Role of Angiotensin II Type 1A Receptors in Cardiovascular Reactivity and Neuronal Activation After Aversive Stress in Mice

Pamela J. Davern, Daian Chen, Geoffrey A. Head, Carolina A. Chavez, Thomas Walther, Dmitry N. Mayorov

Abstract—We determined whether genetic deficiency of angiotensin II Type 1A (AT1A) receptors in mice results in altered neuronal responsiveness and reduced cardiovascular reactivity to stress. Telemetry devices were used to measure mean arterial pressure, heart rate, and activity. Before stress, lower resting mean arterial pressure was recorded in AT1A−/− (85±2 mm Hg) than in AT1A+/+ (112±2 mm Hg) mice; heart rate was not different between groups. Cage-switch stress for 90 minutes elevated blood pressure by +24±2 mm Hg in AT1A+/+ and +17±2 mm Hg in AT1A−/− mice (P<0.01), and heart rate increased by +203±9 bpm in AT1A+/+ and +121±9 bpm in AT1A−/− mice (P<0.001). Locomotor activation was less in AT1A−/− (3.0±0.4 U) than in AT1A+/+ animals (6.0±0.4 U), but differences in blood pressure and heart rate persisted during nonactive periods. In contrast to wild-type mice, spontaneous baroreflex sensitivity was not inhibited by stress in AT1A−/− mice. After cage-switch stress, c-Fos immunoreactivity was less in the paraventricular (P<0.001) and dorsomedial (P=0.001) nuclei of the hypothalamus and rostral ventrolateral medulla (P<0.001) in AT1A−/− compared with AT1A+/+ mice. Conversely, greater c-Fos immunoreactivity was observed in the medial nucleus of the amygdala, caudal ventrolateral medulla, and nucleus of the solitary tract (P<0.001) of AT1A−/− compared with AT1A+/+ mice. Greater activation of the amygdala suggests that AT1A receptors normally inhibit the degree of stress-induced anxiety, whereas the lesser activation of the hypothalamus and rostral ventrolateral medulla suggests that AT1A receptors play a key role in autonomic cardiovascular reactions to acute aversive stress, as well as for stress-induced inhibition of the baroreflex. (Hypertension. 2009;54:1262-1268.)

Key Words: receptors ■ angiotensin II ■ stress ■ blood pressure ■ heart rate ■ immunohistochemistry ■ mice

Cardiovascular reactivity, a rapid sympathetically mediated increase in blood pressure in response to aversive stress, is considered a risk factor for both hypertension and heart disease.1,2 To date, the central mechanisms that control cardiovascular reactivity are not fully elucidated. However, it is plausible that the regulation of stress reactivity occurs at least at 3 major central nervous system levels. The first level includes the formation of an emotional reaction to a stimulus by cortico-limbic structures. The second consists of the activation of autonomic and endocrine outputs to the periphery by hypothalamic-brain stem circuits. The third involves suppression of negative feedback signals from baroreceptors, which would otherwise effectively counteract cardiovascular activation.

Angiotensin II (Ang II) is increasingly recognized as an important modulator of cardiovascular reactivity to stress at several central nervous system levels. First, Ang II is implicated in modulating anxiety3 and may thereby influence the integration of emotional and behavioral reactions to aversive stimuli at the limbic level. Second, Ang II is critically involved, at the hypothalamic-brain stem level, in the regulation of autonomic arousal associated with aversive events.4 In particular, we have shown recently that pharmacological blockade of Ang II type 1 (AT1) receptors in the rostral ventrolateral medulla (RVLM) abolished the pressor response to emotional stress,5 and inhibition of AT1 receptors in the dorsomedial hypothalamus (DMH) attenuates this response in rabbits.6 Third, Ang II may modulate afferent inputs from baroreceptors and other peripheral receptors in the brain stem nucleus of the solitary tract (NTS)7 and, thus, influence feedback autonomic control during stress. Finally, circulating Ang II may affect central pressor responses through receptors localized in brain circumventricular organs, which lack the blood-brain barrier.8 It is, thus, conceivable that central Ang II is capable of multiple simultaneous actions on cardiovascular reactivity. In the rodent, there are 2 forms of the AT1 receptor, and the predominant subtype in the central nervous system appears to be the AT1A subtype.9 The development of a specific AT1A receptor–deficient mouse offered us the opportunity to further explore the role of these receptors in mediating the cardiovascular response to acute stress.

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In the present study, we examined the effect of AT1A receptor deficiency on cardiovascular reactivity and neuronal activation, as detected by c-Fos immunohistochemistry, in response to acute aversive stress in mice. We used cage-switch, which is an olfactory-mediated psychosocial stress model that has been shown previously to produce a sustained 90-minute–long cardiovascular arousal and to increase in locomotor activity in mice.10 We hypothesized that altered cardiovascular reactivity in AT1A−/− mice may also relate to the differential responsiveness to stress in one or several of the aforementioned brain structures and sought to clarify their functional importance in the actions of Ang II as a stress mediator. We used a telemetry monitoring system to measure functional importance in the actions of Ang II as a stress mediator. We used a telemetry monitoring system to measure cardiovascular changes in conscious freely behaving animals and also to take into account confounding effects of locomotion on cardiovascular function.11

Methods

Animals

Experiments were conducted in conscious AT1A−/− mice (n=18) and AT1A+/+ mice (n=18), and the generation of these mice has been described earlier.12 Ten animals were used for the cardiovascular responses, and 2 of these were included in the c-Fos experiments, with an additional 8 animals to make a total of 10 (n=5 for stress and n=5 no stress). These mice were housed at the Baker IDI Heart and Diabetes Institute and were given ad libitum access to food and water. Animals were kept on a 12:12 hour light-dark cycle (6 AM to 6 PM light). The experiments were approved by the Alfred Medical Research Education Precinct Animal Ethics Committee and conducted in accordance with the Australian Code of Practice for Scientific Use of Animals.

Experimental Protocol

Under Halothane open-circuit anesthesia (Fluothane, AstraZeneca, 5% induction and 2% maintenance), AT1A−/− and AT1A+/+ mice were implanted with a PA-C10 telemetry device (Data Sciences International), with the catheter inserted into the carotid artery and the transmitter body implanted along the right flank.13 After ≥1 week of recovery, recordings of systolic arterial pressure, diastolic arterial pressure, calculated mean arterial pressure (MAP), calculated heart rate (HR), and locomotor activity were measured continuously at rest and throughout the stress protocol. Signals were sampled at 1000 Hz using an analog-to-digital data acquisition card (National Instruments 6024E), as described previously.14 Cage-switch stress was conducted over a 90-minute period by removing mice from their original cage and placing them into a cage occupied previously by a different male mouse. A second analysis was performed to calculate MAP and HR during the stress when the activity signal was below the “no-movement” threshold for ≥6 seconds. Some of the AT1A−/− and AT1A+/+ mice subjected to 90-minute cage-switch stress (n=5 per group) or no stress (left in their original cages for 90 minutes; n=5 per group) were immediately perfused and their brains removed for immunohistochemical analysis of brain sites recognized as being rich in AT1 receptors.15

Cardiovascular Reactivity to Locomotor Activity

For each mouse, least-squares regression slopes for the relationships between MAP and activity and HR and activity were calculated.

Assessment of Baroreflex by Sequence Technique

See the online Data Supplement, available at http://hyper.ahajournals.org.

c-Fos Immunohistochemistry and Analysis

See the online Data Supplement.

Statistical Analysis

Cardiovascular data were analyzed by 2-way (repeated-measure) ANOVA to determine the effects of an AT1A receptor deficiency and stress on cardiovascular parameters and locomotion. Values were
expressed as mean±SEM or mean difference±SE of the difference. Statistical evaluation of c-Fos counts was performed by 1-way multi-factor (strain, stress) ANOVA, determining the effect of strains within factors of stress and no stress, as well as the overall effect of stress across groups. Values were considered significant when P<0.05.

Results

Cardiovascular and Locomotor Responses

Figure 1 illustrates the values for MAP, HR, and locomotor activity during the basal and experimental periods. Before stress exposure, lower resting MAP was recorded in AT1A−/− mice (85±2 mm Hg) than in AT1A+/+ mice (112±2 mm Hg), whereas HR levels and locomotor activity were not different between groups. Cage-switch stress elicited prompt and sustained pressor and tachycardia responses, as well as locomotor activation, in AT1A+/+ mice (+24±2 mm Hg, +203±9 bpm, and +5.5±0.6 U, respectively, averaged over 90 minutes; n=10). The MAP, HR, and locomotor activity increased by +17±2 mm Hg, +121±9 bpm, and 2.5±0.4 U, respectively, averaged over the 90-minute period in AT1A−/− mice (n=10), which was less than that observed in AT1A+/+ mice (P<0.01). However, when expressed as a percentage of basal levels, the pressor responses (>90 minutes) were identical in both strains (21.6±1.4 versus 21.1±2 mm Hg in AT1A+/+ and AT1A−/− mice, respectively). Comparing only the initial 20-minute period, the changes in blood pressure and activity were similar in both strains, but the tachycardia response to cage-switch stress was attenuated in AT1A−/− mice by 25.3% (P<0.001).

A further analysis of selected periods of little activity was determined by a minimum activity threshold (Figure 2). Average values for activity during stress were reduced to 0.07±0.04 and 0.05±0.01 U over the 90-minute period in AT1A+/+ and AT1A−/− mice, respectively, and represented 1.2% and 2.0% of the normal activity level. During these still periods, the pressor and tachycardia responses to cage-switch stress in AT1A−/− mice were 57% and 66% of those observed in AT1A+/+ mice, respectively (P<0.01; Figure 2; n=8 per group).

Relationship Between Locomotor Activity and MAP

There was a positive relationship between activity and MAP during cage-switch stress, which was similar between AT1A+/+ (slope: 4.2±1.0 mm Hg per unit of activity; r=0.52; n=10) and AT1A−/− mice (slope: 5.7±0.6 mm Hg per unit of activity; r=0.63; n=10; P=0.25 for difference between strains). Similarly, the HR relationship with activity during cage-switch stress was also similar in both strains (AT1A+/+ slope: 24.8±5.7 mm Hg per unit of activity, r=0.62, n=10; AT1A−/− mice slope: 32.1±4.2 mm Hg per unit of activity, r=0.79, n=10).

Spontaneous Baroreceptor Reflex Sensitivity

Baroreflex sensitivity as assessed as the slope of spontaneous up and down sequences was similar in AT1A−/− (n=9) compared with AT1A+/+ mice (n=10). The number of baroreflex-dependent sequences and the baroreflex activation index (percentage of baroreflex to total number of sequences) was also similar (Figure 3). Cage-switch stress was accom-
panied by a marked decrease in baroreflex sensitivity of AT$_{1A}^{-/-}$ mice, as well as a marked increase in the number of baroreflex sequences \((P<0.05)\). Conversely, AT$_{1A}^{+/+}$ mice exhibited no baroreceptor reflex gain inhibition during stress, nor did they increase the number of sequences per minute during stress (Figure 3). Thus, the level of baroreflex sensitivity during stress was \(69\%\) greater in AT$_{1A}^{+/+}$ compared with AT$_{1A}^{-/-}$ mice (average of up and down sequences, \(P<0.0004\)). Stress did not alter the baroreflex activation index in either group (Figure 3). A control experiment was also performed with no cage-switch or disturbance that showed no changes to baroreflex gain, numbers of sequences, or baroactivation index (Figure 4; \(n=10\) per group).

c-Fos Immunohistochemistry

Cage-switch stress was accompanied, in AT$_{1A}^{+/+}$ mice, by an increase in c-Fos expression in all of the brain regions studied, except the subfornical organ (Table). This stress-induced increase in c-Fos levels was reduced by 73\% in the central nucleus of the amygdala (CeAm; Figure 5A and 5B), 65\% in the raphe pallidus, 47\% in the paraventricular nucleus (PVN; Figure 5C and 5D), 44\% in the RVLM \((P<0.001)\), and by 69\% in the bed nucleus of the stria terminalis, and 47\% in the DMH \((P<0.001)\) in AT$_{1A}^{+/+}$ mice compared with AT$_{1A}^{-/-}$ mice (\(n=5\); Table). Conversely, c-Fos expression after cage-switch stress was greater by 126\% in the NTS, 99\% in the medial nucleus of the amygdala (MeAm; Figure 5G and 5H), 53\% in the caudal ventrolateral medulla \((P<0.001)\), and by 85\% in the organum vasculosum of the lamina terminalis \((P=0.02)\) in AT$_{1A}^{-/-}$ mice compared with AT$_{1A}^{+/+}$ mice (Table). c-Fos expression was also observed in other brain regions recognized as being rich in AT$_{1A}$ receptors,\(^{15}\) including the median preoptic nucleus in the forebrain and area postrema in the hindbrain; but cell counts were quite low, and we did not observe a

![Figure 3. Average baroreflex gain (bpm per millimeter of mercury) is determined from the slope from down and up sequences (top). The total number of sequences (middle) and baroreflex activation index (number of baroreflex-related sequences as the percentage of total sequences) in AT$_{1A}^{+/+}$ mice (left; \(n=10\)) and AT$_{1A}^{-/-}$ mice (right; \(n=9\)) before and during cage-switch stress. \(*P<0.05\) for effect of stress.](image1)

![Figure 4. Average baroreflex gain (bpm per millimeter of mercury) is determined from the slope from down and up sequences (top). The total number of sequences (middle) and baroreflex activation index (number of baroreflex related sequences as the percentage of total sequences) in AT$_{1A}^{+/+}$ mice (left; \(n=10\)) and AT$_{1A}^{-/-}$ mice (right; \(n=10\)) before and during a period of no stress.](image2)
Despite the higher c-Fos–positive cell counts in the MeAm, the pressor, tachycardic, and locomotor responses were less important role for AT1A receptors in the DMH, because.

We observed a 2-fold greater level of c-Fos immunoreactivity in AT1A−/− mice in the MeAm, which is a critical region known to be activated by an aversive olfactory stimulus in the rodent. Our recent findings suggest an important role for AT1A receptors in the DMH, because.

<table>
<thead>
<tr>
<th>Brain Region</th>
<th>AT1A+/* (n=5)</th>
<th>AT1A−/* (n=5)</th>
<th>AT1A+/* vs AT1A−/*</th>
<th>Effect of Stress</th>
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<td>NTS</td>
<td>4.2±1.2</td>
<td>3.6±1.0</td>
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<td>MeAm</td>
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<td>CVLM</td>
<td>2.7±0.7</td>
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<td>NS</td>
<td>P&lt;0.001</td>
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<td>OVLT</td>
<td>0±0</td>
<td>0±0</td>
<td>NS</td>
<td>P&lt;0.001</td>
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<tr>
<td>CeAm</td>
<td>1.3±0.3</td>
<td>0.7±0.3</td>
<td>NS</td>
<td>P&lt;0.001</td>
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<tr>
<td>RPa</td>
<td>1.8±0.5</td>
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<td>NS</td>
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<td>PVN</td>
<td>1.7±0.6</td>
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<td>RVLM</td>
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<tr>
<td>BST</td>
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<td>1.4±0.7</td>
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<td>P&lt;0.001</td>
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<td>DMH</td>
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<td>5.0±1.9</td>
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<td>P&lt;0.001</td>
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<tr>
<td>AP</td>
<td>1.8±0.6</td>
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<td>NS</td>
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<td>MnPO</td>
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<td>0.2±0.1</td>
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<td>P&lt;0.001</td>
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<tr>
<td>SFO</td>
<td>0.1±0.1</td>
<td>0.2±0.1</td>
<td>NS</td>
<td>P&lt;0.001</td>
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</table>

Mean No. of activated neurons, as detected by c-Fos-immunoreactivity, in nonstressed mice (left) and mice exposed to 90-mins of cage-switch stress (right). CVLM indicates caudal ventrolateral medulla; OVLT, vascular organ of the lamina terminalis; RPa, raphe pallidus; BST, bed nucleus of the stria terminalis; AP, area postrema; MnPO, median preoptic nucleus; SFO, subfornical organ; NS, not significant.

Significant difference between groups for these regions (Table). There was little c-Fos expression in most brain regions of control unstressed animals in either group (n=5 per group). Activation was observed in the DMH, but this was one tenth of the effect of cage-switch stress.

Discussion

The major findings from the present study were that the sustained pressor, tachycardic, and locomotor activity responses to a 90-minute cage-switch stress were attenuated in AT1A−/− compared with normal AT1A+/+ mice together with a reduced number of c-Fos–stained cells in specific brain regions, including the DMH, RVLM, PVN, and raphe pallidus. These regions are known to be important for the expression of the autonomic responses to stress. Together, these findings suggest that activation of AT1A receptors, presumably by Ang II, normally facilitate the autonomic manifestations through the hypothalamic-brain stem pathways. This suggests that the lesser pressor response to stress in AT1A−/− is not attributed to a “nonspecific” effect of lower blood pressure or to possible differences in spinal16 or ganglionic17 transmission or peripheral vascular reactivity.

We observed a 2-fold greater level of c-Fos immunoreactivity in AT1A−/− mice in the MeAm, which is a critical region known to be activated by an aversive olfactory stimulus in the rodent18 and also for mediating the pressor responses to stress.19 Thus, the stimulus may have even been greater in the AT1A−/− mice. Despite the higher c-Fos–positive cell counts in the MeAm, the pressor, tachycardic, and also locomotor responses were less in the AT1A−/− mice. Therefore, an even greater attenuation might be expected with an equal level of emotional response.

Our study provides evidence for a link between the physiological response to stress and the activation of the MeAm and DMH involving the AT1A receptor. However, we cannot determine from our study whether this is a direct effect of the absence of receptors in the region or an indirect one from an altered input to these regions. Furthermore, the c-Fos technique is limited in that it only detects neurons that are activated and not those that are inhibited. There is electrophysiological evidence for an angiotensinergic projection from the MeAm to the anterior hypothalamic area that involves AT1 receptors20; however, we are not aware of whether a projection to the DMH has been studied. Contrasting the findings in the MeAm, we observed lesser c-Fos expression in the CeAm and bed nucleus of the stria terminalis, which are the only regions in the basal forebrain that have been shown to respond to an increase in blood pressure.21 Thus, the lower c-Fos counts in these regions might reflect the lesser overall increase in blood pressure and HR in the AT1A receptor–deficient mice. Although the c-Fos counts are likely to reflect a somewhat greater contribution from the first 20-minute period when there was a little difference in the rise in blood pressure between strains, there is still a significant contribution from the later times when a difference was observed.

Previous studies have shown an increase in blood pressure during experimentally induced acute aversive stress in several species, including mice,14 rats22,23 and rabbits.5,6,24 The DMH has been described as the core of the hypothalamic defense area because of its essential involvement in regulating the cardiovascular responses to stress.6,24 In addition, it is the common output center both for the vasconstrictor (via the RVLM) and for the expression of the tachycardia via spinally projecting raphe pallidus neurons receiving inputs from the DMH.25 Furthermore, the DMH has high levels of AT1 receptor binding and AT1A receptor mRNA,26 which makes it a most likely site for the effect of AT1A receptor deletion on the cardiovascular responsivity to aversive stress. Our recent findings suggest an important role for AT1A receptors in the DMH, because microinjections of candesartan into the DMH dose-dependently attenuated the hypertensive effect of aversive stress in rabbits.24 Like the DMH, the RVLM is rich in AT1 receptors,27 and functional studies have identified that sympathetic premotor neurons in the RVLM are heavily inner-
vated by excitatory inputs arising from the DMH. Earlier, we showed that blockade of AT₁ receptors in the RVLM of rabbits attenuates pressor responses induced by aversive (air-jet) stress. This is further supported by other studies that showed that ICV administration of the angiotensin peptide antagonist saralasin inhibited the pressor response to restraint stress in rats. In the present study, reduced cardiovascular reactivity (pressor response and tachycardia) and attenuated neuronal activation in the DMH compared with AT₁A² mice is observed in AT₁A⁻/⁻ mice (n=5; A, C, E, and G) and AT₁A⁻/⁻ mice (n=5; B, D, F, and H) after 90 minutes of cage-switch stress. c-Fos immunoreactivity can be seen throughout many nuclei in the CeAm, PVN, and DMH in AT₁A⁻/⁻ mice (A, C, and E) with less activation in AT₁A⁻/⁻ mice in each of these brain regions (B, D, and F). By contrast, greater c-Fos expression can be seen in the MeAm of AT₁A⁻/⁻ mice (H) compared with AT₁A⁻/⁻ mice (G). Scale bar: 100 μm.

Figure 5. Photomicrographs of coronal sections through the CeAm (A and B), PVN (C and D), DMH (E and F), and MeAm (G and H) of AT₁A⁻/⁻ mice (n=5; A, C, E, and G) and AT₁A⁻/⁻ mice (n=5; B, D, F, and H) after 90 minutes of cage-switch stress. c-Fos immunoreactivity can be seen throughout many nuclei in the CeAm, PVN, and DMH in AT₁A⁻/⁻ mice (A, C, and E) with less activation in AT₁A⁻/⁻ mice in each of these brain regions (B, D, and F). By contrast, greater c-Fos expression can be seen in the MeAm of AT₁A⁻/⁻ mice (H) compared with AT₁A⁻/⁻ mice (G). Scale bar: 100 μm.

Our results are also relevant to the autonomic regulation by the baroreflex, because greater c-Fos expression was observed in the caudal ventrolateral medulla and NTS accompanied by an absence of stress-induced baroreflex inhibition in AT₁A⁻/⁻ mice compared with AT₁A⁺/⁺ mice. These brain regions are important for baroreflex regulation, and the greater c-Fos expression quantitatively reflects a difference in baroreflex responsivity between strains during stress. Previous studies have demonstrated that endogenously formed Ang II acts in the NTS to release NO, which leads to reduced neuronal activity and inhibition of the baroreflex pathways. These data suggest that activation of AT₁A receptors, possibly in the NTS, may play a key role in the stress-induced inhibition of the HR baroreflex in mice. Greater neuronal activation in the caudal ventrolateral medulla of AT₁A⁻/⁻ mice, a major relay site in baroreflex transmission, could further contribute to the lack of baroreflex function during stress in these animals. No differences in baroreflex gain were observed...
in AT1A−/− mice at rest suggesting that, under normal conditions, there is little activation of AT1A receptors.

Perspectives
Our study using genetic deletion of AT1A receptors in mice suggests that brain Ang II is an important modulator of pressor, cardiac, and locomotor responses to acute aversive stress. Although our study does not specify the precise role of these receptors at the level of individual nuclei, the differential pattern of c-Fos between the strains suggests that AT1A receptors may play several discrete roles. We propose that, under conditions of acute stress, AT1A receptors in the limbic system or cortex may play an important role in suppressing the fear/anxiety response but may act within limbic and hypothalamic nuclei to facilitate both cardiovascular and locomotor activation. Because both HR and pressor responses were similarly affected by the absence of AT1A receptors, the regions likely responsible are the DMH and RVLM, both of which are known to be important for the regulation of these responses. We speculate that AT1 receptor activation may, therefore, reinforce the cardiovascular adaptive reactions of increasing cardiac output and directing blood to muscles for flight or fight. In addition, Ang II can also limit the negative feedback of the pressor response by the baroreflex that would try to counteract the rise in pressure, possibly by modulating the pathway from the hypothalamus to the NTS. Conversely, AT1A receptors in circumventricular organs appear to play a limited role in the acute stress-induced cardiovascular activation.

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Disclosures
None.

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