretion determined via ELISA (n=6 per group). Data are shown as

the mean ±SEM. Statistical significance: Two-way ANOVA: *P<0.05, **P<0.01, ***P<0.001 *vs.* respective shams; †P<0.05, ††P<0.01, †††P<0.001 *vs.* control+DOCA.

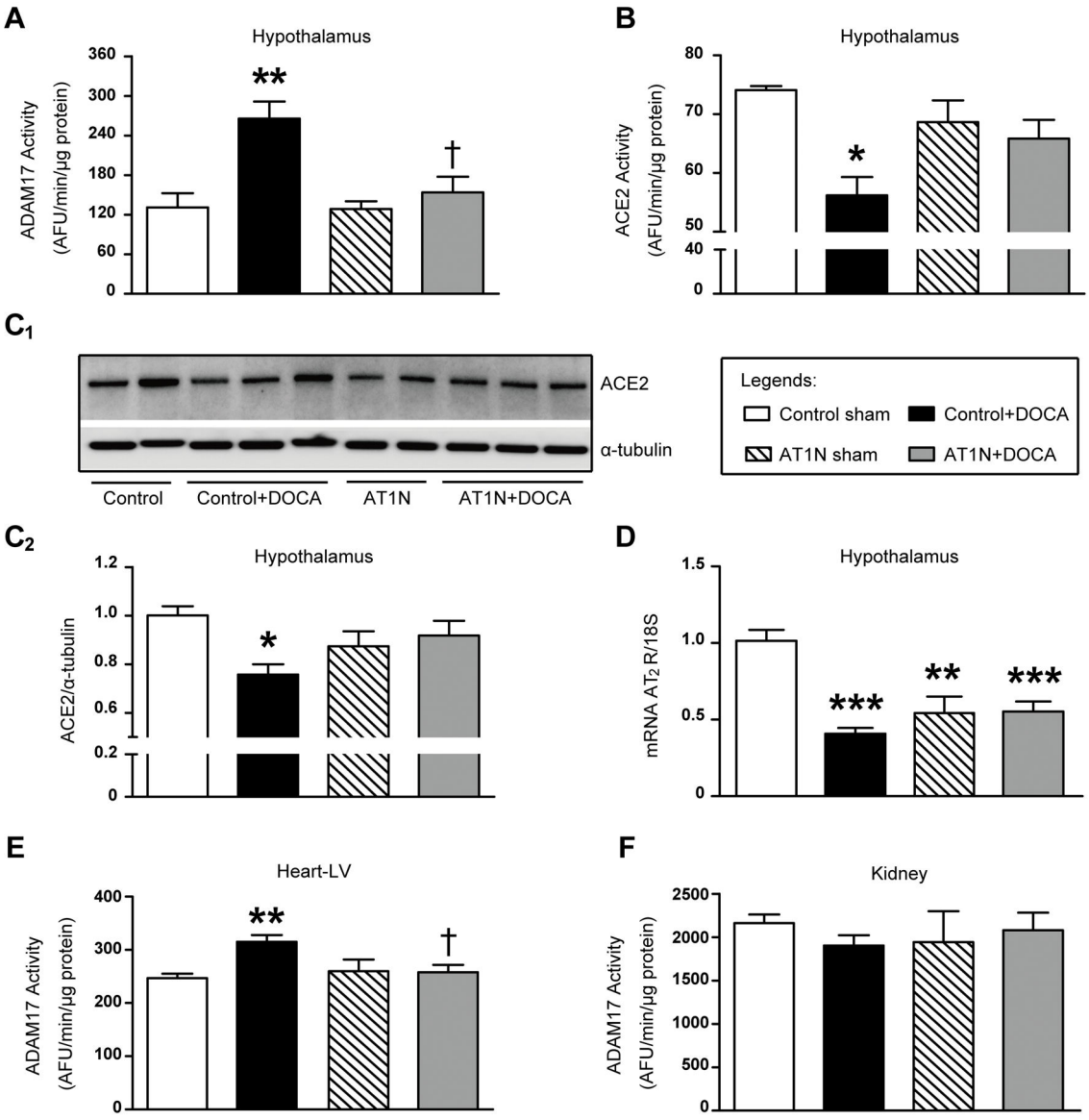

Figure 4. Neuronal $AT_{1a}R$ play a critical role in ADAM17 activation and ACE2 down-regulation during DOCA-salt hypertension

After 18 days of DOCA-salt or sham treatment, ADAM17 (**A**) and ACE2 (**B**) activities were assessed in hypothalami isolated from control and AT1N mice (Controls: n=8 and AT1N: n=6). (**C_1**) Representative Western blot for mouse ACE2 in 2–3 independent hypothalamus homogenates per group. (**C_2**) Quantitative data for mouse ACE2 protein expression in the hypothalamus (n=6/group). (**D**) qRT-PCR results for angiotensin II receptors type 2 (AT_2R) gene expression in hypothalamus from control and AT1N mice (n=6/group). ADAM17 activity was assessed in left ventricles (**E**, Heart-LV) and kidneys (**F**) collected from control and AT1N mice after 18 days of DOCA-salt or sham treatment (n=4/group). Data are shown as mean ±SEM. Statistical significance: Two-way ANOVA: *P<0.05, **P<0.01, ***P<0.001, *vs.* respective shams; †P<0.05, ††P<0.01 *vs.* control+DOCA. AFU indicates arbitrary fluorescence units.

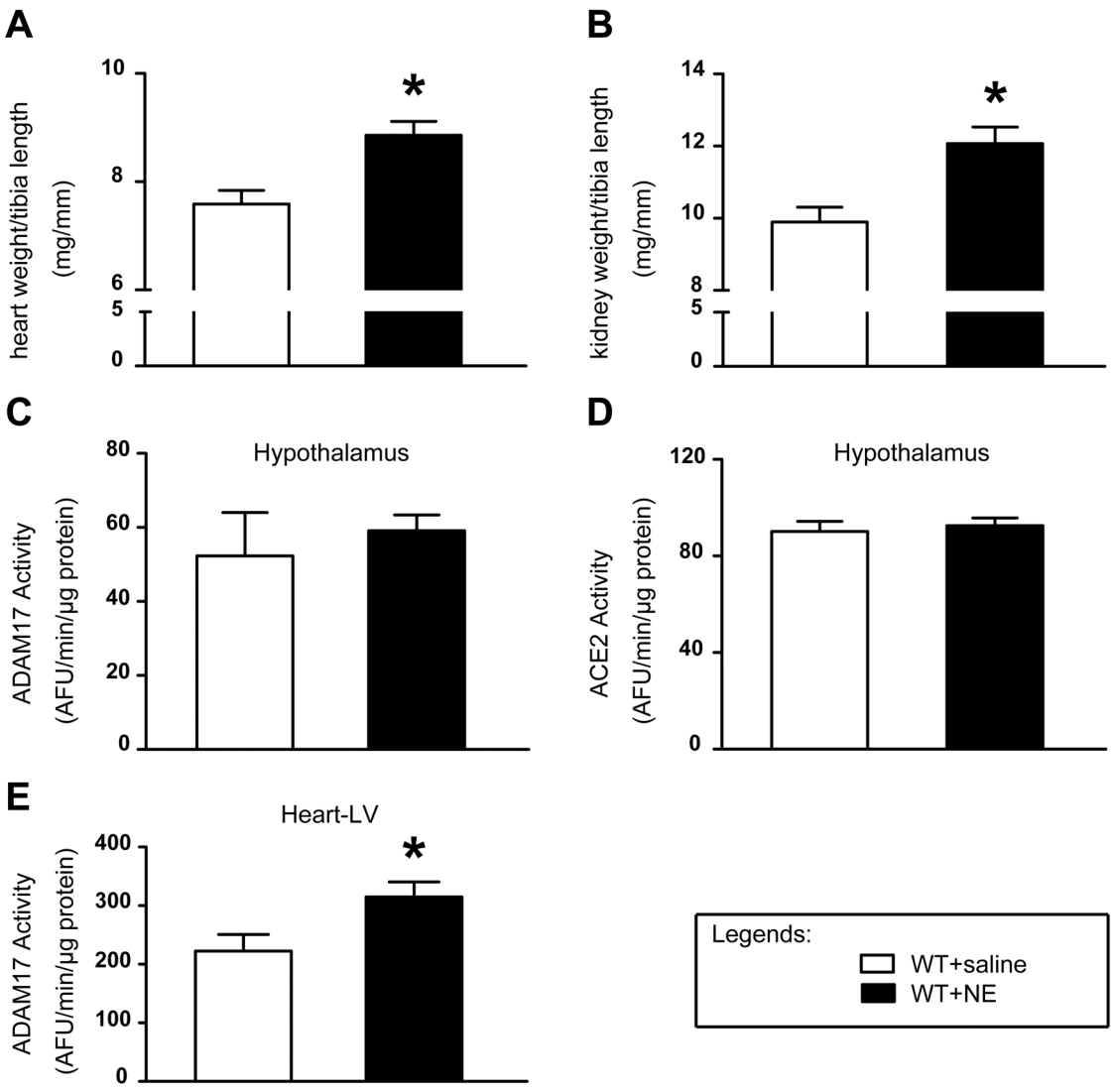

Figure 5. Norepinephrine (NE)-induced hypertension does not induce ADAM17 activation or affect ACE2 activity in the hypothalamus

Both heart weight (**A**) and kidney weight (**B**) were significantly increased after 2 weeks of subcutaneous NE infusion (3.8 μg/kg/min) indicating cardiac and renal hypertrophy. ADAM17 (**C**) and ACE2 (**D**) activity assays were performed using hypothalami samples isolated from NE- or saline-treated wild type (WT) mice. ADAM17 activity (**E**) was elevated in the left ventricle following NE infusion (n=5–6/group). Data are shown as mean ±SEM. Student's t-test: **P*<0.05 *vs.* WT+saline.

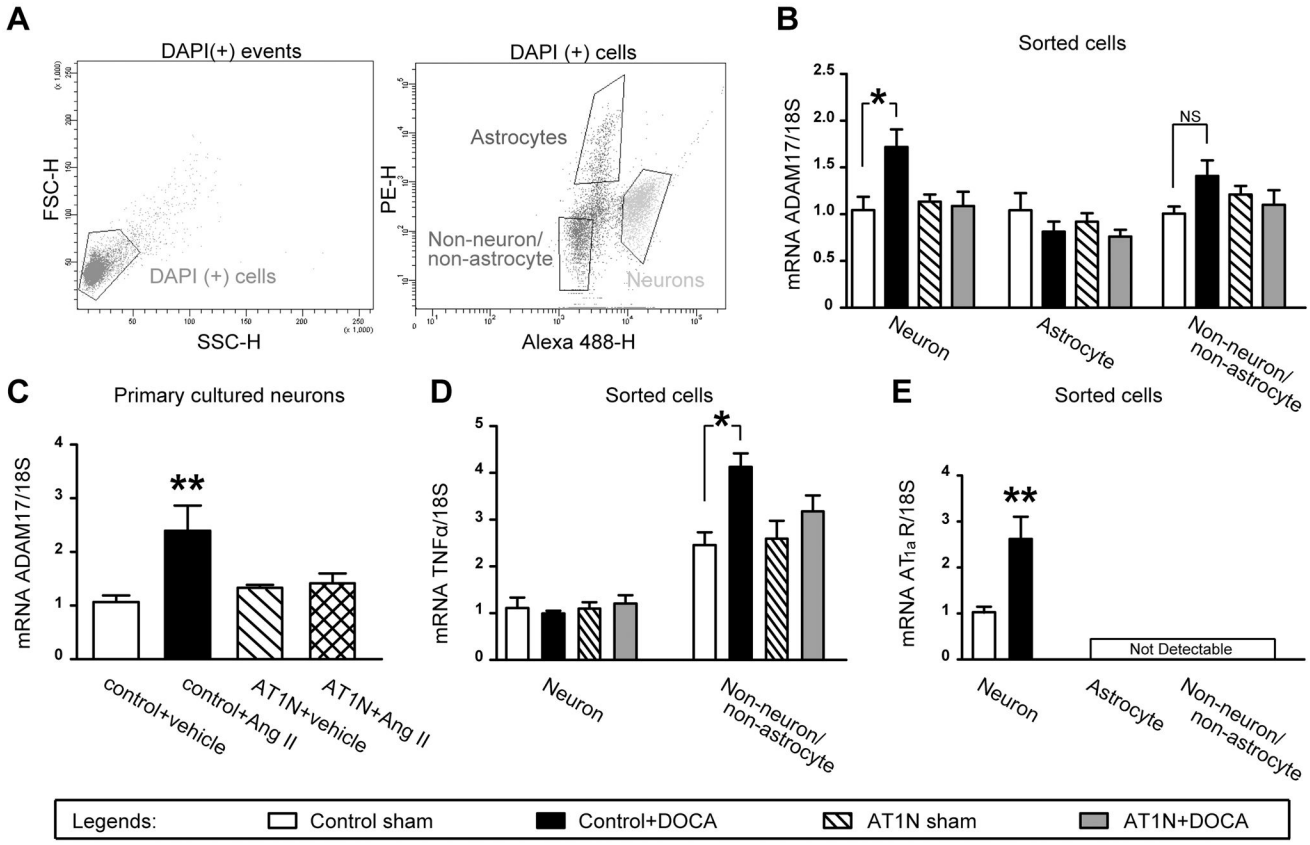

Figure 6. Neuronal ADAM17 is selectively up-regulated by $AT_{1a}R$ in DOCA-salt hypertension

(**A**) Representative sorting strategy for fluorescence-activated cell sorting (FACS) in hypothalamic cells. The scatter gate (DAPI+ cells) was created based on the forward scatter heights (FSC-H) and side scatter heights (SSC-H) of the sorted cells (DAPI+ events). Respective gates for neurons (NeuN-Alexa488+ cells), astrocytes (GFAP-PE+ cells), and non-neuron/non-astrocyte cells were created based on the PE heights (PE-H) and Alexa 488 heights (Alexa 488-H). (**B**) qRT-PCR measurement of ADAM17 was performed in 3 sorted hypothalamic cell populations from control and AT1N mice after DOCA-salt or sham treatment (n=5/group). (**C**) qRT-PCR measurement of ADAM17 was performed in cultured primary neurons isolated from AT1N pups or control littermates, in the presence of Ang II (100 nM, 2 h) or vehicle treatment (n=6/group). (**D**) qRT-PCR measurement of TNFα in 2 sorted hypothalamic cell populations from control and AT1N mice after DOCA-salt or sham treatment (n=5/group). (**E**) qRT-PCR measurement of $AT_{1a}R$ in 3 sorted hypothalamic cell populations from control mice after DOCA-salt or sham treatment (n=5/group). Data are shown as mean ±SEM. Statistical significance: One-way ANOVA or Student's *t*-test: * $P<0.05$ and ** $P<0.01$ *vs.* control sham or control+vehicle. *NS*: not significant.

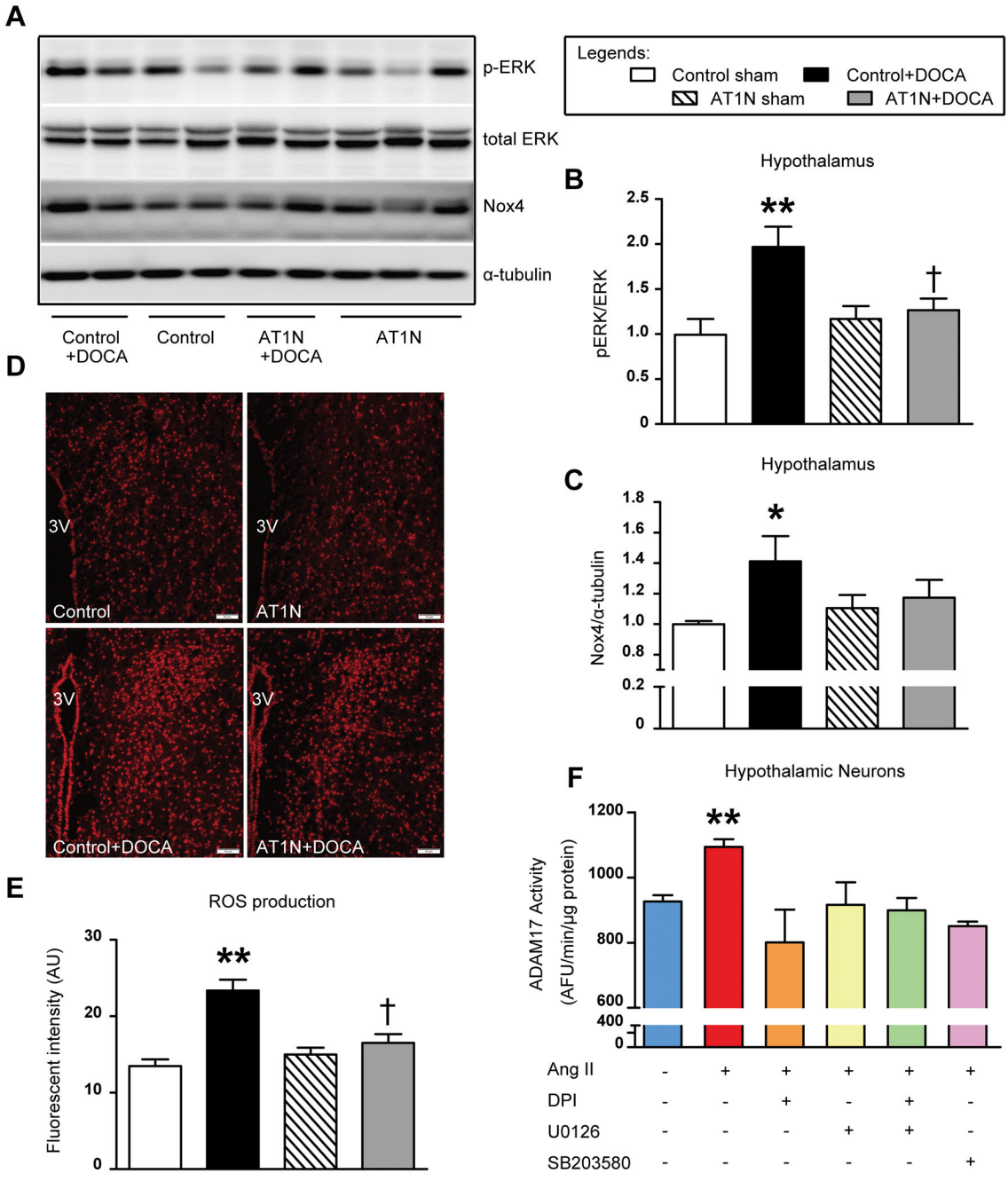

Figure 7. $AT_{1a}R$ downstream signaling mediates neuronal ADAM17 activation

(**A**) Representative Western blot for phosphorylated ERK (p-ERK) and Nox4 in 2–3 independent hypothalamus homogenates per group. Summary of p-ERK (**B**) and Nox4 (**C**) Western blot data based on densitometric analysis (n=5/group). (**D**) Representative results for dihydroethidium (DHE) staining in the PVN region of DOCA-salt- or sham-treated mice. (**E**) Quantitative results for the DHE staining (n=4/group) and AU indicates arbitrary units. (**F**) ADAM17 activity assay performed in cultured primary hypothalamic neurons isolated from wild type neonates. The cultured neurons were treated with Ang II (300 nM, 18 hour),

1 hour after pre-treatment with diphenyleneiodonium (DPI, a flavoenzymes inhibitor, 10 μM), U0126 (MEK inhibitor, used to block ERK phosphorylation, 10 μM), SB203580 (a p38MAPK blocker, 10 μM) and DPI +U0126 respectively for 18 h (n=4–6 independent cultures/group). Statistical significance: One-way or two-way ANOVA: $*P<0.05$ and $**P<0.01$ *vs.* control sham or vehicle; †P<0.05 *vs.* control+DOCA.