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Journal Title: Genes, Brain and Behavior
Volume: Volume 14, Number 7
Publisher: Wiley | 2015-09-01, Pages 526-533
Type of Work: Article | Post-print: After Peer Review
Publisher DOI: 10.1111/gbb.12235
Permanent URL: https://pid.emory.edu/ark:/25593/rr9cq

Final published version: http://dx.doi.org/10.1111/gbb.12235

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Accessed March 7, 2019 6:50 PM EST
Angiotensin Type 1a Receptors on Corticotropin-Releasing Factor Neurons Contribute to the Expression of Conditioned Fear

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Abstract

Although generally associated with cardiovascular regulation, angiotensin II receptor type 1 (AT₁R) blockade in mouse models and humans has also been associated with enhanced fear extinction and decreased post-traumatic stress disorder (PTSD) symptom severity, respectively. The mechanisms mediating these effects remain unknown, but may involve alterations in the activities of corticotropin-releasing factor (CRF)-expressing cells, which are known to be involved in fear regulation. To test the hypothesis that AT₁R signaling in CRFergic neurons is involved in conditioned fear expression, we generated and characterized a conditional knockout mouse strain with a deletion of the AT₁R gene from its CRF-releasing cells (CRF-AT₁R⁻/⁻). These mice exhibit normal baseline heart rate, blood pressure, anxiety, and locomotion, and freeze at normal levels during acquisition of auditory fear conditioning. However, CRF-AT₁R⁻/⁻ mice exhibit less freezing than wild type mice during tests of conditioned fear expression—an effect that may be caused by a decrease in the consolidation of fear memory. These results suggest that central AT₁R activity in CRF-expressing cells plays a role in the expression of conditioned fear, and
identify CRFergic cells as a population on which AT1R antagonists may act to modulate fear extinction.

Introduction

Angiotensin II, a component of the renin-angiotensin system, and its receptor, the angiotensin II receptor type 1a (AT1aR), have been studied extensively in the context of blood pressure regulation (Mehta and Griendling, 2007, Castrop, 2015, Chen and Coffman, 2015) and the stress response (Chen et al., 2012, Krause et al., 2011, Armando et al., 2007). However, recent studies in humans and mice have suggested roles for AT1aR activation in post-traumatic stress disorder (PTSD) and auditory fear conditioning, respectively. For example, treatment of humans with angiotensin receptor blockers (ARBs) has been associated with a decrease in the hyperarousal and intrusive symptoms of PTSD (Khoury et al., 2012, Nylocks et al., 2015). Similarly, either acute or chronic administration of the ARB losartan in mice has been found to enhance the extinction of fear memory, but not fear acquisition or extinction training (Marvar et al., 2013). However, these previous investigations involved systemic administration of ARBs, and therefore did not address whether ARBs are acting centrally and which cell types they are acting on to create these behavioral effects.

To address this, we investigated whether AT1aR deletion from a genetically defined neural population—corticotropin-releasing factor (CRF)-expressing cells—affect the expression of conditioned fear. CRF is a hormone secreted by the paraventricular nucleus of the hypothalamus (PVN) during times of stress, and is a component of the hypothalamic-pituitary-adrenal axis (Rivier and Plotsky, 1986). CRF mRNA is also enriched in the central amygdala (CeA) (Pitts et al., 2009) and bed nucleus of the stria terminalis (BNST) (Beckerman et al., 2013)—regions implicated in the expression of conditioned fear (Sullivan et al., 2004, Wilensky et al., 2006). In support of a functional link between AT1aR activity and central CRFergic tone, previous work has shown that chronic systemic ARB administration can prevent the decrease in PVN CRF release induced by isolation stress (Armando et al., 2007) and that whole-brain AT1aR knockout decreases CRF mRNA expression in the PVN (Yamamoto et al., 2011).

Based on these findings, we hypothesized that AT1aRs on CRFergic cells are involved in the expression of conditioned fear, and predicted that an AT1aR knockout confined to CRFergic cells would impair cued fear expression. Furthermore, because AT1aR and CRF are both expressed in moderate levels in the CeA (Ciccocioppo et al., 2014, Shekhar et al., 2005, Von Bohlen Und Halbach and Albrecht, 1998b) and PVN (Premer et al., 2013, Aguilera et al., 1995a), we expected that this knockout would be pronounced in these areas.

Materials and Methods

Animals

Mice with a floxed AT1aR gene (AT1aRflox/flox; JAX Stock #016211) (Rateri et al., 2011) were bred with mice that selectively express Cre recombinase in CRFergic cells (CRF::Cre;
JAX Stock #011087) (Martin et al., 2010, Gafford et al., 2014, Gafford et al., 2012) to generate offspring with a conditional knockout of the AT$_{1a}$R confined to CRF-expressing cells. Offspring were genotyped for the floxed and wild type AT$_{1a}$R alleles and the Cre recombinase gene using PCR on DNA extracted from ear punches (Figure S1). Male littermates between 8 and 12 weeks that were positive for Cre recombinase and also homozygous for either the floxed (CRF-AT$_{1a}$R$^{−/−}$) or wild type (AT$_{1a}$R$^{+/+}$) AT$_{1a}$R allele were used for the behavioral tests. The same mice were used for the behavioral and cardiovascular tests, but only 8 out of 15 AT$_{1a}$R$^{+/+}$ and 10 out of 16 CRF-AT$_{1a}$R$^{−/−}$ mice that underwent the behavioral tests also underwent the cardiovascular ones. Bacterial artificial chromosome mice that express GFP in AT$_{1a}$R-expressing cells (AT$_{1a}$R-GFP) (MMRRC ID# 036905-UCD) (Gonzalez et al., 2012, Marques-Lopes et al., 2015) were used for immunohistochemical studies, but not for behavioral ones. All procedures were conducted by male experimenters (Sorge et al., 2014) and were approved by the Institutional Animal Care and Use Committee of Emory University.

**Immunohistochemistry**

AT$_{1a}$-GFP mice were perfused transcardially with 4% paraformaldehyde in 0.1M phosphate buffer, and their brains were extracted and fixed overnight in the same fixative. The next day, 40 μm coronal sections were cut using a vibratome. Sections were permeabilized using Triton X-100 and blocked with normal horse serum and bovine serum albumin. GFP and CRF staining was performed by simultaneously incubating sections in 1:2000 and 1:200 dilutions of chicken anti-GFP (Abcam, ab13970) and rabbit anti-CRF (Abcam, ab11133) antibodies, respectively, for 48 hours, followed by simultaneous incubation in 1:500 and 1:100 dilutions of goat anti-chicken IgY Alexa Fluor 488 (Abcam, ab150169) and goat anti-rabbit IgG Alexa Fluor 568 (Life Technologies, A-11011) antibodies, respectively, for 48 hours. Sections were mounted on Superfrost Plus slides (Fisher Scientific, 12-550-15) and air-dried before being coverslipped with Mowiol mounting medium (Sigma-Aldrich, 81381). Sections were imaged using a 20X oil immersion objective (Leica HC PL APO 20X/0.75 IMM) on a Leica SP8 confocal microscope.

**In vitro receptor autoradiography**

Mice were anesthetized using isoflurane and their brains were removed, quickly frozen, and stored at −80 °C. The brains were sectioned coronally at a thickness of 20 μm with a cryostat and thaw-mounted onto charged microscope slides in a sequential manner such that adjacent sections were mounted on different slides in repeating sets of 5. The sections were air-dried for 5–20 minutes and kept refrigerated for no more than 2 weeks. Receptor autoradiography was carried out as described previously (Speth et al., 1999) using $^{125}$I-sarcosine$^1$, isoleucine$^8$ angiotensin II ($^{125}$I-SI Ang II), prepared as described previously (Speth and Harding, 2001) through the Peptide Radiiodination (Shared Resource at Georgetown University), with the following modifications. The concentration of $^{125}$I-SI Ang II was ~250 pM, PD123319 was present at a concentration of 10 μM to saturate all AT$_2$Rs, non-specific binding in sections adjacent to the “total binding” sections was determined in the presence of an AT$_1$R saturating concentration of losartan (10 μM), and the film images were scanned into MCID 7.0 at 2400 dpi resolution and quantified relative to brain paste standards with known concentrations of $^{125}$I. For each assay, an AT$_{1a}$R$^{+/+}$ and a CRF-AT$_{1a}$R$^{−/−}$ brain

*Genes Brain Behav. Author manuscript; available in PMC 2016 September 01.*
were run in parallel to control for any day-to-day variations in the radioligand or assay conditions. To control for the possibility that the area delineated as having high binding corresponding to the regions of interest differed in size, measurements of both the density of binding and the density times area of binding were determined for group comparisons. Another set of adjacent sections were stained with thionin to provide anatomical registration for the autoradiographic images so as to localize areas of high $^{125}$I-SI Ang II binding to specific brain regions.

**Anxiety and locomotion tasks**

Mice performed elevated plus maze and open field tasks to measure their baseline levels of anxiety (Pellow et al., 1985) and locomotion (Tatem et al., 2014), respectively. The elevated plus maze task involved allowing each mouse to explore a plus-shaped maze with two walled arms and two open arms elevated 50 cm from the ground for 5 minutes. As the automated scoring system often tracked the mouse’s position to locations outside the maze, data were scored manually using the center of the mouse’s body to determine its location in the maze. The open field task involved allowing each mouse to explore a 27.3 cm$^2$ arena (Med Associates Inc.) for 10 minutes. Data were scored automatically using the Med Associates Activity Monitor software.

**Blood pressure and heart rate measurements**

Two weeks after the extinction test, a tail-cuff sphygmomanometer (Hatteras Instruments, MC4000) was used to collect baseline blood pressure and heart rate measurements as previously described (Marvar et al., 2010).

**Auditory fear conditioning and extinction**

During habituation, mice were pre-exposed to conditioning cages for 2 days before training. Mice were then trained with an auditory fear conditioning paradigm, as previously described (Choi et al., 2010), that consisted of one day of 5 tone/shock pairings (30 second, 6 kHz, 75 dB tones co-terminating with 500 ms, 1 mA footshocks; 60 second inter-trial interval; room light on). For extinction, 24 hours later mice received 30 tone presentations (30 second, 6 kHz, 75 dB tones; 60 second inter-trial interval) in a different context (room light off, red cage light on, plexiglass floor). Freezing data were scored using the Actimetrics FreezeFrame 3 software.

**Statistical analyses**

Data are expressed as mean ± SEM and values of $p < 0.05$ were considered statistically significant. Averaged freezing data were analyzed using an unpaired, two-tailed Student’s t-test, as these were the first experiments conducted, and it was necessary to test for effects in both directions. Binned freezing data were analyzed using a repeated measures ANOVA (Prism 6.0) with a Bonferroni post-hoc analysis. $^{125}$I-SI Ang II binding levels were analyzed using a paired, one-tailed Student’s t-test, as the anticipated direction of the effect had been determined a priori based on genetic information.
Results

AT$_{1a}$R and CRF co-localize in the PVN and CeA

To determine whether AT$_{1a}$R and CRF are co-expressed, we performed a dual-label IHC on the brains of AT$_{1a}$R-GFP mice. Co-localization was observed in the PVN and CeA—areas heavily implicated in the stress response and fear expression, respectively (Figure 1). CRF cell-specific AT$_{1a}$R knockout (CRF-AT$_{1a}$R($^{-/-}$)) mice were then generated by crossing homozygous floxed AT$_{1a}$R mice with CRF::Cre mice (see Methods).

AT$_{1a}$R expression is reduced in the PVNs of CRF-AT$_{1a}$R($^{-/-}$) mice

To assess whether CRF-AT$_{1a}$R($^{-/-}$) mice express significantly less of the AT$_{1}$R than wild type (AT$_{1a}$R($^{+/+}$)) mice, we conducted an in vitro quantitative densitometric AT$_{1}$R autoradiography analysis. Densitometric analysis of specific $^{125}$I-SI Ang II binding in the PVN indicated that the density of AT$_{1}$Rs was significantly higher in AT$_{1a}$R($^{+/+}$) brains (164±36 fmol/mg initial wet weight) than CRF-AT$_{1a}$R($^{-/-}$) brains (121±28 fmol/mg) (Figure 2). A lower level of $^{125}$I-SI Ang II binding was observed in the basolateral amygdala (BLA) but no significant difference in binding density was observed; 21±7 fmol/mg for AT$_{1a}$R($^{+/+}$) and 21±9 fmol/mg for CRF-AT$_{1a}$R($^{-/-}$) brains.

AT$_{1a}$R knockout from CRFergic cells reduces the expression of conditioned fear

To test for an effect of CRFergic cell-specific AT$_{1a}$R knockout on the acquisition, consolidation, or extinction of fear memory, we subjected mice to auditory fear conditioning and extinction protocols. AT$_{1a}$R($^{+/+}$) and CRF-AT$_{1a}$R($^{-/-}$) mice showed similar levels of freezing during auditory fear conditioning on a trial-by-trial basis (Figure 3A). In a test of fear expression 24 hours later, CRF-AT$_{1a}$R($^{-/-}$) mice displayed significantly less overall freezing than AT$_{1a}$R($^{+/+}$) ($t(30) = 2.1; *p < 0.05$) (Figure 3B). Furthermore, when analyzed over the course of the fear expression test (on a 10 CS bin-by-bin basis) using a repeated measures ANOVA, there was a significant main effect by group ($F_{(1, 30)}=4.31, p=0.046$), but no interaction for the 3 bins of 10 CS presentations ($F_{(2, 60)}=0.02, p=0.974$) (Figure 3C). Collectively, these data indicate a decrease in fear expression in CRF-AT$_{1a}$R($^{-/-}$) mice.

Baseline blood pressure, heart rate, anxiety, and locomotion are not affected by knockout of the AT$_{1a}$R from CRFergic cells

To evaluate whether this knockout affects baseline levels of anxiety, locomotion, and cardiovascular measures, we subjected AT$_{1a}$R($^{+/+}$) and CRF-AT$_{1a}$R($^{-/-}$) mice to elevated plus maze and open field tasks, and measured blood pressure and heart rate. AT$_{1a}$R($^{+/+}$) and CRF-AT$_{1a}$R($^{-/-}$) mice spent similar amounts of time in the open arms of the elevated plus maze (though there was a non-significant ($p=0.13$) trend toward reduced open arm exploration in the CRF-AT$_{1a}$R($^{-/-}$) mice) (Figure 4A), traveled similar distances in the open field (Figure 4B), and showed no differences in baseline blood pressure or heart rate (Figure 4C–D).
Discussion

The AT\textsubscript{1a}R is expressed in many organs, including the heart, kidney, blood vessels, and brain, and inhibition of this receptor is widely used for the treatment of hypertension and cardiovascular disease (Mehta and Griendling, 2007, Wright and Harding, 1995). However, previous studies by our lab (Khoury et al., 2012, Marvar et al., 2013, Nylocks et al., 2015) and others (Marinzalda Mde et al., 2014, Krause et al., 2011) suggest an important role for this receptor in fear-related pathologies such as PTSD. Our current data extend these findings by demonstrating for the first time that AT\textsubscript{1a}Rs on CRF-expressing cells contribute to conditioned fear expression without affecting fear acquisition or baseline anxiety, locomotion, blood pressure, or heart rate.

These results contribute to a growing body of evidence implicating central AT\textsubscript{1}R activity in the expression of conditioned fear and anxiety (Khoury et al., 2012, Marvar et al., 2013, Marinzalda Mde et al., 2014, Shekhar, 2014, Shekhar et al., 2006, Johnson et al., 2013, Nylocks et al., 2015, Saavedra et al., 2006), and identify CRFergic cells as a population on which AT\textsubscript{1}R antagonists may act to create these effects. Further, they indicate that the fear expression-attenuating effects of AT\textsubscript{1}R inactivation may be caused by a decrease in fear memory consolidation (i.e., the transfer of a memory from a short-term to a long-term store in a transcription-dependent manner)—rather than an inability to acquire conditioning—as AT\textsubscript{1}R\textsuperscript{+/+} and CRF-AT\textsubscript{1a}R\textsuperscript{−/−} mice showed similar levels of freezing during auditory fear conditioning, but 24 hours later, CRF-AT\textsubscript{1a}R\textsuperscript{−/−} mice displayed significantly less overall freezing than wild types. Considering that both groups acquired similar amounts of fear, this trend may be attributable to an impairment in memory consolidation in CRF-AT\textsubscript{1a}R\textsuperscript{−/−} mice. This interpretation is consistent with the previous finding that administration of an AT\textsubscript{1}R antagonist immediately before fear expression testing had no effect on expression (Marvar et al., 2013), indicating that this manipulation is not directly affecting conditioned responding.

The AT\textsubscript{1a}R is known to interact with multiple signaling pathways involved in memory consolidation (Higuchi et al., 2007, Guo et al., 2001) and the brain renin-angiotensin system has been implicated in both synaptic plasticity (Tchekalarova and Albrecht, 2007, Von Bohlen Und Halbach and Albrecht, 1998a) and some forms of memory consolidation (Kerr et al., 2005, Wright et al., 2002, Frenkel et al., 2005). The molecular mechanisms by which AT\textsubscript{1}/AT\textsubscript{2}/AT\textsubscript{4}R activation influences the long-term changes underlying memory consolidation remain poorly understood, but likely involve coupling of these G protein-coupled receptors to G\textsubscript{q} subunits, resulting in activation of PLC and subsequent intracellular Ca\textsuperscript{2+} release (Guo et al., 2001), which is known to facilitate synaptic plasticity (Sheng and Kim, 2002). Further, AT\textsubscript{4}R activation has been implicated in spatial memory formation (Wright et al., 1999) and may contribute to fear memory consolidation. Therefore, future studies aimed at elucidating the role of brain angiotensin II signaling in memory consolidation should use chemogenetic techniques (Sternson and Roth, 2014) to determine the role of G\textsubscript{q} activation in this process, and should focus on providing a more mechanistic understanding of how AT\textsubscript{1} receptor activation influences the intracellular signaling cascades involved in memory consolidation and how this interaction differs for the consolidation of fear and extinction memories.

*Genes Brain Behav.* Author manuscript; available in PMC 2016 September 01.
Additionally, the downstream effects of AT1aR knockout from CRFergic cells are not known. CRF receptor activation has been implicated in fear memory formation (Rainnie et al., 2004), but it is unclear how knockout of the AT1aR affects CRF release or if a change in CRF release is the primary mechanism by which this knockout reduces fear expression. Further, because there may be considerable functional heterogeneity in both the CRF-releasing (Valentino et al., 2001) and CRF-responsive (Kirby et al., 2000) populations, it is possible that AT1aR knockout affects various functionally, genetically, and spatially defined subsets of neurons differently (Lew et al., 2003, Hirawa et al., 1999).

It is worth noting that CRF-AT1aR(−/−) animals showed a trend toward less open arm exploration in the elevated plus maze test. This result, while not statistically significant, would seem to indicate slightly enhanced baseline anxiety levels in these mice, considering that their baseline locomotion levels (as measured by the total distance they traveled during the open field test) do not differ from AT1aR(+/+) animals. However, because the strains do not differ in baseline blood pressure or heart rate, it is likely that this trend does not indicate increased anxiety in CRF-AT1aR(−/−) mice; still, the technique used to collect these cardiovascular measurements suffers from low sensitivity. Future studies should use more sensitive tests of HPA axis activity such as plasma corticosterone measurements or PVN c-Fos quantification to determine whether this knockout confers a slight increase in baseline anxiety.

The receptor autoradiography analyses demonstrated an incomplete reduction in AT1R binding in the PVNs of CRF-AT1aR(−/−) mice, which may have occurred because AT1Rss are not exclusively expressed by CRFergic PVN neurons. It was not possible to demonstrate a reduction in AT1R binding in the CeAs of CRF-AT1aR(−/−) mice. AT1aR expression in the CeA is normally low, and a small subset of CeA cells express both AT1aR and CRF; as a result, the small differences in CeA AT1aR expression created by our conditional knockout model were likely not detectable with receptor autoradiography.

Given that the aim of our study was to investigate whether AT1aR deletion from a genetically defined neural population affects fear learning, our use of a constitutive knockout mouse model imposes certain limitations that merit mention. Because this knockout is present from birth, it may affect the animals’ normal development (Aguilera et al., 1994); however, because wild type and knockout animals showed similar baseline levels of blood pressure, heart rate, and anxiety, and acquired conditioned fear similarly, we feel that any effects of this knockout on development did not meaningfully affect the behaviors in which we were primarily interested (i.e., fear learning and expression). A further limitation of this model is its inability to provide conclusive evidence regarding which CRFergic populations are contributing to the observed reduction in fear expression.

However, the finding that AT1aR and CRF co-localize in the CeA and PVN indicates that the CRFergic cells in these areas may be involved in creating this phenotype. CeA CRFergic cells have been implicated in fear memory retention (Gafford et al., 2014, Pitts and Takahashi, 2011, Pitts et al., 2009) and CeA AT1aRs have been implicated in anxiety (Marinzalda Mde et al., 2014). Additionally, PVN CRFergic cells are involved in conditioned fear expression (Otagiri et al., 2000) and anxiety (Bale et al., 2002), and are regulated by angiotensin II (Aguilera et al., 1995b).
Future studies involving site-specific AT$_{1A}$R knockouts will be required in order to determine which brain areas (e.g., CeA, PVN, BNST) contain the AT$_{1A}$R- and CRF-expressing cells most involved in creating this effect on conditioned fear memory. However, the identification of a genetically defined subset of neurons on which AT$_{1}$R antagonists act to influence fear expression will allow for more targeted future studies and potential new treatments of normal and dysregulated fear memory.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

**Acknowledgments**

We would like to thank Donald Rainnie (Department of Psychiatry and Behavioral Sciences, Emory University School of Medicine) for the anti-CRF antibody, Colin Young (Department of Pharmacology and Physiology, The George Washington University School of Medical and Health Sciences) for his comments on the manuscript, and Teresa A. Milner (Director of the Neuroanatomy EM Core, Feil Family Brain and Mind Research Institute, Weill Cornell Medical College) for the AT$_{1A}$-GFP brain sections and her comments on figure design. This research project was supported by the Emory University Integrated Cellular Imaging Microscopy Core and the following sources of funding: NIH-NHLBI 113905 (R.C.S., A.L., L.C.), Nova Southeastern University President’s Faculty Development Grant (R.C.S., A.L., P.J.M.), Nova Southeastern University Cardiovascular Neuroscience Program (R.C.S., A.L., L.C.), Shared Resource Center supported by NIH-P30 CA51008 and by NCATS 8 UL1 TR000101 (R.C.S.) and NIH R00 HL107675-03 (P.J.M).

**References**


Figure 1. AT\textsubscript{1a}R and CRF co-localize in subsets of PVN and CeA neurons
Dense AT\textsubscript{1a}R and CRF expression is observed in the PVN and CeA of AT\textsubscript{1a}R-GFP mice. Arrows in (d) and (h) indicate co-localization.
Figure 2. Autoradiographic analysis of AT$_1$R binding

AT$_1$R autoradiography revealed a decrease in AT$_1$R expression in the PVNs of CRF-AT$_1$R$^{(−/−)}$ mice compared to controls. Data are presented as mean ± SEM. * p<0.05.
Figure 3. AT$_{1a}$R knockout from CRFergic cells has no effect on acquisition of conditioned fear, but leads to decreased fear expression and enhanced extinction retention
(a) AT$_{1a}$R$^{(+/-)}$ and CRF-AT$_{1a}$R$^{(-/-)}$ mice show similar levels of freezing during fear acquisition ($n=15–17$). (b and c) During extinction, CRF-AT$_{1a}$R$^{(-/-)}$ mice show significantly less freezing than AT$_{1a}$R$^{(+/-)}$ mice overall and during the last 10 CS presentations ($n=15–17$).
Figure 4. AT$_{1a}$R knockout from CRFergic cells does not affect baseline levels of anxiety, locomotion, blood pressure, or heart rate
(a) AT$_{1a}$R$^{(+/-)}$ and CRF-AT$_{1a}$R$^{(-/-)}$ mice spend similar amounts of time on the open arms of an elevated plus maze ($n$=15–16) and (b) travel similar total distances during an open field test ($n$=15–17). Both groups also have similar (c) mean arterial pressure and (d) heart rate at baseline ($n$=8–10). Data are presented as mean ± SEM.