Role of locus coeruleus expressing angiotensin type 1 receptors (AT_1R) 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 pre-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 (AT_1R)-eGFP and Cre mice combined with neuroanatomical and behavioral approaches, we examined the role of LC expressing AT_1R in fear- and anxiety-related behavior.

Methods: RNAscope® *in situ* hybridization assay was used to analyze cellular mRNA expression of AGT in the LC while *in vivo* chemogenetics combined with behavioral testing in AT_1R-Cre mice mouse was used to examine the role of LC-AT_1R cells in fear memory and stress-induced anxiety. Further, dual immunohistochemistry plus cre-inducible anterograde tracing was used to characterize LC AT_1R^+ cells and limbic circuit connections.

Results: AGT mRNA and AT_1R-$eGFP^+$ immunoactivity was found localized to the LC. The majority of AT_1R-$eGFP^+$ neurons (94%) in LC were co-localized with tyrosine hydroxylase, a marker for norepinephrine-containing neurons. Anterograde labeling revealed that the AT_1R^+ neurons sends projections to amygdala, an important brain structure for modulating fear memory and stress induced anxiety responses. The AT_1R^+ neurons in LC were silenced using Cre-dependent inhibitory designer receptor exclusively activated by designer drug (hM4Di DREADD) expression followed by Clozapine-n-oxide (CNO) administration. Silencing the LC AT_1R-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, $F_{(5, 70)} = 3.681$, $p<0.001$, n=8). Furthermore, restraint stress-induced anxiety behavior was attenuated by LC AT_1R^+ neuron inhibition, as shown by increased center entries (28.6±6.7 Saline v.s. 53.6±6.6 CNO, $p<0.05$, n=8) and center distance (3.2± .8 Saline v.s. 6.9±1.0 CNO, $p<0.05$, n=8) in the open field test, and increased open arm entries (4.8±0.9 Saline v.s. 10.4±1.3 CNO, $p<0.01$, n=8) and % time in the open arm (4.1±1.3 Saline v.s. 13.0±2.3 CNO, $p<0.01$, n=8) in the elevated plus maze test.

Conclusion: These findings provide evidence for a novel angiotensinergic LC cell type and position the LC AT_1R as a potential mediator of noradrenergic regulation in learned fear and stress-induced anxiety. Future studies are needed to fully characterize the underlying neurocircuits and neuropeptide modulators that likely interact with the LC AT_1R expressing neurons during stress-related behaviors.

Methods:

Animals: 10-12-week-old adult male C57BL/6J mice, AT_1R-GFP reporter mice, AT_1R-cre mice or tdTomato-flox mice were used for this study.

Virus: The AAV-DIO-GFP virus was used for anterograde tracing and the AAV-DIO-hM4Di-mcherry virus was used for AT_1R^+ neuron inhibition.

RNAscope: The RNAscope assay was performed according to the manufacture'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 AT_1R-Cre mice at 4.95 mm caudal, ±0.8mm lateral 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 400nl was injected at a rate of 100 nl/min.

Fear Conditioning: During cued fear conditioning, mice received 5 paired conditioned stimulus (CS) tone (30s, 12kHz, 70 db) + unconditioned stimulus (US) shock (1s, 0.75 mA) trials with a 5 min inter-trial interval (ITI) in the startle boxes. 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.

Acknowledgements and Funding:

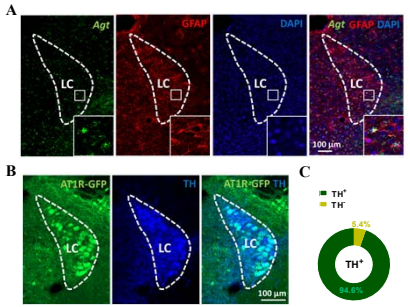

Fig. 1: AGT mRNA expressing astrocytes and AT_1R^+ norepinephrine neurons were found in the LC.
(A) Representative images through the LC of the C57 mouse co-staining AGT mRNA and astrocyte marker GFAP. **(B)** Representative images through the LC of the AT_1R-GFP mice with co-staining against norepinephrine neuron marker TH. **(C)** Pie chart depicting the percentage value of AT_1R-GFP^+ neurons that co-expressed TH. *AGT*, angiotensinogen; *GFAP*, Glial fibrillary acidic protein; *TH*, Tyrosine Hydroxylase.

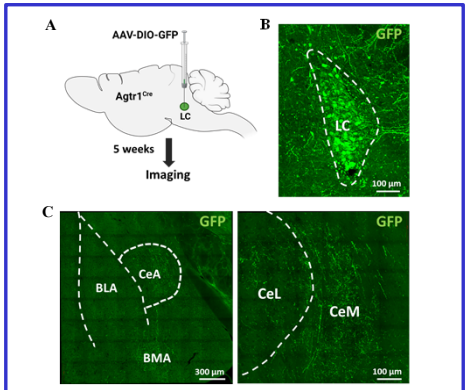

Fig. 2: AT_1R^+ neurons in the LC project to central and basomedial amygdala.
(A) Experimental protocol for anterograde tracing. **(B)** AT_1R-cre induced GFP signal in the LC. **(C)** Representative coronal sections through the amygdala of the virus injected mice showing the GFP^+ terminals projected from LC AT_1R^+ neurons. *DIO*, double-floxed inverse open reading frame; *CeA*, central amygdala; *BLA*, basolateral amygdala; *BMA*, basomedial amygdala; *CeL*, lateral division of the central amygdala; *CeM*, medial division of the central amygdala.

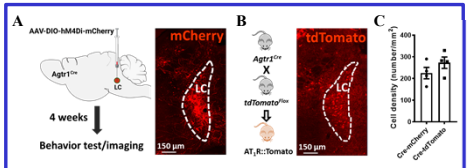

Fig. 3: AT_1R-cre induced mCherry expression in the LC after AAV-hM4Di-mCherry virus injection.
(A) AT_1R-cre induced mCherry signal was found in the LC of AT_1R-cre mice after Gi-coupled DREADDs (pAAV-hSyn-DIO-hM4Di-mCherry) virus injection. **(B)** Representative images through the LC of the AT_1R-cre/tdTomato-flox mice. The expression pattern of the AT_1R-cre-induced signal is similar as the virus injected mice. **(C)** Cell density of the Cre-induced $mCherry^+$ cells after virus injection and Cre-induced $tdTomato^+$ cells in in the AT_1R-cre/tdTomato-flox mice.

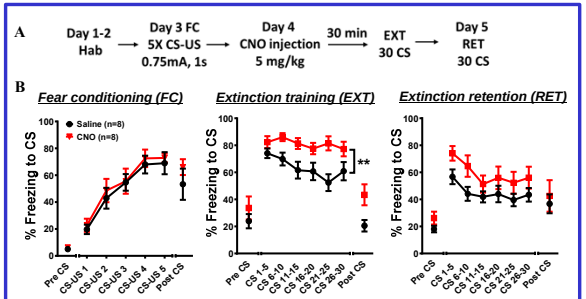

Fig. 4: Chemogenetic silencing of the LC-AT_1R^+ neurons impaired the fear extinction
(A) Auditory fear conditioning protocol. **(B)** Impaired extinction learning to conditioned fear after silencing the LC AT_1R^+ neuron during extinction training. *Hab*, habitation; *FC*, fear conditioning; *CS*, conditioned stimulus; *US*, unconditioned stimulus; *CNO*, Clozapine-n-oxide; *EXT*, extinction traning; *RET*, extinction retention.

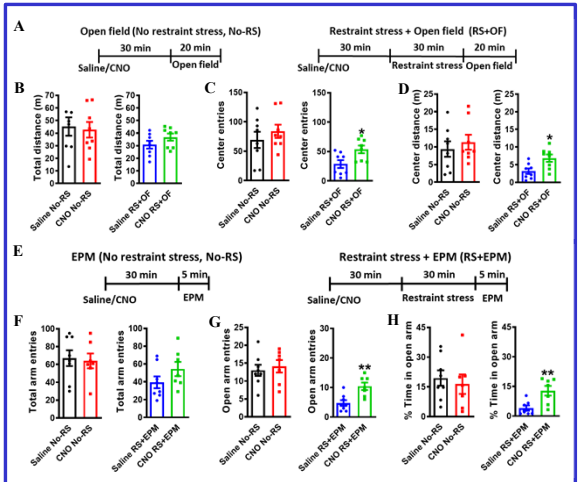

Fig. 5: Chemogenetic silencing of the LC- AT_1R^+ neurons attenuate stress-induced anxiety.
(A) Experimental protocol for the open field test. **(B-D)** AT_1R^+ neuron silencing didn't affect mice locomotor activity but blocked restraint stress-reduced anxiety in the open field test. **(E)** Experimental protocol for elevated plus maze test. **(F-G)** Inhibition of LC AT_1R^+ neurons didn't affect mice's general anxiety but attenuated the restraint stress-reduced anxiety in the elevated plus maze test. *OF*, open field test; *RS*, restraint stress; *EPM*, elevated plus maze test; *CNO*, Clozapine-n-oxide.

Summary and conclusion:

- The angiotensin system component AGT and AT_1R were found in the LC (**Fig. 1**).
- LC AT_1R^+ neurons project to CeA and BMA (**Fig. 2**).
- Chemogenetic silencing of the LC-AT_1R^+ neurons impaired the fear extinction (**Fig. 4**).
- Chemogenetic silencing of the LC- AT_1R^+ neurons attenuate stress-induced anxiety (**Fig. 5**).

❖ These data provide evidence for a novel angiotensinergic LC cell type and position the LC AT_1R as a potential mediator of noradrenergic regulation in learned fear and stress-induced anxiety.