Role of locus coeruleus expressing angiotensin type 1 receptors (AT1R) neurons in fear learning and stress-induced anxiety

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Abstract:
Background: The renin-angiotensin system (RAS) has been implicated in stress-related disorders, however the central mechanisms responsible for this remains unknown. The locus coeruleus (LC), a major noradrenergic nucleus of the brain, plays a critical role in modulating anxiety-like behaviors. The LC has also been previously shown to express angiotensinogen (AGT), the pro-cursor for angiotensin, as well as strong expression of angiotensin II receptors, but its role in stress-related disorders has not been examined. Using angiotensin II type 1 receptor (AT1R)-eGFP and Cre mice combined with neuroanatomical and behavioral approaches, we examined the role of LC expressing AT1R in fear- and anxiety-related behavior.

Methods: RNAseq was performed on AT1R-eGFP mice to determine changes in gene expression. Immunohistochemistry was used to determine the localization of AT1R+ neurons. These data were validated using chemogenetic silencing with clozapine-n-oxide (CNO) administration. To determine the role of LC in fear learning, mice were subjected to fear conditioning and extinction training. Mice were then exposed to the open field or elevated plus maze (EPM) to test their anxiety.

Results: AT1R mRNA and AT1R-eGFP immunoreactivity was found localized to the LC. The majority of AT1R+ neurons (94%) in LC were co-localized with tyrosine hydroxylase, a marker for noradrenergic-containing neurons. Immunohistochemistry revealed that the AT1R+ neurons sends projections to amygdala, an important brain structure for modulating fear memory and stress-induced anxiety responses. The AT1R+ neurons in LC were silenced using Cre-dependent inhibitory designer receptor exclusively activated by designer drug (DREADD) expression followed by Clozapine-n-oxide (CNO) administration. Silencing the LC AT1R+ expressing neurons prior to fear extinction training impaired the extinction of learned fear as shown by increased percent freezing during the training (time × drug interaction, F(16, 80) = 3.681, p<0.001, n=6). Furthermore, restraint stress-induced anxiety behavior was attenuated by LC AT1R+ neuron inhibition, as shown by increased center entries (28.6 ± 5.6 saline v.s. 53.6 ± 6.6 CNO, p<0.05, n=6) and center distance (3.2 ± 0.8 saline v.s. 4.9 ± 1.0 CNO, p<0.05, n=6) in the open field test, and increased open arm entries (48.6 ± 9.9 saline v.s. 10.4 ± 1.1 CNO, p<0.01, n=6) and time in the open arm (4.1 ± 1.3 saline v.s. 15.0 ± 2.3 CNO, p<0.01, n=6) in the elevated plus maze test.

Conclusion: These findings provide evidence for a novel angiotensinergic LC cell type and position the LC AT1R as a potential mediator of noradrenergic regulation in learned fear and stress-induced anxiety. Future studies are needed to fully characterize the underlying neurotransmitters and neuropeptide modulators that likely interact with the LC AT1R expressing neurons during stress-related behaviors.

Methods:
Animals: 10-12-week-old adult male C57BL/6J mice, AT1R-GFP reporter mice, AT1R-cre mice or tdTomato-flx mice were used for this study.

Virus: The AA-V-DIO-GFP virus was used for anterograde tracing and the AA-V-DIO-KDEL-hm4Di-mCherry virus was used for AT1R+ neuron inhibition.

RNAseq: The RNAseq assay was performed according to the manufacturer’s instructions and images were analyzed via Zeiss spinning disk confocal microscope.

Immunostaining: Mice were perfused with 4% PFA, and brains were cut into 30 μm thickness free floating sections for antibody incubation. After staining, images were captured with the Zeiss spinning disk confocal microscope.

Surgery: Viruses were bilaterally injected into the LC of AT1R-Cre mice at 4.95 mm lateral to bregma, 3.5 mm posterior to bregma, and 4.4 mm below the skull surface with an UltraMicroPump III and microprocessor controller (World Precision Instruments, FL). A total volume of 400 nl was injected at a rate of 100 nl/min.

Fear Conditioning: During each fear conditioning, mice received 5 paired conditioned stimulus (CS) tone (10s, 12kHz, 70 db) + unconditioned stimulus (US) shock (1s, 0.75 mA) trials with a 5 min inter-trial interval (ITI) in the open field. Percent time spent freezing to the tones was measured. For extinction training and retention, mice were put into modular test chambers 24 and 48 hrs after fear conditioning. Mice were exposed to 30 trials of the 30s CS tone with a 30s ITI and fear expression during the tone presentations was measured as freezing behavior.

Anxiety test: Mice were kept in home cage or placed into the restraint stress tubes for 30 min and then open field or elevated plus maze (EPM) were used to test their anxiety level. For open field test, mice were placed in the open field box for 20 min, and the EPM tests were performed for 5 min.

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Fig. 1: AT1R mRNA expression astrocytes and AT1R+ noradrenergic neurons were found in the LC.

(A) Representative images through the LC of the C57 mouse co-staining AGT mRNA and astrocyte marker GFAP. (B) Representative images through the LC of the AT1R-GFP mice with co-staining against noradrenergic neuron marker TH. (C) Pie chart depicting the percentage value of AT1R-GFP+ neurons that co-expressed TH, AGT, angiotensinogen, GFAP, Glial fibrillary acidic protein, TH, Tyrosine Hydroxylase.

Methods:
(A) Experimental protocol for the open field test. (B) AT1R-cre neuron silencing didn’t affect mice locomotor activity but blocked restraint stress-induced anxiety in the open field test. (C) Experimental protocol for the elevated plus maze test. (D) CNO inhibition of LC AT1R+ neurons didn’t affect mice’s general anxiety but attenuated the restraint stress-reduced anxiety in the elevated plus maze test. (E-F) Open field test, RS, restraint stress; EPM, elevated plus maze test; CNO, Clozapine-n-oxide.

Summary and conclusion:
- The angiotensin system component AGT and AT1R were found in the LC (Fig. 1).
- LC AT1R+ neurons project to CeA and BMA (Fig. 2).
- Chemogenetic silencing of the LC-AT1R+ neurons impaired the fear extinction (Fig. 4).
- Chemogenetic silencing of the LC AT1R+ neurons attenuate stress-induced anxiety (Fig. 5).
- These data provide evidence for a novel angiotensinergic LC cell type and position the LC AT1R as a potential mediator of noradrenergic regulation in learned fear and stress-induced anxiety.