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Angiotensin II Type 2 Receptor–Expressing Neurons in the Central Amygdala Influence Fear-Related Behavior

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ABSTRACT

BACKGROUND: The renin-angiotensin system has been implicated in posttraumatic stress disorder; however, the mechanisms responsible for this connection and the therapeutic potential of targeting the renin-angiotensin system in posttraumatic stress disorder remain unknown. Using an angiotensin receptor bacterial artificial chromosome (BAC) and enhanced green fluorescent protein (eGFP) reporter mouse, combined with neuroanatomical, pharmacological, and behavioral approaches, we examined the role of angiotensin II type 2 receptor (AT_2R) in fear-related behavior.

METHODS: Dual immunohistochemistry with retrograde labeling was used to characterize AT_2R-eGFP$^+$ cells in the amygdala of the AT_2R-eGFP-BAC reporter mouse. Pavlovian fear conditioning and behavioral pharmacological analyses were used to demonstrate the effects of AT_2R activation on fear memory in male C57BL/6 mice.

RESULTS: AT_2R-eGFP$^+$ neurons in the amygdala were predominantly expressed in the medial amygdala and the medial division of the central amygdala (CeM), with little AT_2R-eGFP expression in the basolateral amygdala or lateral division of the central amygdala. Characterization of AT_2R-eGFP$^+$ neurons in the CeM demonstrated distinct localization to gamma-aminobutyric acidergic projection neurons. Mice receiving acute intra–central amygdala injections of the selective AT_2R agonist compound 21 prior to tests for cued or contextual fear expression displayed less freezing. Retrograde labeling of AT_2R-eGFP$^+$ neurons projecting to the periaqueductal gray revealed AT_2R-eGFP$^+$ neuronal projections from the CeM to the periaqueductal gray, a key brain structure mediating fear-related freezing.

CONCLUSIONS: These findings suggest that CeM AT_2R-expressing neurons can modulate central amygdala outputs that play a role in fear expression, providing new evidence for a novel angiotensinergic circuit in the regulation of fear.

Keywords: Amygdala, Angiotensin II, AT_2R, Pavlovian fear conditioning, PTSD, Mouse

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Current evidence-based treatment options for posttraumatic stress disorder (PTSD), including psychotherapy and pharmacological approaches, provide limited benefits in a substantial proportion of individuals (1–4), with treatment mechanisms that are not fully understood. An improved neurobiological understanding of this disorder is therefore critical for the development of new treatment and prevention strategies. A growing body of evidence implicates the renin-angiotensin system (RAS), a regulator of blood pressure and fluid homeostasis, as a potential therapeutic target for PTSD (5–9). Retrospective clinical studies suggest that RAS blockade reduces the severity of PTSD symptoms (5,6). More recently, the angiotensin receptor blocker losartan was shown to affect amygdalar activity and emotional processing in high–trait anxiety individuals (8,10). Preclinical studies confirm and extend these findings by demonstrating that angiotensin II (Ang II) type 1 receptor (AT_1R) inhibition (7,11,12) or deletion of AT_1R from select neuronal populations in the brain facilitates fear extinction (13). Collectively, these data suggest that the brain RAS may be an important therapeutic target in PTSD. However, the underlying mechanisms and the potential roles of other central Ang II receptors remain unclear.

Ang II is the principal effector peptide of the RAS and mediates its effects by binding to two primary receptor subtypes: the AT_1R and the Ang II type 2 receptor (AT_2R) (14,15). AT_1R and AT_2R subtypes are expressed throughout the brain in similar regions but on different cellular populations (16–19). In addition, the Ang II peptide can have both direct excitatory and inhibitory effects in the brain (20,21). While the AT_1R in hypothalamic, forebrain, and brainstem regions mediating cardiovascular and neuroendocrine regulation is widely studied (22–24), much less is known regarding brain AT_2R function.

AT_2R is considered to be a protective arm of the RAS (25,26) because it exerts functional effects (e.g., anti-inflammatory, antiproliferative, antihypertensive) that oppose those produced by the AT_1R. Preclinical studies using the selective nonpeptide

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AT_2R agonist compound 21 (C21) have shown therapeutic potential for the reduction of hypertension, stroke, and inflammation (27–29). In addition, activation of the brain AT_2R with C21 is both neuroprotective (30–32) and cognition enhancing (33). Further studies using AT_2R-deficient mice (*Agtr2*$^{-/-}$) suggests a role for this receptor in mediating emotional stress and stress-related learning tasks (34–36).

To further understand these mechanisms, and to build on the growing evidence linking brain Ang II receptors to fear and anxiety (7,12,13,36,37), we sought to investigate the role of the brain AT_2R in fear memory and behavior. Here we examined the distribution, characteristics, and connectivity of AT_2R-expressing cells within the brain, testing the hypothesis that site-specific activation of brain AT_2R attenuates the behavioral and physiological responses to conditioned fear. We proposed that endogenous Ang II acting on the brain AT_1R and AT_2R differentially modulates fear memory through a balance of strengthening excitatory, and/or weakening inhibitory, synaptic inputs that regulate fear memory. Further understanding of the brain angiotensin system in fear learning may provide novel and improved therapeutic strategies for PTSD.

METHODS AND MATERIALS

Animals

All procedures were approved by the Institutional Care and Use Committee of The George Washington University and were in compliance with National Institutes of Health guidelines. Adult male C57BL/6J mice (8–10 weeks old) from Jackson Laboratory (Bar Harbor, ME) were housed in a temperature- and humidity-controlled room on a 12-hour light/dark cycle with water and food available ad libitum. AT_2R-enhanced green fluorescent protein (eGFP) reporter mice that express eGFP in cells that express AT_2R (17) were used for immunohistochemical and tracing studies. In addition, AT_2R-eGFP mice were crossed with *Gad2-T2A-NLS-mCherry* mice (#023140; Jackson Laboratory), which express mCherry in the nuclei of gamma-aminobutyric acidergic (GABAergic) neurons.

Immunohistochemistry

To examine the distribution and characteristics of eGFP AT_2R-expressing cells within amygdala, mice were anesthetized with ketamine/xylazine and perfused transcardially with saline followed by 4% paraformaldehyde. The brains were then postfixed overnight in 4% paraformaldehyde and transferred into a 30% sucrose solution. After a 2-day dehydration period, the brains were embedded in optimal cutting temperature compound (Thermo Fisher Scientific, Waltham, MA) and stored at -80°C until sectioned using a cryostat (CryoStar NX50; Thermo Fisher Scientific). Additional details are provided in the Supplement.

Animal Surgery

Mice were anesthetized with ketamine/xylazine (intraperitoneal), and stainless steel guide cannulas (0.24-mm inner diameter, 0.46-mm outer diameter; Plastics One, Roanoke, VA) were bilaterally implanted into the central amygdala (CeA) at 0.8 mm caudal to bregma, ±2.9 mm lateral to bregma, and 4.9 mm below the skull surface. Cannulas were fixed in position with dental cement anchored to the skull by a stainless steel screw screwed into the cranium. Mice were individually housed for a 10-day postoperative period and handled daily for 5 days to familiarize them with the injection procedure.

Drug and Retrograde Tracer Administration

The specific nonpeptide AT_2R agonist C21 (Vicore Pharma, Gothenburg, Sweden) (28), which has a plasma half-life of approximately 4 to 6 hours (31,38), was infused into the CeA bilaterally (0.06 mg/mL) with an UltraMicroPump III and microprocessor controller (World Precision Instruments, Sarasota, FL) through the internal cannula (0.1-mm inner diameter, 0.2-mm outer diameter; Plastics One). The intra-CeA C21 concentration was determined based on previous in vivo application concentration ranges (38–40). A total volume of 200 nL was injected at a rate of 100 nL/min. For retrograde tracing studies, cholera toxin B (CTB) was injected directly into the periaqueductal gray (PAG) of AT_2R-eGFP reporter mice. Additional details can be found in the Supplement.

Pavlovian Fear Conditioning (Cued and Contextual)

Cued fear conditioning was performed to examine the effects of an intra-CeA C21 injection on cue-dependent fear memory as previously described (7,41). Briefly, mice received 5 conditioned stimulus (CS)/unconditioned stimulus (US) pairings of a 30-second auditory cue (6 kHz, 75 db) coterminating with a mild footshock (0.5 seconds, 0.5 mA) and an intertrial interval of 3 minutes. Extinction training was initiated 24 hours after fear conditioning. Animals first received bilateral C21 or vehicle injections into the CeA as described above, and 10 minutes later animals were introduced to the extinction chamber. After a 5-minute pre-CS period, mice received 20 CS trials (30 seconds each) with 30-second intertrial intervals. For extinction tests, mice were placed into the same chamber 24 hours after extinction training was initiated and were exposed to an additional 20 CS trials.

In a separate group of animals, contextual fear conditioning was performed to examine the effects of intra-CeA C21 on context-dependent fear memory. Following a 3-minute preshock period, animals received a series of 7 electric foot shocks (0.8 mA, 1 second) spaced by random intervals varying between 15 and 120 seconds over 7 minutes. The same procedure was repeated 24 hours later on conditioning day 2. After another 24-hour period (day 3), animals received bilateral C21 or vehicle injections into the CeA and 10 minutes later were reintroduced to the conditioning chamber for a 30-minute fear expression test. After another 24 hours (day 4), the same procedure was repeated without any drug injection. Percentage freezing behavior was recorded and quantified using Freezeframe 3.32 (Coulbourn Instruments, Holliston, MA).

Generalized Anxiety Measures

Open field test and elevated plus maze (EPM) tests were used to determine the effect of intra-CeA AT_2R activation on basal anxiety levels. Mice were placed in the center of the open field 10 minutes after C21 or vehicle injection and were allowed to freely explore for 30 minutes. Their activities during this period were recorded and analyzed using the TRU SCAN Activity Monitoring System (Coulbourn Instruments, Whitehall, PA). For

EPM testing, mice were put onto the center area of the maze facing the same closed arm 10 minutes after C21 or vehicle injection and were recorded for 5 minutes. Total arm entries, open arm entries, and the percentage of time spent in the open arm were analyzed using the TopScan software suite (CleverSys, Reston, VA).

Corticosterone Enzyme-Linked Immunosorbent Assay

Plasma corticosterone was measured 30 minutes following contextual fear recall with a commercially available enzyme-linked immunosorbent assay kit (LDN, Nordhorn, Germany). See Supplement for details.

Data Presentation and Statistical Analysis

Data in this study are expressed as mean ± SEM, with $p < .05$ being considered as statistically significant. All statistical analyses were performed using Prism 6.0 (GraphPad Software, San Diego, CA). Unpaired two-tailed Student's t tests were used when comparing two groups. When comparing more than two groups, a one-way analysis of variance followed by Newman-Keuls multiple comparisons post hoc test was used.

RESULTS

Intra-CeA C21 Administration Reduces Fear Expression Following Cued and Contextual Fear Conditioning

AT_2R-eGFP reporter mice expressed a high level of eGFP expression in the amygdala, particularly within the central (CeA: 154.5 ± 7.9 cells/mm^2) and medial (medial amygdala: 221.2 ± 15.3 cells/mm^2) subnuclei, with lower eGFP in the basolateral amygdala (11.7 ± 1.8 cells/mm^2) (Figure 1A–C). To determine whether CeA-AT_2R contributes to expression and extinction of fear memory, we examined the effects of the AT_2R agonist C21 (28) (Figure 1D). Prior to intra-CeA drug injections, both groups exhibited a progressive increase in freezing behavior during the 5 CS/US pairings (Figure 1F). The next day animals received CeA microinjections of either saline or C21 and were exposed to extinction training. A reduction in initial fear expression was observed in the C21 group during the first 5 CS block (vehicle CS 1–5: 75 ± 5% freezing; C21 CS 1–5: 55 ± 7% freezing; $p < .05$), while no differences in percentage freezing were found between groups (vehicle CS 6–20: 52 ± 5; C21 CS 6–20: 38 ± 6; $p > .05$) during CS 6 to 20. Over the course of extinction training (CS 1–20) a within-session extinction effect was observed, as freezing behavior in the vehicle group significantly decreased (vehicle CS 1–5: 75 ± 5% freezing; vehicle CS 6–20: 52 ± 5% freezing; $p < .05$) over time. However this within-group reduction did not occur in the C21-treated animals, likely because these animals exhibited significantly lower freezing from the start of the session (Figure 1G).

To determine whether intra-CeA C21 could modify long-term extinction memory, a final extinction test 24 hours later (day 3) in the absence of the drug was administered. This day 3 test reflects extinction recall, and no between-group differences in freezing were observed at this time point (vehicle CS 1–5: 66 ± 6; C21 CS 1–5: 51 ± 9; $p > .05$; vehicle CS 6–20: 35 ± 5; C21 CS 6–20: 32 ± 6; $p > .05$) (Figure 1H). These findings suggest that intra-CeA C21 administration suppresses fear expression to a conditioned auditory cue, while extinction recall 24 hours later is unaffected by C21.

Next, to determine whether C21 inhibits the expression of contextual fear memory, a separate group of mice were fear conditioned and 24 hours later were reexposed to the conditioned context following intra-amygdala C21 infusions (Figure 1I, J). Animals that received C21 showed a reduced freezing response upon reexposure to the conditioning context relative to vehicle controls (vehicle 0–5 minutes: 69 ± 4; C21 0–5 minutes: 54 ± 4; $p < .05$; vehicle 6–30 minutes: 43 ± 5; C21 6–30 minutes: 30 ± 3; $p < .05$) (Figure 1L). One full day (24 hours) later, and in the absence of drug, group differences persisted throughout the contextual memory test (vehicle 0–5 minutes: 52 ± 7; C21 0–5 minutes: 28 ± 5; $p < .05$) (Figure 1M). Together these findings suggest that intra-CeA C21 has enduring effects following contextual but not cued fear conditioning.

Activation of CeA-AT_2R With C21 Decreases Plasma Corticosterone Following Context Reexposure

Activation of the hypothalamic-pituitary-adrenal axis, reflected by an increase in corticosterone levels, occurs in response to stress and conditioned fear stimuli (42). In a separate group, we therefore evaluated the effects of intra-CeA C21 during conditioned context reexposure (Figure 2A, B). In addition to increased freezing behavior in the conditioned context, there was a significant rise in plasma corticosterone (control: 65.8 ± 2.6 ng/mL; context: 98.3 ± 10 ng/mL; $t_6 = 3.14$, $p = .02$) (Figure 2C). This response was blunted in mice receiving intra-CeA C21 prior to context reexposure (vehicle: 104.2 ± 3.9 ng/mL; C21: 76.0 ± 8.1 ng/mL; $t_{13} = 3.262$, $p = .006$) (Fig 2D). These data suggest that intra-CeA C21 modulates the hypothalamic-pituitary-adrenal axis by attenuating the release of corticosterone in response to conditioned fear context reexposure.

Activation of CeA-AT_2R With C21 Does Not Alter General Anxiety-like Behavior or Locomotor Activity

Treatments that affect anxiety-like behavior or motor activity can confound measurements of freezing behavior. Therefore, to determine whether AT_2R activation in the CeA has off-target (i.e., locomotor) effects that might account for the differences observed in our fear conditioning experiments, we injected C21 into the CeA prior to EPM and open field tests (Figure 2E). C21 did not affect the total distance traveled (vehicle: 35.7 ± 2.6 m; C21: 42.1 ± 3.6 m; $t_{21} = 1.471$, $p = .16$) or the number of center entries (vehicle: 104 ± 10; C21: 117 ± 9; $t_{21} = 0.975$, $p = .34$), although there was a statistical trend for an increase in center time in the open field (vehicle: 618 ± 66 seconds; C21: 808 ± 91 seconds; $t_{21} = 1.741$, $p = .10$) (Figure 2F; see also Supplemental Figure S2). The EPM revealed no group differences in the percentage of time spent in the open arms (vehicle: 19.7 ± 4.7%, $n = 9$; C21: 14.4 ± 3.6%; $t_{17} = 0.912$, $p = .37$), the number of open arm entries (vehicle: 12 ± 1, $n = 9$;

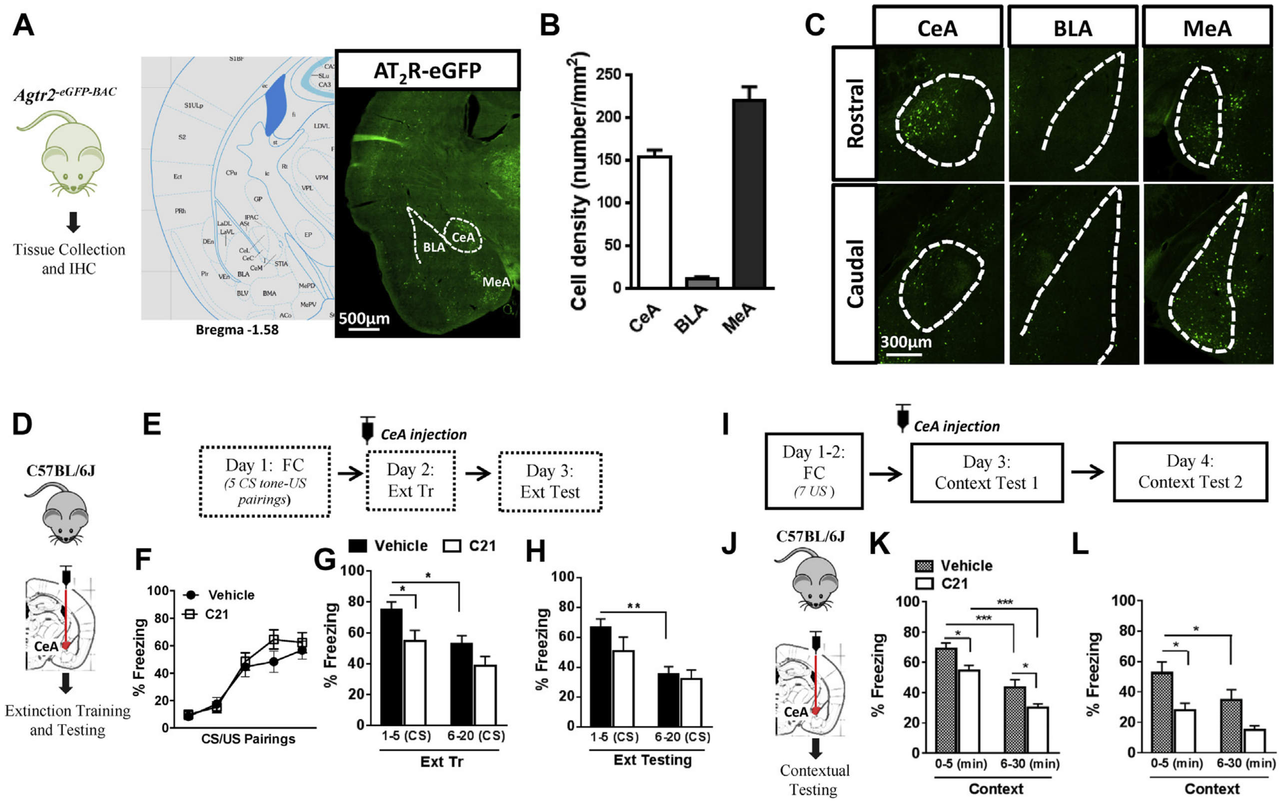

Figure 1. Intra-central amygdala (CeA) angiotensin II type 2 receptor (AT$_2$R) activation reduces fear expression. **(A)** Representative coronal sections through the amygdala of the AT$_2$R-enhanced green fluorescence protein (eGFP) reporter mice. **(B)** Quantification of AT$_2$R-eGFP$^+$ cells in different amygdala subdivisions. **(C)** Representative rostral-caudal images of AT$_2$R-eGFP$^+$ cells from the amygdala of AT$_2$R-eGFP reporter mice. All staining was performed on 4 mouse brains ($n = 4$). **(D, E)** Cued fear conditioning (FC) protocol. **(F)** Freezing behavior during cue-dependent fear conditioning. **(G, H)** Freezing behavior during extinction training (Ext Tr) **(G)** and extinction test (Ext Test) **(H)** ($n = 11$–12; $^*p < .05$, $^{**}p < .01$). **(I, J)** Contextual FC protocol. **(K, L)** Freezing behavior during context test day 1 **(K)** and day 2 **(L)** ($n = 16$–17 for test day 1 and $n = 9$–10 for test day 2; $^*p < .05$, $^{***}p < .001$). BAC, bacterial artificial chromosome; BLA, basolateral amygdala; C21, compound 21; CS, conditioned stimulus; IHC, immunohistochemistry; MeA, medial amygdala; US, unconditioned stimulus.

C21: 11 ± 2; $t_{17} = 0.489$, $p = .63$), or the total number of open/closed arm entries (vehicle: 25 ± 2; C21: 26 ± 2; $t_{17} = 0.193$, $p = .85$) (Figure 2G). Despite a trend for increased time spent in the center of the open field, there were no group differences in all other parameters measured, suggesting that intra-CeA C21 does not alter locomotor or generalized anxiety behavior.

Distribution and Characterization of AT$_2$R$^+$ Cells in the Amygdala

To further investigate the distribution of AT$_2$R throughout the CeA, immunohistochemistry was performed on CeA sections from AT$_2$R-eGFP mice (Figure 3A). As seen in Figure 3B, C, AT$_2$R-eGFP$^+$ cells were highly expressed in the medial (CeM) region (225 ± 9.4 cells/mm^2), but not in the lateral (CeL) region (3.2 ± 0.8 cells/mm^2), of the CeA. Sections were costained with antibodies for protein kinase C delta (PKC-δ), a protein expressed in the lateral portion, but not in the medial portion, of the CeA (Figure 3E, H) (43). The lack of colocalization between eGFP and PKC-δ (0/789 cells) in the CeL further suggests that expression of AT$_2$R within the CeA is restricted to the CeM (Figure 3D–I).

To determine whether AT$_2$R in the CeM was of neuronal or glial origin, AT$_2$R-eGFP$^+$ cells were costained with a neuronal marker (neuronal nuclei), an astrocytic marker (glial fibrillary acidic protein), an oligodendritic marker (adenomatous polyposis coli), and a microglia/macrophage-specific protein (ionized calcium binding adapter molecule 1). AT$_2$R-eGFP$^+$ cells were highly expressed with neuronal nuclei (457/462 cells, 99%), whereas only 2 of 266 (0.75%) colocalized with adenomatous polyposis coli (Supplemental Figure S1). No colocalization was observed with glial fibrillary acidic protein or ionized calcium binding adapter molecule 1 staining (Supplemental Figure S1). AT$_2$R-eGFP$^+$ mice were then crossed with glutamatic acid decarboxylase (GAD)-mCherry animals to determine whether AT$_2$R is expressed on GABAergic neurons (Figure 3J) as identified in other brain regions (26). As shown in Figure 3K–N, the vast majority of eGFP$^+$ cells were colocalized with GAD-mCherry, suggesting that the population of AT$_2$R-eGFP$^+$ cells in the CeM predominantly consists of GABAergic neurons.

Additional immunohistochemistry was performed to classify AT$_2$R-eGFP$^+$ cells as either projection neurons or interneurons. Brain sections were stained with five interneuronal

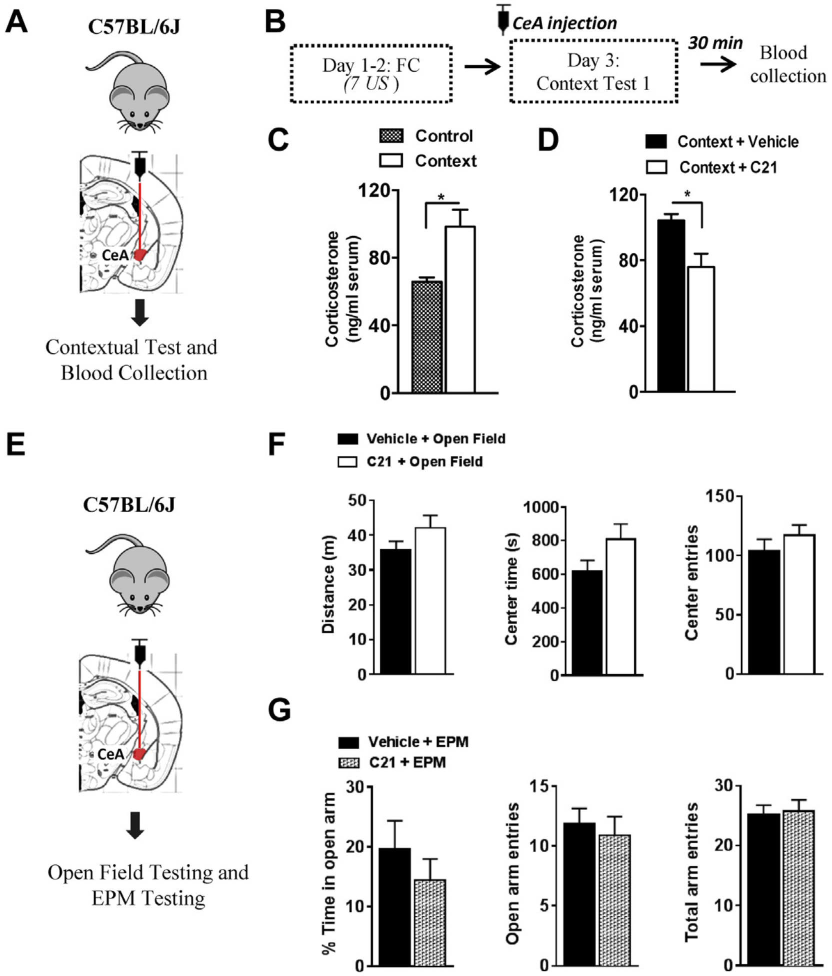

Figure 2. The effects of intra-central amygdala (CeA) angiotensin II type 2 receptor (AT$_2$R) activation on basal anxiety measures and plasma corticosterone levels. **(A, B)** Experimental protocol for plasma corticosterone test. **(C)** Plasma corticosterone levels in contextual fear conditioned (FC) and the non–foot shock control mice ($n = 4$; *$p < .05$). **(D)** Plasma corticosterone levels in vehicle- and compound 21 (C21)-injected groups ($n = 7$–8; *$p < .05$). **(E)** Experimental protocol for open field and elevated plus maze (EPM) test. **(F)** Total distance traveled (left), time in center (middle), and center entries (right) in vehicle- and C21-injected groups ($n = 10$–13). **(G)** Percentage of time in open arms (left), open entries (middle), and total arm entries of the EPM (right), in vehicle- and C21-injected groups ($n = 9$–10). US, unconditioned stimulus.

markers: calbindin, calretinin, neuronal nitric oxide synthase, parvalbumin, and somatostatin (Figure 4A–E). In total, 17% of eGFP$^+$ cells expressed interneuron markers (Figure 4F). Because some cells may express multiple markers, it is likely that the actual percentage of interneurons is less than 17%. This suggests that the remaining cells that did not stain for any markers are likely GABAergic projection neurons.

CTB Retrograde Tracing and Labeling of the CeM to PAG Projection

Given the role of CeA-PAG connections in defensive responses (e.g., freezing), we next determined whether the PAG receives input from AT$_2$R-GFP$^+$ projection neurons. Alexa Fluor–conjugated CTB was injected into the PAG of AT$_2$R -eGFP-bacterial artificial chromosome mice (Figure 5A, B). After 2 weeks, tissue was collected and coronally sectioned for immunohistochemistry. CTB injection sites were verified by fluorescence in the PAG (Figure 5C, D). Cell bodies containing CTB were observed in the CeM and assessed for colocalization with eGFP (Figure 5E–J). The detection of multiple CTB/eGFP$^+$ cells in the CeM suggests that a portion of the CeM-eGFP+ GABAergic projection neurons may modulate freezing behavior controlled by CeM-PAG.

DISCUSSION

Current available treatment options for PTSD provide limited benefits to a substantial proportion of individuals (1–4), and the need for more effective therapeutic strategies remains. Recent clinical studies identify the brain RAS as a potential therapeutic target, and preclinical studies in mice have begun to explore potential neurobiological mechanisms (7,12,13,44,45). In this study, we determined that AT$_2$R-eGFP$^+$ GABAergic projection neurons are highly expressed in the CeM and that select pharmacological activation of AT$_2$R in the CeA reduces conditioned fear expression, likely via an AT$_2$R-CeM-PAG projection.

Our previous studies using Pavlovian fear conditioning demonstrated that AT$_1$R blockade facilitates fear extinction (7,13), which is the gradual reduction of a learned response following the withdrawal of reinforcement and the basis of exposure therapy for anxiety-related disorders such as PTSD (46). Importantly, Pulcu *et al.* recently showed that AT$_1$R

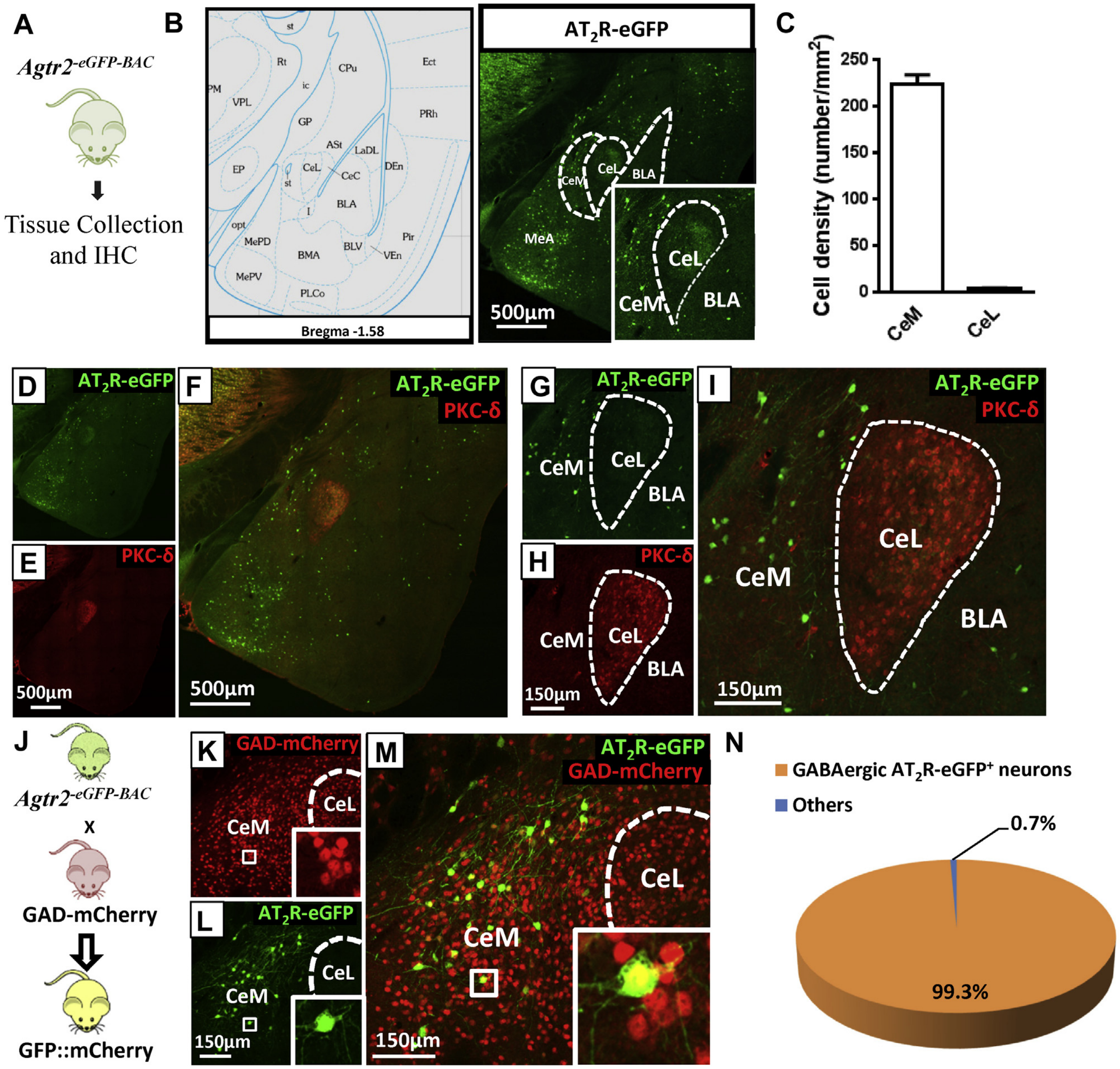

Figure 3. Angiotensin II type 2 receptor (AT_2R)-enhanced green fluorescent protein (eGFP)–expressing gamma-aminobutyric acidergic (GABAergic) neurons are present in medial division of the central amygdala (CeM) but not in lateral division of the central amygdala (CeL). **(A)** Experimental protocol. **(B)** Representative coronal sections through the amygdala of the AT_2R-eGFP reporter mice. The inset shows the enlargement of the central amygdala (CeA) area. **(C)** Quantification of AT_2R-$eGFP^+$ cells in CeM vs. CeL. **(D–I)** Representative images of the amygdala depiction AT_2R-$eGFP^+$ cells **(D, G)**, protein kinase C delta $(PKC\text{-}\delta)^+$ cells **(E, H)**, and the merged image **(F, I)**. **(D–F)** Low-magnification images showing the whole amygdala. **(G–I)** High-magnification images showing only the CeA. **(J)** AT_2R-eGFP reporter mice were bred with glutamic acid decarboxylase (GAD)-mCherry mice. **(K–M)** Representative images of the CeA depiction $mCherry^+$ neurons **(K)**, AT_2R-$eGFP^+$ neurons **(L)**, and the merged image **(M)**. **(N)** Pie chart depicting the percentage value of AT_2R-$eGFP^+$ neurons that coexpressed GAD-mCherry. Images were taken from either the rostral or caudal portion of the CeA, and analysis was performed on 36 brain sections from 6 mice. BAC, bacterial artificial chromosome; BLA, basolateral amygdala; IHC, immunohistochemistry; MeA, medial amygdala.

blockade, with losartan, in healthy volunteer participants modifies the valence to emotional learning, thereby supporting a potential therapeutic role in human exposure therapy (10). More recent studies in mice have demonstrated that AT_1Rs are expressed by neuronal phenotypes (e.g., corticotropin-releasing factor-expressing neurons) associated with stress responses in hypothalamic and limbic brain regions (e.g., the CeA) (16), thereby altering the expression and extinction of fear (13). To complement the growing body of evidence linking brain angiotensinergic activity with conditioned fear and anxiety (7,13,37,47), here we sought to examine the brain AT_2R, about which far less is known regarding the emotional

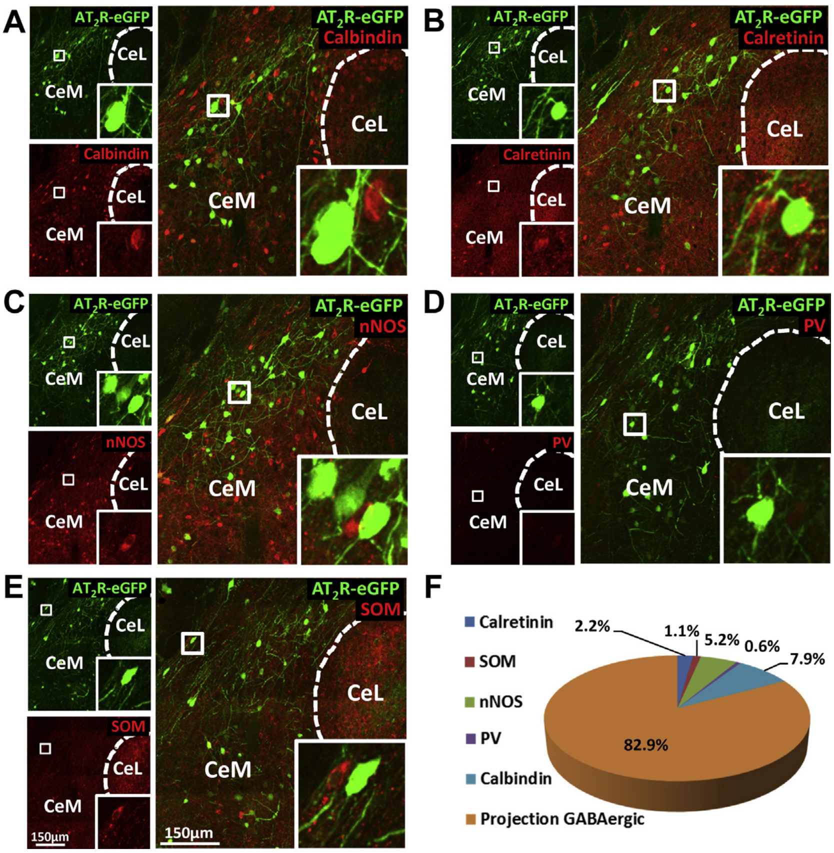

Figure 4. Angiotensin II type 2 receptor (AT$_2$R)-enhanced green fluorescent protein (eGFP) neurons in medial division of the central amygdala (CeM) are predominantly projection neurons. **(A–E)** Representative coronal sections through the central amygdala of the AT$_2$R-eGFP reporter mice with costaining against interneuron markers calbindin **(A)**, calretinin **(B)**, neuronal nitric oxide synthase (nNOS) **(C)**, parvalbumin (PV) **(D)**, and somatostatin (SOM) **(E)**. The insets show enlargements of the boxed areas. **(F)** Pie chart depicting the percentage value of AT$_2$R-eGFP$^+$ neurons that coexpressed the corresponding interneuron marker. Images were taken from either the rostral or caudal portion of the central amygdala, and 7 to 10 brain sections from 3 or 4 mice were used for analysis. CeL, lateral division of the central amygdala; GABAergic, gamma-aminobutyric acidergic.

responses to stress (48–50) and which we propose to have a modulatory role in AT$_1$R-mediated regulation of fear learning and recall of an emotional threat memory.

Consistent with previous results using the AT$_2$R-eGFP$^+$ reporter mouse (17), immunohistochemical analysis revealed dense localization of eGFP in two subnuclei of the amygdala: the medial region (medial amygdala), which is generally associated with innate fear and defensive behavior, and the CeA, which is the primary nucleus mediating behavioral and autonomic responses to fear (51,52). The CeA is a striatal-like structure that can further be divided into capsular, medial (CeM), and lateral (CeL) divisions (53). The CeL contains a number of distinct populations [for reviews, see (43,54)], with the two most notable expressing somatostatin and PKC-δ types (55,56). PKC-δ$^+$ cells, which are restricted to the CeL, can directly inhibit CeM output neurons and reduce fear responses to conditioned stimuli (57). To determine the distribution patterns of AT$_2$R-eGFP$^+$ cells, we identified the CeL/CeM boundary by staining for PKC-δ. We found negligible overlap between areas positive for PKC-δ and eGFP expression, confirming that AT$_2$R expression within the CeA occurs primarily in the medial division. While neurons within the CeA are primarily considered inhibitory (58), the phenotype of AT$_2$R-eGFP$^+$ neurons in this region is unknown. Using our AT$_2$R-eGFP$^+$-GAD-mCherry mice, we determined that these AT$_2$R cells are GABAergic projection neurons that likely project to regions outside of the CeM to mediate their inhibitory effects.

Based on our findings that AT$_2$R-eGFP$^+$ neurons are uniquely positioned to modulate CeM output, we examined the effects of intra-CeA C21 injections prior to conditioned fear responses in two different models of Pavlovian fear conditioning. Contextual conditioning and auditory fear (cued) conditioning vary in their recruitment of areas such as the hippocampus, thereby reflecting different upstream pathways of memory formation and maintenance. Both types of memory, however, require the CeA to affect changes in behavior during fear memory recall (59,60). Our behavioral experiments indicate that AT$_2$R activation reduced freezing behavior across both contextual and auditorily cued models of fear conditioning. We also observed a sustained reduction in freezing in C21-infused animals during the second day of context reexposure, indicating a long-lasting effect on memory. Interestingly, this effect was observed only in the contextual model of fear conditioning. While there was a trend for reduced freezing in the C21 group at the start of the cued extinction test, the difference was not statistically significant. This is likely due to the fact that responses to cue-conditioned stimuli are more resistant to extinction than responses to context-conditioned stimuli (59). These findings suggest that like its AT$_1$R counterpart (7,13), central AT$_2$R activity could modulate the strength of consolidation of extinction memories. This may occur through an AT$_2$R-mediated effect on pathways previously implicated in the pathogenesis of trauma-related memory (e.g., PTSD) such as brain-derived neurotrophic factor/tropomyosin

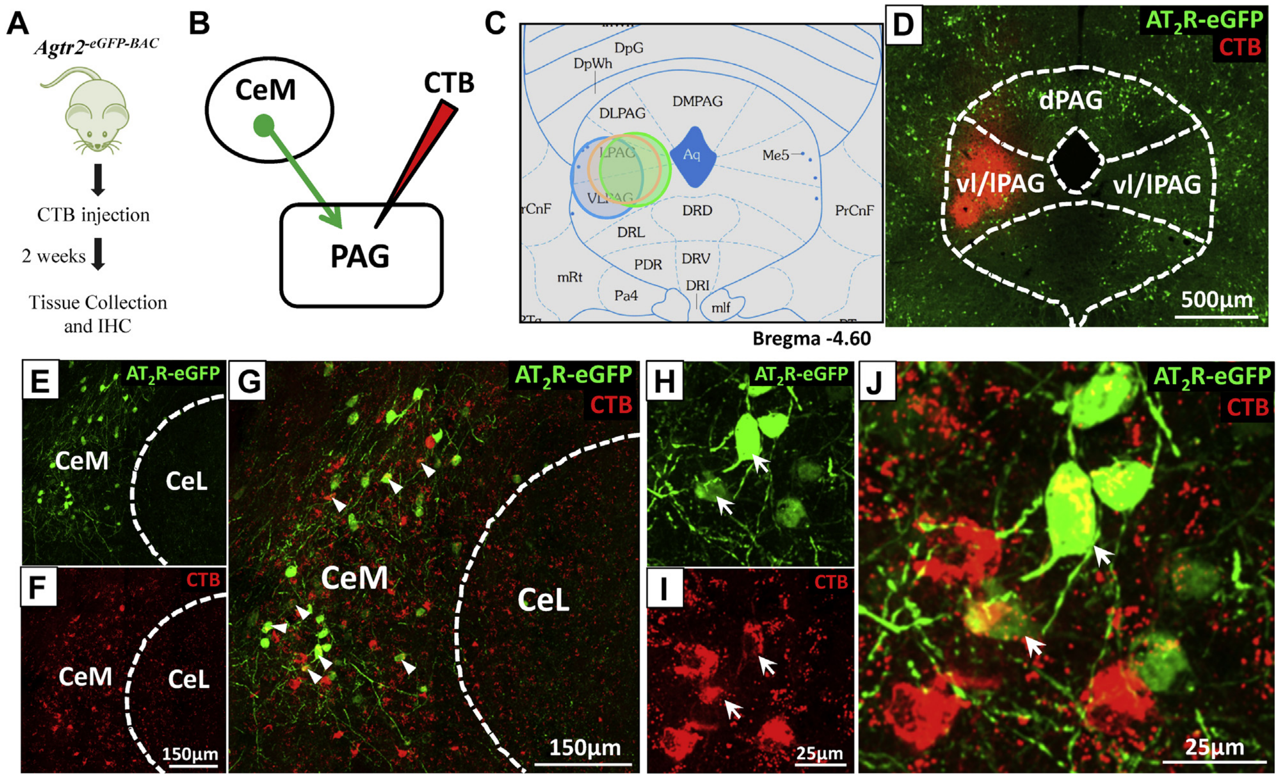

Figure 5. Angiotensin II type 2 receptor (AT_2R)-enhanced green fluorescent protein (eGFP) neurons in medial division of the central amygdala (CeM) project to periaqueductal gray (PAG). **(A, B)** Experimental protocol for retrograde tracing from CeM using Alexa red fluorescent cholera toxin B (CTB). **(C)** Atlas section through the PAG depicting the localization and spread of CTB injection sites in the 3 mice used for this study. **(D)** Representative coronal sections through the PAG of the AT_2R-eGFP reporter mice with the CTB injection. **(E–J)** Projection images of the central amygdala depiction AT_2R-eGFP$^+$ neurons **(E, H)**, CTB$^+$ PAG-projecting neurons **(F, I)**, and the merged image **(G, J)**. **(E–G)** Low-magnification images. **(H–J)** High-magnification images. Arrows indicate example double-labeled cells. BAC, bacterial artificial chromosome; CeL, lateral division of the central amygdala; dPAG, dorsal PAG; IHC, immunohistochemistry; vl/lPAG, ventrolateral and lateral PAG.

receptor kinase B (61,62) or pituitary adenylate cyclase-activating polypeptide (63). Moreover, in the perspective of recent work identifying the complex and tightly controlled CeA microcircuits that contribute to fear learning (64,65), these results may also indicate a functional role of AT_2R-CeM cells that extends beyond that of simply gating the behavioral and endocrine responses during conditioned threat exposure.

Numerous lesioning (66–68) and pharmacological (69–71) studies have identified the PAG as an essential site of converging signals regulating flight and freezing responses, and input to the PAG from regions such as the CeA has been recently shown to mediate freezing through disinhibition of ventrolateral PAG outputs to the medulla (72). Given the necessity of CeA-PAG connectivity to orchestrate adaptive behavioral responses, we targeted the PAG with the retrograde tracer CTB to determine whether it receives direct AT_2R^+-GABAergic input. Our retrograde tracing studies identified an AT_2R-eGFP$^+$ projection from the CeM to the PAG, suggesting that AT_2R activation in the CeM may modulate fear expression via direct projections to the PAG. Future studies will determine the extent to which this particular projection controls freezing behavior, because it is likely that CeM-AT_2R^+ neurons also project to other regions, such as the hypothalamus and dorsal vagal complex, that could simultaneously affect defensive behavior as well as cardiovascular and autonomic adjustments to conditioned fear (43,73).

In addition to behavioral adjustments, the CeA also mediates physiological responses to fear stimuli, and lesioning of the CeA is known to attenuate both circulating corticosterone and renin levels to conditioned stress (74). Consistent with previously reported sympathoinhibitory effects of central AT_2R activation (75,76), C21-infused mice exhibited reduced corticosterone responses to contextual fear recall. Importantly, AT_2R activation had no significant effect on exploratory behavior or anxiety-like behavior, suggesting that the reductions in conditioned freezing behavior cannot be attributed to nonspecific anxiolytic drug effects and that AT_2R activation in the CeM does not play a significant role in basal anxiety levels.

A potential mechanism through which AT_2R activation in the CeM may modulate fear expression is by altering the firing rate of AT_2R^+ neurons that control freezing behavior. Although we did not examine this in the current study, C21 has been previously shown to facilitate potassium channel function and thereby decrease spontaneous neuronal discharge (75,77). Alternatively, AT_2R stimulation could modify freezing behavior by enhancing the inhibitory effects of GABA on signaling pathways that control defensive reactions (66). Interestingly, activation of AT_2R in GABAergic cells projecting to the paraventricular nucleus of the hypothalamus was shown to reduce the activity of target cells within the paraventricular nucleus, inhibiting their release of vasopressin (78). While the effects of AT_2R activation on neuronal firing rate and GABA sensitivity within the amygdala are not currently known, the resulting behavioral changes are likely mediated by downstream effector regions such as the PAG as shown here.

The current study demonstrates that activation of the AT_2R in the CeM attenuates freezing behavior in response to conditioned auditory and contextual fear, while simultaneously mitigating fear-induced increases in circulating levels of corticosterone, without altering exploratory or anxiety-like behavior. In addition, we show that the AT_2R^+ cell population in the CeM is made up of GABAergic projection neurons, some of which project to the PAG. These results offer functional and anatomical evidence to support a role for brain AT_2R in fear expression while expanding the neurobiological understanding of brain angiotensin receptors as a potential therapeutic target for PTSD and adding to the emerging understanding of neuropeptide modulation of the CeA (79,80).

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