Angiotensin type 1a receptors in the paraventricular nucleus of the hypothalamus control cardiovascular reactivity and anxiety-like behavior in male mice

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Wang L, Hiller H, Smith JA, de Kloet AD, Krause EG. Angiotensin type 1a receptors in the paraventricular nucleus of the hypothalamus control cardiovascular reactivity and anxiety-like behavior in male mice. Physiol Genomics 48: 667–676, 2016. First published July 28, 2016; doi:10.1152/physiolgenomics.00029.2016.—This study tested the hypothesis that deletion of angiotensin type 1a receptors (AT1a) from the paraventricular nucleus of hypothalamus (PVN) attenuates anxiety-like behavior, hypothalamic-pituitary-adrenal (HPA) axis activity, and cardiovascular reactivity. We used the Cre/LoxP system to generate male mice with AT1a specifically deleted from the PVN. Deletion of the AT1a from the PVN reduced anxiety-like behavior as indicated by increased time spent in the open arms of the elevated plus maze. In contrast, PVN AT1a deletion had no effect on HPA axis activation subsequent to an acute restraint challenge but did reduce hypothalamic mRNA expression for corticotropin-releasing hormone (CRH). To determine whether PVN AT1a deletion inhibits cardiovascular reactivity, we measured systolic blood pressure, heart rate, and heart rate variability (HRV) using telemetry and found that PVN AT1a deletion attenuated restraint-induced elevations in systolic blood pressure and elicited changes in HRV indicative of reduced sympathetic nervous activity. Consistent with the decreased HRV, PVN AT1a deletion also decreased adrenal weight, suggestive of decreased adrenal sympathetic outflow. Interestingly, the altered stress responsivity of mice with AT1a deleted from the PVN was associated with decreased hypothalamic microglia and proinflammatory cytokine expression. Collectively, these results suggest that deletion of AT1a from the PVN attenuates anxiety, CRH gene transcription, and cardiovascular reactivity and reduced brain inflammation may contribute to these effects.

corticosterone; corticotropin-releasing hormone; neuroinflammation; stress; anxiety

ANXIETY IS THE MOST COMMON neuropsychiatric illness with nearly 30% of the adult population in the US reporting a lifetime prevalence of some type of anxiety disorder (35). In addition to excessive uncontrollable anxiety, patients with anxiety disorders frequently present with impairment of the hypothalamic-pituitary-adrenal (HPA) axis (reviewed by Ref. 20) and overactivation of the sympatho-cardiovascular system (57). While the etiology of anxiety disorders are not fully understood, research from several groups has implicated the renin-angiotensin system (RAS) in the underlying pathophysiology (36, 48, 59).

Angiotensin II (ANG II) is the effector peptide of the RAS and exerts the majority of its physiological effects via activation of angiotensin type 1 receptor in humans or its homolog, angiotensin type 1a receptors (AT1a), in rodents. Preclinical studies utilizing laboratory rodents have found that activation of AT1a in the brain promotes stress-response and anxiety-like behavior and exposure to chronic stress augments the expression of AT1a within the paraventricular nucleus of hypothalamus (PVN) (2, 42). The PVN contains neuronal phenotypes critically involved in the regulation of the HPA axis and sympathetic nervous system activity. Specifically, the PVN contains neurosecretory neurons synthesizing corticotropin-releasing hormone (CRH) that project to the median eminence and initiate activation of the HPA axis. Additionally, the PVN contains neurons with projections to the brain stem and spinal cord. These projections influence sympathetic nervous activity, and therefore, these PVN neurons are deemed “preautonomic.” Interestingly, icv administration of ANG II increases CRH mRNA in the PVN (3, 12), and losartan delivered with the same route of administration suppresses the increased CRH mRNA and elevated plasma catecholamines that are observed subsequent to immobilization stress (33). Together, these results suggest stress exposure promotes AT1a activation within the PVN to regulate the HPA axis and sympathetic nervous system; however, the long-term physiological and behavioral consequences of ablating AT1a signaling specifically in the PVN has not been evaluated.

In addition to the control of endocrine axes and autonomic function, ANG II and AT1a have been implicated in the neuroinflammation that is associated with several brain-based diseases (59, 69). In this regard, accumulating evidence from several studies has identified a link between inflammation and neuropsychiatric illness (27, 49, 60). Patients with anxiety disorders have increased inflammation (70), and administration of inflammatory endotoxins elicits HPA axis activation and feelings of anxiety in human subjects (55). Studies using laboratory rodents indicate that administration of exogenous proinflammatory cytokines promotes HPA axis activation (19), sympato-cardiovascular excitation (61, 64), and anxiety-like behavior (56), effects that are attenuated by delivery of anti-inflammatory agents (24, 34, 56, 74). Interestingly, nondiscrete central administration of an angiotensin receptor antagonist ameliorates neuroinflammation (5) and reduces anxiety-like behavior in rodents (59). Whether abrogation of AT1a signaling specifically in the PVN attenuates local neuroinflammation, which in turn is predictive of altered stress responsiveness, is unknown.

To study the physiological and behavioral consequences of chronic inhibition of hypothalamic AT1a, we utilized the Cre/LoxP system to generate mice that have AT1a selectively

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deleted from the PVN (PVN AT1a KO mice). Specifically, mice expressing Cre recombinase driven by Sim1 (Sim1-Cre) were bred to mice with the gene coding for AT1a flanked by LoxP sites (AT1a flox/flox). Sim1 is a transcription factor that is mainly expressed in the PVN (46), and therefore, Sim1 can be used to drive the expression of Cre that deletes the floxed AT1a gene specifically from the PVN. Here, we use PVN AT1a KO mice and their littermate AT1a flox/flox controls to test the hypothesis that long-term deletion of AT1a from the PVN attenuates stress-induced HPA axis activation, cardiovascular reactivity, and anxiety-like behavior.

MATERIALS AND METHODS

Animals. The details about the generation, validation, and characterization of the PVN AT1a KO mice have been described previously (14). In brief, PVN AT1a KO mice were male mice and homologous for floxed AT1a (AT1a flox/flox) and expressing Sim1-Cre. It was previously determined that Sim1-Cre on its own did not affect stress responsiveness (23), and consequently, littermate male mice that were homologous for floxed AT1a (AT1a flox/flox mice) but did not express Sim1-Cre were used as controls. All mice were at least 8 wk old at the initiation of the studies, individually housed, and given ad libitum access to pelleted rodent chow and water. All procedures were approved by the University of Florida Institutional Animal Care and Use Committee.

Dual in situ hybridization and immunohistochemistry. To confirm that the PVN AT1a KO mice indeed have AT1a specifically deleted from the PVN and to further investigate the phenotype of cells where the deletion of AT1a occurs in the PVN, we performed dual RNAscope in situ hybridization (ISH; Advanced Cell Diagnostics, Hayward, CA) for AT1a as well as immunohistochemistry (IHC) for HuC/D (neuronal specific marker) and Iba-1 (microglia marker) using monoclonal anti-HuC/D (1:500; Life Technologies, Eugene, OR) for CORT with an I125 kit (MP Biomedicals, Orangeburg, NY) as previously described (37–39).

Assessment of the cardiovascular response to stress. AT1a fibroblast cells were collected immediately after the onset of restraint to reflect basal CORT levels. Thirty minutes after the onset of restraint, blood samples were again collected, and mice were released from the restrainers. Recovery blood samples were collected at 60 and 120 min after the onset of restraint. Blood samples were centrifuged at 6,500 rpm for 15 min at 4°C. Plasma was extracted and stored at −80°C until undergoing radioimmunoassay for CORT with an I125 kit (MP Biomedicals, Orangeburg, NY) as previously described (37–39).

Testing anxiety-like behavior using elevated plus maze. Anxiety-like behavior was tested in the elevated plus maze (EPM) between 8000 and 1200 with mice (AT1a flox/flox mice, n = 36; AT1a KO mice, n = 35) that had not previously been exposed to stress. The EPM consisted of two opposing open arms (31 × 6 cm) and two opposing closed arms (31 × 6 cm) elevated 41 cm from the floor. Mice were placed in the center of the EPM and were then allowed to explore the maze freely for 5 min. To minimize the effect of olfactory cues, the maze was cleaned with 30% ethanol after each test. Testing sessions were recorded by a ceiling-mounted camera connected to a personal computer running TopScan software (CleverSys, Reston, VA).

Restraint induced HPA activation. AT1a flox/flox mice (n = 18) and PVN AT1a KO mice (n = 17) underwent a 30 min restraint challenge to evaluate the influence of AT1a expressed within the PVN on systemic corticosterone (CORT) subsequent to an acute psychogenic stressor. Mice were restrained in clear plastic ventilated tubes, and tail vein blood samples (30 µl) were collected immediately after the onset of restraint to reflect basal CORT levels. Thirty minutes after the onset of restraint, blood samples were again collected, and mice were released from the restrainers. Recovery blood samples were collected at 60 and 120 min after the onset of restraint. Blood samples were centrifuged at 6,500 rpm for 15 min at 4°C. Plasma was extracted and stored at −80°C until undergoing radioimmunoassay for CORT with an I125 kit (MP Biomedicals, Orangeburg, NY) as previously described (37–39).

Image analysis. Fluorescent images were captured with the same optimized exposure time under which fluorescence was abundantly detected in sections hybridized with the Ubc (positive control) but was undetectable in sections hybridized with DapB (negative control). The integrated density of AT1a was counted using ImageJ. The frequency of colocalizations of AT1a mRNA with either HuC/D-positive cells or Iba-1-positive cells was counted manually. It was considered a colocalization when at least two AT1a mRNA molecules were detected within a cell.
The HRV was calculated as the LF-to-HF ratio. Cardiovascular data collected at 10 s were condensed into 10 min bins. Condensed data were then averaged for genotypes to generate curves representing the systolic blood pressure, heart rate, and HRV of AT$_{1a}$ flox/flox mice and PVN AT$_{1a}$ KO mice.

**RNA isolation and cDNA synthesis.** AT$_{1a}$ flox/flox mice ($n=9$) and AT$_{1a}$ KO mice ($n=8$) were euthanized immediately after they were taken out of the housing room. Brains were extracted and flash-frozen in dry ice-cooled 2-methylbutylate. Hypothalami were dissected from flash-frozen brains in a cryostat as described previously (14). The procedure area and instruments were cleaned with RNaseZap (Ambion, Foster City, CA) to minimize RNA degradation. Collected tissues were lysed in Buffer RLT (Qiagen, Valencia, CA), and RNAs were isolated using an RNeasy Mini Kit (Qiagen). RT reactions were performed with iScript (Bio-Rad Laboratories, Hercules, CA). Semiquantitative real-time PCR. Diluted (1:5) cDNA samples, mixed with TaqMan Gene Expression Master Mix and TaqMan Probes (Applied Biosystems, Foster City, CA), were analyzed for gene expression on the StepOne Real-Time PCR system (Applied Biosystems). All samples were run in duplicate. Genes of interest included corticotropin-releasing hormone (CRH; Mm01329577), tumor necrosis factor alpha (TNF-$\alpha$; Mm00443260), interleukin 6 (IL-6; Mm00446190), interleukin 1 beta (IL-1B; Mm00434228), and cluster of differentiation molecule 11b (CD11b; Mm00434455). Ribosomal protein L32 (Mm02528467) was used as a housekeeping gene. To quantify the real-time PCR results, the expression of the gene of interest in each genotype was first normalized to the expression of L32. Subsequently, the normalized expression level was plotted as the percentage of the AT$_{1a}$ flox/flox group.

**Statistical analysis.** All data were analyzed and graphed using Prism 5 (GraphPad Software, La Jolla, CA) and are presented as means and SE. Statistical significance was determined by the appropriate analysis of variance or a one-tailed Student’s t-test.

**RESULTS**

Coexpression of Sim1-cre and AT$_{1a}$ flox/flox selectively decreases the expression of AT$_{1a}$ in neurons located within the PVN. We found that AT$_{1a}$ mRNA was predominantly localized to cells that were positive for HuC/D (Fig. 1C), indicating that within the PVN, AT$_{1a}$ are expressed on neurons. The PVN AT$_{1a}$ KO mice had significantly fewer HuC/D-positive cells that expressed AT$_{1a}$ mRNA ($t_{(4)}=8.90$, $P<0.0001$) in the PVN compared with that of AT$_{1a}$ flox/flox mice (Fig. 1G), demonstrating effective Sim1-Cre-mediated deletion of AT$_{1a}$ from PVN neurons. Moreover, relative to AT$_{1a}$ flox/flox mice, PVN AT$_{1a}$ KO mice had reduced density of AT$_{1a}$ mRNA within the PVN ($t_{(4)}=3.70$, $P<0.05$, Fig. 1H). Importantly, this effect was specific to the PVN because expression of AT$_{1a}$ mRNA in the SFO, BMA, CeA, and LH (Fig. 1, E, F, and H) was similar between PVN AT$_{1a}$ KO mice and AT$_{1a}$ flox/flox controls.

**Deletion of AT$_{1a}$ from the PVN reduces anxiety-like behavior.** To determine whether deletion of AT$_{1a}$ from the PVN decreases anxiety-like behavior, we tested PVN AT$_{1a}$ KO mice and AT$_{1a}$ flox/flox mice in the EPM. The PVN AT$_{1a}$ KO mice spent more time in the open arms ($t_{(68)}=1.82$, $P<0.05$, Fig. 2, A and B) but had similar open arm entries and travelled similar distance relative to AT$_{1a}$ flox/flox mice (Fig. 2, C and D).

Deletion of AT$_{1a}$ from the PVN reduces anxiety-like behavior and cardiovascular reactivity to stress. We measured systolic blood pressure, heart rate, and HRV in PVN AT$_{1a}$ KO and AT$_{1a}$ flox/flox mice with telemetry to test whether deletion of AT$_{1a}$ from the PVN affects cardiovascular function basally and subsequent to restraint stress. Deletion of AT$_{1a}$ from the PVN did not affect basal systolic blood pressure (Fig. 4A) but significantly attenuated ($t_{(3)}=2.37$, $P<0.05$) the integrated pressor response during restraint (Fig. 4B). However, the heart rate response to restraint was not affected by deletion of AT$_{1a}$ from the PVN (Fig. 4, C and D). We then calculated HRV as the LF-to-HF ratio with increases indicative of elevated sympathetic nervous system activity and parasympathetic withdrawal. Deletion of AT$_{1a}$ in PVN had no effect on basal HRV; however, 10 min after the onset of restraint, HRV was significantly decreased in PVN AT$_{1a}$ KO mice relative to AT$_{1a}$ flox/flox controls ($t_{(3)}=3.32$, $P<0.05$, Fig. 4E), an effect that was also present with the integrated response ($t_{(3)}=2.625$, $P<0.05$, Fig. 4F).

In addition, deletion of AT$_{1a}$ from the PVN significantly decreased adrenal gland weight relative to body weight (AT$_{1a}$ flox/flox mice, 0.473 ± 0.0217%; PVN AT$_{1a}$ KO mice, 0.416 ± 0.0251%; $t_{(33)}=1.72$, $P<0.05$).

**Deletion of AT$_{1a}$ from the PVN attenuates hypothalamic expression of proinflammatory cytokines and decreases activation of microglia.** Deletion of AT$_{1a}$ from the PVN significantly decreased hypothalamic expression of TNF-$\alpha$ ($t_{(15)}=5.40$, $P<0.0001$), IL-1B ($t_{(15)}=4.521$, $P<0.001$), and CD11b ($t_{(15)}=3.149$, $P<0.01$) (Fig. 5A). To determine whether it is possible that ANG II acts on AT$_{1a}$ expressed on microglia to promote their activation and cytokine expression, we performed RNAscope ISH for AT$_{1a}$ mRNA with IHC for Iba-1 (microglia marker) on PVN sections obtained from AT$_{1a}$ flox/flox mice. As depicted in Fig. 5, B–D, AT$_{1a}$ mRNA was not colocalized with Iba-1 in the PVN.

**DISCUSSION**

This study used genetically modified mice to test the hypothesis that chronic deletion of AT$_{1a}$ from the PVN attenuates endocrine, cardiovascular, and behavioral responses to psychogenic stress. Compared with AT$_{1a}$ flox/flox control mice, PVN AT$_{1a}$ KO mice had reduced CRH mRNA expression in the hypothalamus, lowered adrenal weight, diminished cardiovascular responses to restraint stress, and decreased anxiety-like behavior but similar plasma CORT. The altered physiological and behavioral phenotypes of PVN AT$_{1a}$ KO mice were accompanied by decreased indexes of hypothalamic inflammation and microglial infiltration. Collectively, these results suggest that deletion of AT$_{1a}$ from the PVN attenuates anxiety-like behavior and cardiovascular reactivity in response to psychogenic stressors, effects that may be the result of decreased hypothalamic inflammation and CRH expression.
Fig. 1. Coexpression of Sim1Cre and AT1a flox/flox decreases the expression of angiotensin type 1a receptor (AT1a) mRNA in neurons within the paraventricular nucleus of hypothalamus (PVN). ×2.5 images of a coronal sections through the PVN (dotted outline) of an AT1a flox/flox mouse (A) and a PVN AT1a KO mouse (B). C: ×20 image showing AT1a mRNA (depicted as punctate red dots) frequently colocalize with HuC/D (cyan, marker for neurons) in the PVN (enclosed with white dashed line) of an AT1a flox/flox mouse. Inset, high magnification image of the region enclosed with yellow dashed line in C. D: ×20 image showing that AT1a mRNA is not detected in the PVN of a PVN AT1a KO mouse (cyan indicates HuC/D). Inset, high magnification image of the region enclosed with yellow dashed line in D. ×20 image showing that AT1a mRNA (depicted as punctate red dots) frequently colocalize with HuC/D (cyan) in the SFO (enclosed with white dashed line) of an AT1a flox/flox mouse (E) and a PVN AT1a KO mouse (F). G: compared with AT1a flox/flox mice, PVN AT1a KO mice had significantly decreased number of HuC/D-positive cells (neurons) that express AT1a mRNA. H: quantification of the density of AT1a mRNA signal in different brain nuclei of AT1a flox/flox mice and PVN AT1a KO mice. SFO, subfornical organ; BMA, basomedial amygdala; LH, lateral hypothalamus; CeA, central nucleus of amygdala. Bars represent means and SE. *P < 0.05, ***P < 0.001.
We have previously confirmed that breeding Sim1-Cre mice to AT1a flox/flox mice significantly decreases ANG II binding to AT1a in the PVN without affecting binding in the SFO, supraoptic nucleus, median preoptic nucleus, and periventricular nucleus (14). The same study determined that AT1a expression was slightly, but significantly, decreased in the renal cortex, but this effect did not alter renal function (14). In the present study, Sim1-Cre also deleted AT1a from the PVN, but we conducted additional analyses to determine the cellular phenotype in which this deletion occurs. The colocalization of AT1a mRNA with HuC/D was significantly decreased in PVN AT1a KO mice, and AT1a mRNA and Iba-1 colocalizations were absent in AT1a flox/flox mice. These results indicate that Sim1-Cre-mediated deletion of AT1a predominantly occurs in neurons. Previous reports indicate that, in addition to the PVN, Sim1 is found in the LH and amygdala (4), and these brain nuclei express AT1a (25). Therefore, it is possible Sim1-Cre alters AT1a expression in the LH and amygdala; however, AT1a mRNAs in the LH, BMA, and CeA were similar between PVN AT1a KO mice and AT1a flox/flox mice. Collectively, these results indicate that Sim1-Cre deletes AT1a from PVN neurons, and consequently, any observed effects can be attributed to this specific deletion.

Central AT1a antagonism affects systemic RAS activity (44, 45), which in turn, affects renal handling of sodium, and therefore, it is possible that deleting AT1a from the PVN alters hydromineral balance. In this regard, we have previously found that under basal conditions, PVN AT1a KO and AT1a flox/flox mice have similar plasma renin activity, hematocrit, plasma protein concentration, plasma sodium concentration, and plasma glucose concentration (14). Additionally, under basal conditions, body weight and water and saline intakes are also similar (14). Collectively, these results suggest that the decreased anxiety-like behavior and inhibited stress-responsivity that we observed in PVN AT1a KO mice are not secondary to altered systemic RAS activity or impaired hydromineral balance but, rather, are the result of eliminating AT1a signaling from a brain nucleus that is known to coordinate the stress response.

The neuropeptide CRH is an established mediator of the stress response (16, 29, 65), and AT1a-expressing neurons within the PVN are implicated in its regulation. For example, studies conducted by Oldfield and colleagues (52) discovered that within the PVN, AT1a are expressed on parvocellular...
Fig. 4. Deletion of AT1α from the PVN attenuates cardiovascular reactivity to stress. Compared with AT1α flox/flox mice, PVN AT1α KO mice had decreased pressor responses (A, B) but had similar heart rate responses to restraint (C, D). Additionally, relative to AT1α flox/flox mice, PVN AT1α KO mice also had altered heart rate variability (HRV) 10 min after restraint (E) that was also evident in the integrated HRV response (F). Bars represent means and SE. *P < 0.05.

neurons with identified projections to the median eminence. Such neurons are known to produce CRH, and, indeed, mRNAs for CRH and AT1α colocalize in cells residing in the PVN (3). More recently, genetic reporting for AT1α was used in conjunction with IHC to demonstrate that AT1α-expressing neurons in the PVN colocalize with CRH immureactivity (31). In regard to functionality, acute injection or chronic infusion of exogenous ANG II into the brain increases CRH mRNA in the PVN (3, 12), indicating that AT1α stimulation in the PVN may regulate CRH gene transcription but does not contribute to stress-induced activation of the HPA axis. Another implication of these results is that AT1α may be expressed on CRH neurons in the PVN that send their axons to brain regions other than the median eminence to affect cardiovascular and/or behavioral responses to stress.

In addition to sending axons to the median eminence, CRH neurons within the PVN project to hind-brain nuclei such as the nucleus of the solitary tract (NTS) (58). The NTS is heavily involved in the regulation of sympathetic nervous activity and cardiovascular function and expresses receptors for CRH (58, 68). Central administration of CRH significantly increases plasma catecholamines and blood pressure by activating CRH receptors residing in the brain (51). We hypothesized that the decreased CRH gene transcription that we observed in the PVN AT1α KO mice may attenuate sympathetic drive during restraint stress, resulting in reduced cardiovascular reactivity. Relative to AT1α flox/flox controls, PVN AT1α KO mice had decreased adrenal weight, blunted systolic blood pressure and heart rate variability indicative of reduced cardiac sympathetic outflow in response to acute restraint stress. These results are consistent with other studies demonstrating that microinjection of the AT1α antagonist, losartan, into the PVN decreased the pressor response to acute restraint stress (10), but microinjection of ANG II into the PVN augments renal sympathetic nerve activity by activating AT1α (75). Collectively, these results suggest that AT1α expressed on CRH neurons in the PVN can be inhibited to alleviate the increased sympathetic nervous activity that accompanies psychogenic stress exposure.

In addition to increasing plasma catecholamines and blood pressure, central administration of CRH also increases anxiety-like behavior (21, 41). Mice genetically engineered to overexpress CRH display increased anxiety-like behavior (66), and administration of a CRH type 1 receptor antagonist relieves anxiety-like behavior in behavior in rats (26, 28). Oral administration of candesartan, an AT1α antagonist permeable to the blood-brain barrier, inhibits the expression of CRH in the hypothalamus (54) and decreases anxiety-like behavior in the EPM (59), suggesting that AT1α can be antagonized to suppress anxiety by altering the function of CRH neurons residing in the PVN. To the best of our knowledge, our study demonstrates for the first time that AT1α in the PVN specifically upregulates hypothalamic CRH mRNA expression.
time that specific deletion of AT₁a from PVN neurons decreases hypothalamic CRH mRNA expression as well as anxiety-like behavior as assessed in the EPM. These results conflict with those of a recent report demonstrating that selective deletion of AT₁a from CRH cells had no effect on anxiety-like behavior in the EPM (31). While these discrepancies may be explained by differences in mouse strains, testing environment, and the degree of AT₁a inhibition, it is clear that within the brain AT₁a interacts with cells that produce CRH (3, 33), and there is strong preclinical and clinical evidence that preventing this interaction may influence the etiology of stress-related disorders (9, 14, 36, 59).

In addition to upregulating AT₁a mRNA in the PVN (42), acute psychogenic stress also increases the concentration of ANG II in the brain and the periphery (73). Bloodborne ANG II can act on circumventricular organs, brain regions with an incomplete blood-brain barrier, to stimulate AT₁a that regulate stress responsivity (22, 67) (38). In contrast, under nonpathological conditions the hypothalamus is protected by the blood-brain barrier, which prevents hydrophilic peptides, like ANG II, from accessing AT₁a expressed in the PVN (6). Consequently, it is unlikely that the elevated hypothalamic ANG II that occurs subsequent to stress exposure has systemic origins, but rather, is synthesized in the brain. Consistent with this notion, microinjection of lisinopril, an ACE inhibitor that suppresses the synthesis of endogenous ANG II, into the PVN reduces the pressor response to acute restraint stress (10).

Thus, the cardiovascular and behavioral effects observed in PVN AT₁a KO mice are likely due to decreased binding of brain-derived ANG II to AT₁a expressed on CRH neurons in the PVN.

In addition to decreasing CRH mRNA, deletion of AT₁a from the PVN suppressed indexes of neuroinflammation and microglia infiltration. Clinical and preclinical studies have identified neuroinflammation as a potential contributor to the etiology of affective disorders (reviewed by Refs. 11, 30). For example, depressed patients present with higher concentrations of IL-1β in cerebrospinal fluid compared with healthy controls (43), and studies conducted in rodents found that exposure to psychological stressors significantly increased microglial infiltration and central expression of proinflammatory cytokines (7, 8, 63). Conversely, administration of exogenous proinflammatory cytokines mimics the anxiogenic and pressor responses observed during stress exposure (56, 64), and these effects are diminished by delivery of anti-inflammatory agents (32, 34, 50, 56, 64). Therefore, it is possible that the decreased neuroinflammation and microglia infiltration that occurred in PVN AT₁a KO mice contribute to their dampened stress responsiveness.

Our neuroanatomical studies failed to observe colocalization of AT₁a mRNA and the microglia marker, Iba-1, within the PVN of the AT₁a flox/flox control mice. Although unexpected, this result is consistent with other studies demonstrating that, under basal conditions, AT₁a mRNA is not present in microglia (47), and application of candesartan to primary microglia cultures does not affect microglia activation or the release of proinflammatory cytokines under basal conditions (5). These results then beg the question: How does deletion of AT₁a from neurons suppress the activation of microglia as well as the expression of proinflammatory cytokines? In this regard, microglia reside in close proximity to AT₁a-expressing neurons in the brain (13), and consequently, AT₁a-expressing neurons and microglia are positioned to affect each other’s function through paracrine signaling. Here, we propose that proinflammatory cytokines derived from AT₁a-expressing neurons in the PVN...
may serve as this paracrine signal. Bath application of ANG II to neuronal cultures stimulate the release of TNF-α and IL-1β (1), but application of candesartan suppresses ANG II-induced release of these proinflammatory cytokines (5). Microglia express receptors for proinflammatory cytokines (17, 53), and stimulation of these receptors can trigger microglia to release their own proinflammatory cytokines (40). Thus, activation of AT1a expressed on neurons within the PVN may cause these neurons to release proinflammatory cytokines, which stimulate proinflammatory cytokine receptors expressed on microglia in close proximity, which in turn, elicits their release of proinflammatory cytokines. Deletion of AT1a from neurons within the PVN may prevent this “feed-forward” paracrine signaling, thereby inhibiting microglial infiltration and release of proinflammatory cytokines within the hypothalamus. Additional studies are required to evaluate the validity of this hypothesis.

The present study found that deletion of AT1a from the PVN attenuated CRH mRNA expression, blunted cardiovascular responses to psychogenic stress, and reduced anxiety-like behavior. Although AT1a mRNA was absent in microglia, deletion of AT1a from the PVN reduced hypothalamic indexes of microglia infiltration and proinflammatory cytokine expression, and these effects may contribute to the decreased cardiovascular reactivity and anxiolytic phenotype of PVN AT1a KO mice. Because AT1a are known to regulate CRH transcription and neuroinflammation, which in turn, are implicated in a variety of stress-related neuropsychiatric disorders, it is possible that augmented AT1a signaling within the central nervous system facilitates the onset of such disorders. Consequently, brain angiotensin receptor signaling may be a viable therapeutic target for the alleviation of stress-related diseases such as anxiety disorders.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

L.W., A.D.d.K., and E.G.K. conceived and designed the research; L.W., H.H., J.A.S., A.D.d.K., and E.G.K. performed the experiments; L.W., H.H., A.D.d.K., and E.G.K. analyzed the data; L.W., H.H., A.D.d.K., and E.G.K. interpreted the results of experiments; L.W., A.D.d.K., and E.G.K. prepared figures; L.W., A.D.d.K., and E.G.K. wrote the manuscript; J.A.S., A.D.d.K., and E.G.K. performed experiments; L.W., H.H., A.D.d.K., and E.G.K. edited and revised the manuscript; L.W., H.H., J.A.S., and A.D.d.K. approved the final version of the manuscript.

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ROLE OF PVN AT1a IN STRESS AND ANXIETY


