Clinical Relevance and Role of Neuronal AT$_1$ Receptors in ADAM17-Mediated ACE2 Shedding in Neurogenic Hypertension

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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 type 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 angiotensin II into angiotensin-(1–7) in human cerebrospinal fluid. We also observed an increase in ACE2 activity in the cerebrospinal fluid of hypertensive patients, which was correlated with systolic blood pressure. Moreover, the increased level of tumor necrosis factor-α in those cerebrospinal fluid samples confirmed that ADAM17 was upregulated in the brain of hypertensive patients. To further assess the interaction between brain renin–angiotensin system and ADAM17, we generated mice lacking angiotensin II type 1 receptors specifically on neurons. Our data reveal that despite expression on astrocytes and other cells types in the brain, ADAM17 upregulation during deoxycorticosterone acetate–salt hypertension occurs selectively on neurons, and neuronal angiotensin II type 1 receptors are indispensable to this process. Mechanistically, reactive oxygen species and extracellular signal-regulated kinase were found to mediate ADAM17 activation.

Conclusions: Our data demonstrate that angiotensin II type 1 receptors promote ADAM17-mediated ACE2 shedding in the brain of hypertensive patients, leading to a loss in compensatory activity during neurogenic hypertension.

Key Words: ACE2 ■ central nervous system ■ hypertension ■ renin–angiotensin system ■ TNF-α convertase enzyme

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$_1$R). 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 angiotensin-(1–7) [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, paraventricular nucleus of hypothalamus, nucleus of the solitary tract, and rostral ventrolateral medulla. Regardless of the enzyme’s compensatory effects, during neurogenic hypertension, brain expression and activity of ACE2 are found to be compromised. Previous work has shown that central ACE2 is downregulated in several conditions, including hypertension.
experimental hypertension models, 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 downregulation in hypertension remain unknown.

ADAM17, a member of the disintegrin and metalloproteinase (ADAM) family, is known to cleave a variety of membrane-anchored proteins. We previously demonstrated an ADAM17-mediated mechanism responsible for the impairment of ACE2 compensatory function in the mouse hypothalamus during neurogenic hypertension. Accordingly, on brain RAS overactivation, ADAM17 is upregulated, 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 first associated ADAM17-mediated shedding to the loss of membrane-bound ACE2 and increase in the shed form of ACE2 (sACE2). 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. Because intracerebroventricular losartan blocked ADAM17 upregulation, central AT1R were identified as critical players in ADAM17-mediated ACE2 shedding. However, AT1R are expressed on various cell types in the brain, including neurons, microglia, and astrocytes, which have been shown to contribute to neurogenic hypertension. 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 AT1R are essential for ADAM17-mediated ACE2 shedding in neurogenic hypertension.

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 AT1R deficiency, our data provide strong evidence that neuronal AT1R are required for central ADAM17 upregulation 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 AT1R, such as reactive oxygen species (ROS) and extracellular signal-regulated kinase (ERK),

 ### What Is Known?

- Overactive brain renin–angiotensin system is associated with decreased angiotensin-converting enzyme 2 (ACE2) level and activity in the brain during the development of neurogenic hypertension.
- A disintegrin and metalloproteinase 17 (ADAM17) is a member of the disintegrin and metalloproteinase family that has been shown to be upregulated in the brain in deoxycorticosterone acetate-salt–induced hypertensive mice.

### What New Information Does This Article Contribute?

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

ACE2, an important link between classic renin–angiotensin system and its compensatory axis, is downregulated in various animal models of cardiovascular disease. In the deoxycorticosterone acetate-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 upregulated 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. Angiotensin II type 1 receptors promote ADAM17 upregulation selectively in neurons, through reactive oxygen species and the extracellular signal-regulated kinase/mitogen-activated protein kinase signaling pathway. ADAM17-mediated ACE2 shedding is exacerbated in the brain of hypertensive patients and compromises the compensatory effect of angiotensin-(1–7).
play a critical part in mediating the AT$_2$R-driven increase in ADAM17 activity in neurons.

**Methods**

A detailed Methods section is available in the Online Data Supplement.

**Patients' CSF Samples**

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

**Transgenic Mice and Animal Husbandry**

Experiments were performed in adult (14–16 weeks old, 25–30 g) male neuronal AT$_2$R knockdown mice (AT1N) and their control littermates. All procedures were approved by the Louisiana State University Health Sciences Center-NO Animal Care and Use Committee (No. 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 analysis of variance, or 2-way analysis of variance, 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. 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 pretreated 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 with 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

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**Figure 1.** Angiotensin-converting enzyme type 2 (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 angiotensin (Ang) II into Ang-(1–7) in humans CSF. Measurements of soluble ACE2 (sACE2) activity (C) and tumor necrosis factor-α (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 (1-way analysis of variance [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. rhACE2 indicates recombinant human ACE2.
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, tumor necrosis factor-$\alpha$ (TNF$\alpha$) 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 $\text{AT}_1\text{R}$ Reduces DOCA-Salt–Induced Hypertension and Dysautonomia

In our previous study, intracerebroventricular losartan infusion in DOCA-salt–treated mice significantly attenuated both BP rise and increased ADAM17 expression, suggesting the pivotal role of $\text{AT}_1\text{R}$ in ADAM17 upregulation during RAS overactivity–related neurogenic hypertension. However, in the CNS, $\text{AT}_1\text{R}$ and ADAM17 are colocalized in various cell types that have all been shown to be involved in BP regulation. Therefore, we hypothesized that neuronal $\text{AT}_1\text{R}$ 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 $\text{AT}_1\text{R}$, a new neuronal knockout mouse (AT1N) was generated, in which cre-recombinase expression is driven by the Nefh promoter (Figure 2A and 2B). Using primary neuron cultures, specific cre expression to neurons was verified by double-labeling with MAP2 (Figure 2C). Quantitative reverse transcription polymerase chain reaction performed with these cells confirmed a significant reduction of $\text{AT}_1\text{R}$ mRNA in AT1N mice compared with controls (Figure 2D), thus, validating the knock-down of $\text{AT}_1\text{R}$ in these mice neurons.

DOCA-salt, a well-accepted neurogenic hypertension model, was previously reported by us to upregulate ADAM17 through RAS overactivation. Before the DOCA-salt paradigm, BP and heart rate were not significantly different between AT1N mice and their control littermates, suggesting that neuronal $\text{AT}_1\text{R}$ are not involved in the maintenance of baseline BP and heart rate (Figure 3A through 3C). However, daily BP recording (Figure 3A) uncovered major differences between these 2 genotypes on DOCA-salt treatment. Indeed, while BP progressively increased in control littermates and rose by $\approx 30$ 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

Figure 2. Genotyping and validation of angiotensin II type 1 receptors ($\text{AT}_1\text{R}$) conditional knockout mice. A, Schematic diagram showing the breeding strategy. On introduction of cre-recombinase (Nefh-cre), exon 3 of the $\text{AT}_1\text{R}$ gene is excised specifically in neurons, allowing for the generation of the selective $\text{AT}_1\text{R}$ knockout (AT1N) mice. B, Representative genotyping results for AT1N and control littermates. Accordingly, pups No. 4, 6, and 7 were identified as AT1N: $\text{AT}_1\text{R}^{\text{fl/fl}}$ with cre recombinase (Cre$^{+/0}$), while pups No. 1, 2, 3, and 5 were the control littermates: $\text{AT}_1\text{R}^{\text{fl/fl}}$ 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 $\mu$m. D, Quantitative reverse transcription polymerase chain reaction (qRT-PCR) results showing that $\text{AT}_1\text{R}$ expression is knocked down in the cultured neurons from AT1N mice (Student’s $t$ test: **$P<0.01$ vs control).
Figure 3. Deoxycorticosterone acetate (DOCA)-salt–induced hypertension and dysautonomia are significantly attenuated in mice with neuronal angiotensin II type 1 receptors (AT_{1a}) 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 AT_{1a} knockout (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: 2-way analysis of variance (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.
mice was significantly lower than in controls (21±3 versus 34±4 mm Hg; 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 or autonomic function might be altered after AT1R 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 contributed to the maintenance of hypertension in DOCA-salt–treated animals (*P<0.05 and **P<0.01 versus control). The similarity of intrinsic heart rate between groups, after ganglionic blockade (Figure 3H), suggests that these changes are related to autonomic function and not cardiac function. Deletion of AT1R 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 was altered after DOCA-salt treatment in these animals.

Control+DOCA mice exhibited an ≈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 IA through IC), and norepinephrine (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 ID and IE), suggesting similar blood volumes between control and AT1N mice under DOCA-salt treatment. On the contrary, despite remaining elevated, NE excretion was reduced by >50% in AT1N treated with DOCA-salt (Figure 3I). Taken together, these data highlight the critical role of brain AT1R in promoting cardiac dysfunction through enhanced NE release in DOCA-salt hypertension.

Neuronal AT1R Are Required for ADAM17 Activation During DOCA-Salt Hypertension

After 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 versus control sham; Figure 4A). As a result, both ACE2 activity and expression were attenuated by ≈25% (P<0.05 versus control sham; Figure 4B and 4C), confirming the opposite relationship between ADAM17 and ACE2. AT1R gene expression was reduced as well in hypertensive animals (Figure 4D). Interestingly, AT1R deletion on neurons did not affect baseline ADAM17 activity and had no impact on ACE2 expression or activity in the brain, suggesting that AT1R 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 AT1R gene expression (Figure 4B through 4D), in the hypothalamus of these mice. These data suggest that while constitutive ADAM17 enzymatic activity does not rely on the presence of neuronal AT1R, the DOCA-salt hypertension-mediated rise in its shedding activity requires AT1R 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 AT1R 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 AT1R 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 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 and 5B), supporting a BP increase after NE treatment in these mice. Unlike DOCA-salt treatment, compared with 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 after NE infusion (Figure 5D). These observations demonstrate that ADAM17 activation during DOCA-salt hypertension is mediated by neuronal AT1R 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 AT1R play an essential role in the ADAM17-mediated ACE2 ectodomain shedding after brain RAS overactivation, ultimately contributing to the development of neurogenic hypertension.

Neuronal ADAM17 Is Selectively Upregulated by AT1R in DOCA-Salt Hypertension

To better understand the relationship between neuronal AT1R and ADAM17, a flow cytometry protocol was developed to sort different types of hypothalamic cells from DOCA-salt–treated mice. After labeling with NeuN (neuronal nuclei)-conjugated Alexa Fluor 488 and GFAP (glial fibrillary acidic protein)-conjugated phycoerythrin antibodies, hypothalamic cells were sorted into neuronal, astrocyte, and non-neuronal/nonastrocyte (NNNA) populations (Figure 6A). ADAM17 gene expression was easily detected among the various cell populations but only neuronal ADAM17 was upregulated 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 AT1R.

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 fluorescence-activated cell sorting data, primary neurons cultured from control and AT1N mice were exposed to Ang II (100 nmol/L). After 2 hours, ADAM17 gene expression was significantly elevated by Ang II treatment in control mice (** P<0.01 versus 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 AT1aR (Figure 6D). These data suggest that although 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/Mitogen-Activated Protein Kinase Are Involved in AT1aR-Mediated ADAM17 Activation**

Because basal ADAM17 levels were not affected by neuron-targeted AT1aR knockdown, signals that upregulate ADAM17 during DOCA-salt treatment are thought to be downstream of AT1aR activation. Among those, ERK/mitogen-activated protein kinase (MAPK) activation15–17 and oxidative stress18

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**Figure 4.** Neuronal angiotensin II type 1 receptors (AT1R) play a critical role in a disintegrin and metalloprotease 17 (ADAM17) activation and angiotensin-converting enzyme type 2 (ACE2) downregulation during deoxycorticosterone acetate (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 AT1R knockout (AT1N) mice (controls, n=8 and AT1N, n=6). C1, Representative Western blot for mouse ACE2 in 2 to 3 independent hypothalamic homogenates per group. C2, Quantitative data for mouse ACE2 protein expression in the hypothalamus (n=6/group). D, Quantitative reverse transcription polymerase chain reaction (qRT-PCR) results for angiotensin II receptors type 2 (AT1R) 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: 2-way analysis of variance (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.
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 AT1aR (Figure 7A and 7B). 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 dihydroethidium (DHE) staining, an index of ROS production, in the paraventricular nucleus of hypothalamus (Figure 7D and 7E). Importantly, neither Nox4 expression nor ROS production was affected by DOCA-salt treatment in AT1N mice (Figure 7C through 7E), confirming that these signaling molecules are regulated by AT1aR 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, an MEK inhibitor (used to prevent ERK phosphorylation), SB203580 (a p38-MAPK inhibitor), and diphenyleneiodonium, an NADPH oxidase inhibitor. As shown by us previously in neuronal cell lines,18 ADAM17 activity was significantly upregulated by Ang II treatment (Figure 7F), and this effect was completely blocked by pretreatment with U0126, SB203580, or diphenyleneiodonium. Taken together, our data provide strong evidence that neuronal AT1aR mediate ADAM17 activation through its ERK/MAPK and oxidative stress downstream signals, which are also known to be upregulated during RAS overactivation.

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 substrates19 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 surface21; 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. 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), our data suggest that ACE2 seems 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 AT1R-dependent internalization. While there are no data available for the latter, in humans, elevated sACE2 has been reported in the periphery in heart failure, hypertension, severe acute respiratory syndrome, and type 1 diabetes mellitus. 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 and 1D) 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 because these individuals were taking a RAS blocker or a sympatholytic (Online Table II) in combination with a diuretic 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 be because of decreased local metabolism or changed structure. Whether sACE2 has therapeutic benefits or is merely a degradation product resulting from RAS overactivation 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 overtactive brain RAS. As the main mediator of brain RAS overactivity, the AT1R was previously demonstrated to prompt ADAM17 upregulation both in vitro and in vivo. Moreover, in recent years, cell-specific expression of AT1R in the CNS, has been shown to contribute to neurogenic hypertension and other ailments. Because AT1R and ADAM17 coexist on various cell types, including endothelial cells, vascular smooth muscle cells, glial cells, and neurons, 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 AT1R 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. Accordingly, most of AT1R 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 AT1R (Figure 5A). In addition, it is not secondary to hypertension because NE infusion, by which BP can be increased to >160 mm Hg, failed to increase the sheddase activity in the brain (Figure 5C), while it did promote activation in the heart, as reported previously. Importantly, this new mechanism is dependent on brain RAS overactivation because AT1R 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, coimmunoprecipitates with AT1R on agonist stimulation, and these rafts play a critical role for AT1R-mediated signal transduction. Altogether, it is conceivable that on RAS overactivation, AT1R could move to a closer location from which its downstream signals can affect ADAM17 in a more efficient way.

On acute stimulation, intracellular signals resulting from GPCR (G-protein–coupled receptor) activation or oxidative stress can activate ADAM17 within a few minutes via phosphorylation of its cytoplasmic domain or upregulate ADAM17 availability through increasing its translocation. In addition to its critical role in the migration of AT1R into membrane microdomains, ROS are actually important to Golgi apparatus–mediated ADAM17 transport, which requires a 2-fold increase in NNNA cells. Interestingly, the rise of TNFα mRNA upregulation in neurons after DOCA-salt treatment, while there was almost a 2-fold increase in NNNA cells. 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 infiltrating immune cells, 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 LV, accompanied with upregulated cardiac hypertrophy, which can be blocked by deletion of ADAM17 independently of decreasing BP. In the kidney, ADAM17-mediated ACE2 downregulation has also been found to be altered in mouse and human renal diseases. In this study, we observed an upregulation of ADAM17 activity in the mouse heart after DOCA-salt treatment presumably driven by increased NE level during hypertension (Figures 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, the lack of detectable AT1R 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 because of fluorescence-activated cell sorting resolution. The NNNA population which includes mostly microglia and vascular cells also showed a significant level of ADAM17 mRNA and a trend to upregulation on DOCA-salt treatment. Although AT1R gene expression was undetectable in these cells, likely because of its overabundance in neurons, the rise of TNFα, and maybe ADAM17, mRNA after DOCA-salt treatment highlights a partnership between neuronal AT1R 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 vascular smooth muscle cell, ADAM17-mediated HB-EGF (heparin-binding epidermal growth factor) upregulation is one of the mechanisms of vascular remodeling, 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 (ie, 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 neuroinflammation, most likely from microglia, which can be activated by Ang II and infiltrating immune cells. The cell specificity of ADAM17 is further confirmed by the lack of TNFα mRNA upregulation in neurons after 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 AT1R 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. Although not the focus of this study, their involvement could be an attractive hypothesis to explain AT1R-ADAM17 cooperation between neurons and TNFα-producing cells in hypertension.

Limitations

Though we provide strong evidence that AT1R promote ADAM17-mediated ACE2 shedding in the development of neurogenic hypertension, there are some limitations in our study about the experimental methods we chose. (1) While diphenylethionium is wildly used as an inhibitor of flavoenzymes and often accepted as evidence of NADPH oxidase
inhibition, it is a nonspecific inhibitor that binds strongly to flavoproteins, including nitric oxide synthase, NADPH-ubiquinone oxidoreductase, NADPH oxidases, and NADPH cytochrome P450 oxidoreductase. It is well documented that Ang II–associated ROS production is not limited to NADPH oxidase; therefore, using diphenyleneiodonium 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 paraventricular nucleus of deoxycorticosterone acetate (DOCA)-salt- or sham-treated mice. E, Quantitative results for the DHE staining (n=4/group). F, ADAM17 activity assay performed in cultured primary hypothalamic neurons isolated from wild-type neonates. The cultured neurons were treated with angiotensin (Ang) II (300 nmol/L, 18 hour), 1 hour after pretreatment 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: 1-way or 2-way analysis of variance (ANOVA): *P<0.05 and **P<0.01 vs control sham or vehicle; †P<0.05 vs control+DOCA. AU indicates arbitrary units.
of hypothalamus. 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 •OH into ethidium.47 Because the difference in their fluorescence spectra is small, analytical methods, like high-performance liquid chromatography and mass spectrometry, should be applied to verify the specific superoxide signals.48 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 al50 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 (eg, cortex versus 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 AT1R 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.

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Disclosures
None.

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Clinical Relevance and Role of Neuronal AT1 Receptors in ADAM17-Mediated ACE2 Shedding in Neurogenic Hypertension
Jiaxi Xu, Srinivas Sriramula, Huijing Xia, Lisa Moreno-Walton, Frank Culicchia, Oliver Domenig, Marko Poglitsch and Eric Lazartigues

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Supplemental Material for “Clinical relevance and role of neuronal AT1 receptors in ADAM17-mediated ACE2 shedding in neurogenic hypertension”

Supplemental Methods:

Measurement of soluble ACE2 in patients
Cerebrospinal fluid (CSF) samples were obtained from 27 patients undergoing neurosurgery or seeking neurological treatment at the LSU Emergency department. Excess of CSF, collected for diagnostic purposes, was processed for ACE2 activity or measurement of TNFα levels (EH3TNFA, Thermo Fisher). Patients medical records, including current medications and co-morbidities were used, in conjunction with 5 BP measurements collected in the clinic over a 5-hour period before CSF collection, to determine the assignment groups (i.e. normotensive, uncontrolled hypertensive or controlled hypertensive; Supplemental Table 1). All procedures were approved by the LSU Health Sciences Center-NO Institutional Review Board committee (#7832) and the Institutional Biosafety Committee (#12086).

ACE2 activity measurements were performed by Attoquant Diagnostics GmbH (Vienna, Austria), based on a previously described method.1 Briefly, the metabolism of spiked Ang II (1 µg/mL, Fisher Scientific) was assayed in conditioned CSF (40 µL) following ex vivo incubation at 37°C in the presence or absence of the specific ACE2 inhibitor MLN-4760 (10 µM, Merck-Millipore). CSF conditioning included the stabilization of pH at 7.4 and blocking potentially interfering substrate and product degrading enzymatic pathways using a combination of specific enzyme inhibitors including Lisinopril, Chymostatin, Z-Pro-Prolinal, DL-Thiorphan and aminopeptidase inhibitors (Attoquant Diagnostics, Vienna).2 Enzyme activity was calculated by determining the MLN-4760 sensitive part of Ang-(1-7) formation in incubated samples and referred to a rhACE2 (recombinant human ACE2, R&D) standard. Accordingly, 1 unit (U) corresponds to the Ang-(1-7) formation obtained in the presence of 1 pg/mL rhACE2 in the sample. Ang-(1-7) quantification was performed by liquid chromatography tandem-mass spectrometry analysis (LC-MS/MS), as previously described.3, 4 Assay linearity was confirmed in pooled CSF samples spiked with increasing concentrations of rhACE2, assuring efficient stabilization of substrates and products in ACE2 assays.

Transgenic mice and animal husbandry
Experiments were performed in adult (14-16 weeks old, 25-30 g) male neuronal AT1aR knock-down mice (AT1N) and their control littermates. AT1N were obtained by breeding AT1aR floxed mice (kind gift of Dr. Lisa Cassis, University of Kentucky) with Nefh-cre recombinase animals (kind gift of Dr. Yumei Feng, University of Nevada). AT1N mice appear fertile and devoid of gross anatomy defects. All animals were housed in a temperature- and humidity-controlled facility under a 12-hour dark/light cycle, fed standard mouse chow and water ad libitum. Since one copy of Cre expression (heterozygous, Cre+/0) is efficient for removal of target gene, we bred male AT1N mice (AT1aRfl/fl plus Cre+/0) with female AT1aR floxed mice (AT1aRfl/fl) to generate AT1N and control littermates at the same time. 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.

Hypertension models and physiological recordings
Before surgery, mice were anesthetized with isoflurane (2%) in an oxygen flow (1 L/min) and placed on a heating pad to maintain normal body temperature. Post-operative care, included a long-acting buprenorphine-SR injection to relieve pain (50 µg/kg, sc.). AT1N and control mice first underwent uninephrectomy during which an incision was made in the skin in the retro peritoneal region to remove the right kidney. After 1 week of recovery, mice were implanted with telemetry probes (PA-C10, DSI, St Paul, MN) for conscious BP monitoring, as described.5 Following recovery, baseline BP was recorded in AT1N and control cohorts for 4 days, then mice were randomly divided between groups (n=10/group), implanted subcutaneously with a DOCA-silicone sheet containing 1 mg of DOCA/g body weight or sham. Drinking water from DOCA-implanted mice was replaced by 1% NaCl solution. BP was recorded daily for 3 weeks.
post implantation. Autonomic function was assessed, in conscious freely moving mice, before and 3 weeks after DOCA-salt treatment, using a pharmacological method involving ip injection of propranolol (β-blocker, 4 mg/kg), atropine (muscarinic receptor blocker, 1 mg/kg) and chlorisondamine (ganglionic blocker, 5 mg/kg). The drugs were injected in the following order: atropine, propranolol and chlorisondamine. At least a 3-hour recovery was allowed before propranolol administration, while chlorisondamine was given on the following day. Changes in HR (ΔHR) or mean arterial pressure (ΔMAP) were calculated following administration of these drugs.

A subset of control mice (n=11) was implanted with an osmotic mini-pump (Alzet, Model 1002) containing norepinephrine (3.8 μg/kg/min) or vehicle and infused for 14 days. At the end of the protocol, mice were euthanized and brain and plasma were collected and stored at -80 °C until used in the following assays.

**Metabolic cage study**
In another set of experiments, mice (n=9/control group; n=4/AT1N group) were individually housed in metabolic cages. Food and water were available ad libitum. Mice were given both water or 1% NaCl solution in separate burettes and were allowed to adapt to the metabolic cage for 3 days. After the adaptation period, daily water intake and urine volume were measured for 3 days at baseline and during the last week of DOCA-salt treatment period, respectively.

**Hypothalamic primary neurons culture**
Hypothalamic primary neurons were isolated from control or AT1N mouse newborns. Brains from the euthanized pups were dissected immediately and the hypothalami were collected and placed in ice-cold Hank’s balanced salt solution (HBSS) (Gibco 14175-079). Following brief dissociation, the hypothalami were washed and dispersed in HBSS containing 1% trypsin (Sigma-Aldrich T1426) and 1.5 KU/mL DNase I (Sigma-Aldrich D5025), then digested for 10 min at 37 °C. The digested tissues were then dissociated by gentle pipetting in HBSS containing 1.5 KU/mL DNase I. After brief centrifuge, the cell pellets were re-suspended in complete Neurobasal culture medium, supplemented with 2% B27 and 0.5 mM GlutaMax (Gibco), and plated at a density of 10⁶ cells/ml onto poly-L-lysine coated plates or glass slides. Cytosine arabinofuranoside (Ara-C, 2 μM, Sigma-Aldrich C1768) was added to the cultures 48 h after plating to arrest the growth of non-neuronal cells. For in vitro experiments, hypothalamic primary neurons were cultured for at least 10 days and then treated with specific agonist or blockers. All blockers were given 1 hour before Ang II treatment: diphenyleneiodonium (DPI, a flavo enzymes inhibitor, 10 μM), U0126 (a MEK inhibitor, 10 μM), SB203580 (a p38MAPK blocker, 10 μM).

**Western blotting**
Hypothalamic proteins (10-20 μg) were processed for Western blotting as described previously, using rabbit anti-ACE2 (Santa Cruz sc-20998, 1:500), rabbit anti-NAPDH oxidase 4 (Abcam ab109225, 1:1000), rabbit anti-phospho-ERK (Cell Signaling Technology 43775, 1:1000), and rabbit anti-total-ERK (Cell Signaling Technology 9102, 1:1000). α-tubulin (Sigma-Aldrich T6199, 1:5000) was used as a loading control.

**Immunocytochemistry**
Immunofluorescent detection of Cre-recombinase and microtubule-associated protein 2 (MAP2) was performed on cultured primary neurons. Neurons were fixed in 4% paraformaldehyde (PFA) in PBS (0.1 M, pH 7.4) for 15 min and incubated with primary antibodies for 18-36 h at 4 °C. Dilutions of primary antibodies are as follow: Cre-recombinase (Covance MMS-106P, 1:1000), MAP2 (Abcam ab24640, 1:500). Cells were washed with PBS, then incubated with appropriate Alexa-fluor (488 or 546)-conjugated secondary antibodies (Life Technologies, 1:500 dilution) for 2 h at room temperature. Additionally, primary and secondary antibodies were omitted in the incubation to check for non-specific staining. Images were captured using a fluorescence microscope (Olympus BX43) and “CellSens” imaging platform.

**Norepinephrine assay**
Urine samples were collected by bladder massage at the end of the experimental protocol and urinary norepinephrine concentration was measured using a Noradrenaline ELISA kit (Immuno-Biological Laboratories IB89552), according to the manufacturer’s instructions.

ACE2 and ADAM17 activity assay
Hypothalami collected from DOCA-salt- or sham-treated mice were processed for ACE2 and ADAM17 activities, as reported previously. Data are presented as fluorescence units (amount of substrate converted to fluorescent product) per minute and normalized for protein content (AFU/min/μg).

Cells sorting
The following protocol was modified from previously reported method. The hypothalami were finely minced in a drop of PBS-T before transfer to an Eppendorf tube. Following trituration in cold PBS-T, supernatants were combined and filtered through a 70 μm mesh prior to antibody staining. Cells were then incubated with NeuN (Abcam ab177487, 1:500) at 4 °C overnight. After washing with PBS-T, the cells were incubated with Alexa-fluor 488-conjugated secondary antibody (Life Technologies, 1:300 dilution) for 1 h at 4 °C. Cells were then incubated with PE-conjugated GFAP (BD biosciences 561483, 1:300) at 4 °C for 1 h. Fluorescence-assisted cell sorting (FACS) was performed following filtering through a 40 μm mesh. All samples were sorted on a BD FacsAria, through a 70 μm nozzle, into empty Eppendorf tubes. Prior to cell sorting, 50,000-100,000 events were analyzed from each sample to set gating values.

RNA isolation and quantitative reverse transcription polymerase chain reaction (qRT-PCR)
Primary neurons and sorted cells were extracted with TRIzol, phase separated with 1-bromo-3-chloropropane (BCP, MRC BP151) and spun at 13,000 g for 15 min at 4 °C. For sorted cells, the supernatant was added to an equal volume of isopropanol, supplemented with 1 μg of glycogen to aid RNA precipitation. RNA was then washed in ethanol and dissolved in 10 μL nuclease free water for cDNA synthesis. For primary neurons, the supernatant was added to an equal volume of ethanol and then processed using a RNA-MiniPrep kit from ZYMO Research (R2070). Real time RT-PCR amplification reactions were performed with Power SYBR Green RNA-to-C_T one-step kit (Thermo Fisher 4389986). The primer sequences used for qRT-PCR are listed in Supplemental Table 2. Data were normalized to 18S expression by the ΔΔC_T comparative method and expressed as fold change compared to sham.

DHE staining
Mouse brains were rapidly dissected, snap frozen in cold isobutane on dry ice and then embedded in OCT. Brains were cryostat-sectioned to 20 μm and directly mounted onto chilled microscope slides. Sections were thawed at room temperature and rehydrated with PBS before incubation in DHE (5 μM in PBS) for 5 min in the dark. Sections were then washed thrice with PBS. After the final wash, sections were fixed with 4% PFA, cover-slipped and then imaged using Olympus-BX43 fluorescence microscope (Excitation/Emission wavelengths: 518/605 nm). DHE fluorescence was quantified using Image J software, pictures were transformed into 32 bit grey format and background was deducted before quantification.

Data analysis
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.
References:


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Online Figures and Figure Legends:

Online Figure I. DOCA-salt hypertension-associated cardiac dysfunction is attenuated in mice with neuronal AT1aR deficiency. (A) Representative echocardiography recordings. (Cardiac function was assessed via measurement of ejection fraction (B) and fractional shortening (C) in DOCA-salt- or sham-treated control and AT1N mice (n=6/sham group and n=9/DOCA-salt treated group). Increased daily water intake (D) and urine output (E) in DOCA-salt-treated mice was not altered by neuronal AT1aR knockdown (Control: n=9/group and AT1N: n=4/group). Data are shown as the mean ±SEM. Statistical significance: Two-way ANOVA: *P<0.05, **P<0.01, ***P<0.001 vs. sham; †P<0.05 vs. control+DOCA.
Online Figure II. Regulation of ADAM17-mediated ACE2 shedding: During DOCA-salt hypertension, ADAM17-mediated ACE2 shedding is up-regulated via activation of neuronal AT1 receptor.