Stress Accelerates Defensive Responses to Looming in Mice and Involves a Locus Coeruleus-Superior Colliculus Projection

Highlights
- Stressed mice display accelerated defensive responses to looming
- TH+ LC-SC pathway mediates stress-induced accelerated defensive responses
- Stress-induced defensive responses to looming are adrenergic receptor dependent

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In Brief
Li et al. identify a stress-activated TH-positive locus coeruleus-superior colliculus (LC-SC) pathway. This adrenergic-receptor-dependent connection mediates accelerated looming-evoked defensive responses, illustrating the projection-selective circuit mechanism underlying innate defensive behavior modulation.
Stress Accelerates Defensive Responses to Looming in Mice and Involves a Locus Coeruleus-Superior Colliculus Projection

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https://doi.org/10.1016/j.cub.2018.02.005

INTRODUCTION

Defensive behavioral responses to threatening stimuli are crucial to the survival of animals. It is known that specific neural circuits underlie different defensive behaviors [1, 2]. While expression of these responses is considered to be instinctive and unconditional, their magnitude may be affected by environmental and internal factors. It is believed that neural circuit activities can be reconfigured by neuromodulators to produce a variety of outputs to enhance both behavioral flexibility and robustness [3] in a rational strategic reallocation of resources to vital functions. However, the related neural circuit mechanism underlying this modulation of defensive behaviors is still largely unknown.

Looming-evoked defensive behaviors in rodents mimicking response to aerial predators have recently been used to study the neural circuitry underlying instinctive defensive behaviors [4]. We have previously interpreted a cell-specific neural pathway, from the medial region of the intermediate layer of the superior colliculus (SC) that projects to the lateral posterior thalamic nucleus (LP), to mediate innate freezing behavior resulting from looming stimuli [5, 6]. Additionally, another pathway in which PV+ neurons in the superficial layer of SC project to the parabigeminal nucleus (PBGN) mediates innate flight behavior [7]. A further study demonstrated that changing spatial environments can influence defensive behavioral outputs [8], suggesting that environmental contexts are integrated with visual stimuli information during processing of a visual threat.

The modulation of instinctive behaviors is not only important for individual adaptation to challenges but also is suggestive of a general evolutionary drive. The ubiquity of stress has shaped biological machinery, highly conserved across species, that functions to acutely mobilize bodily resources and generate behavioral outputs in response to environmental danger signals. The locus coeruleus noradrenergic (LC-NE) system is the most extensively studied stress-activated modulatory system [9, 10]. LC sends widespread outputs to many regions of the brain and spinal cord to regulate diverse functions, including sleep/wake

SUMMARY

Defensive responses to threatening stimuli are crucial to the survival of species. While expression of these responses is considered to be instinctive and unconditional, their magnitude may be affected by environmental and internal factors. The neural circuits underlying this modulation are still largely unknown. In mice, looming-evoked defensive responses are mediated by the superior colliculus (SC), a subcortical sensorimotor integration center. We found that repeated stress caused an anxiety-like state in mice and accelerated defensive responses to looming. Stress also induced c-fos activation in locus coeruleus (LC) tyrosine hydroxylase (TH)+ neurons and modified adrenergic receptor expression in SC, suggesting a possible Th::LC-SC projection that may be involved in the accelerated defensive responses. Indeed, both anterograde and retrograde neural tracing confirmed the anatomical Th::LC-SC projection and that the SC-projecting TH+ neurons in LC were activated by repeated stress. Optogenetic stimulation of either LC TH+ neurons or the Th::LC-SC fibers also caused anxiety-like behaviors and accelerated defensive responses to looming. Meanwhile, chemogenetic inhibition of LC TH+ neurons and the infusion of an adrenergic receptor antagonist in SC abolished the enhanced looming defensive responses after repeated stress, confirming the necessity of this pathway. These findings suggest that the Th::LC-SC pathway plays a key role in the sophisticated adjustments of defensive behaviors induced by changes in physiological states.
Figure 1. Stressed Mice Displayed Anxiety-like Behavior and Accelerated Defensive Responses to Looming Coupled with Higher c-fos Expression in LC

(A and B) Stressed mice (A) had fewer entries to the open field (OF) center and less time in the OF center than non-stressed mice (B; n = 8 mice for non-stressed control group, n = 12 for stressed group; ***p < 0.001), fewer entries to the open arms of the elevated plus maze (EPM) and less time in the open arms of the EPM (B; n = 7 mice for non-stressed control group and n = 12 for stressed group; **p < 0.01, *p < 0.05; dotted lines indicate open arms of EPM).
states, attention, and memory [11–15]. However, the way in which LC responds to stress stimuli and modulates neural activity in distinct brain regions to produce various behavioral outputs remains elusive. Advances in neural interrogation tools, including viral tracing, optogenetics, and chemogenetics, has made it possible to target LC and its projections with increased accuracy and resolution. Tonic activity of LC-NE neurons by optogenetics induces anxiety-like and aversive behaviors in mice [16, 17]; furthermore, the downstream LC-basolateral amygdala (BLA) projection mediates anxiety-like behavior in an adrenergic-receptor-dependent way [18]. These studies begin to illustrate how the complex LC-NE system selectively mediates specific behaviors through distinct receptor and projection-selective mechanisms.

Here, we report that stress induced an accelerated defensive response to looming stimuli, which is mediated by a novel TH+ LC-SC pathway. This modulation is adrenergic receptor-dependent. We provide neural circuit evidence that instinctive behavior is actually subjected to modulation, thus allowing more flexible circuit dynamics. The accelerated defensive responses induced by looming and associated neural pathways may also shed light on the mechanism of altered reactivity in stress-related brain disorders, such as posttraumatic stress disorder (PTSD).

RESULTS

Stressed Mice Displayed Anxiety-like Behavior and Accelerated Defensive Responses to Looming Coupled with Elevated c-fos Expression in LC

To test possible effects of stress on innate defensive responses, mice were subjected to a 4-day repeated stress protocol. Stressed mice demonstrated anxiety-like behaviors in an open field (OF) test and an elevated plus maze (EPM) (Figures 1A–1C). Compared with non-stressed controls, stressed mice had fewer entries to the OF center (Figure 1B; n = 8 non-stressed controls, n = 12 stressed; Student’s t test, t(11.0) = 5.25, p < 0.001), less time in the OF center (Figure 1B; n = 8 non-stressed controls, n = 12 stressed; Student’s t test, t(9.21) = 5.98, p < 0.001), fewer entries to the open arms of the EPM (Figure 1B; n = 7 non-stressed controls, n = 12 stressed; Student’s t test, t(13.2) = 3.43, p < 0.01) and less time in the open arms of the EPM (Figure 1B; n = 7 non-stressed controls, n = 12 stressed; Student’s t test, t(16.1) = 2.56, p < 0.05). The repeated stress had no effect on locomotion as the non-stressed control group and stressed groups had no difference in locomotor activity in the OF (Figure 1C; n = 8 non-stressed controls, n = 12 stressed; Student’s t test, t(17.8) = 0.296, p = 0.771).

Stress-evoked c-fos expression in several brain regions, including LC, raphe nuclei (DRN), periaqueductal gray (PAG), Edinger-Westphal nucleus (EW), hippocampus (HIPPO), BLA, central amygdala (CEA), bed nucleus of the stria terminals (BNST), lateral septum (LS), anterior cingulate cortex (ACC), and SC (Figure 1D; see also Figure S1; n = 3 mice per group). These brain regions have been implicated in stress-related or anxiety-like behaviors [1]. Notably, c-fos expression in LC of the stressed group was higher than that of non-stressed controls (Figures 1D and 1E; eight sections of LC per group; Student’s t test, t(9.80) = 5.29, p < 0.001).

When presented with an overhead looming stimulus, stressed mice exhibited accelerated defensive responses (Figures 1F–1H). Compared with controls, stressed mice had lower flight latencies (Figure 1H; n = 8 non-stressed controls, n = 12 stressed; Student’s t test, t(65.4) = 3.49, p < 0.001) and a higher amount of time spent in the nest (Figure 1H; n = 8 non-stressed controls, n = 12 stressed; Student’s t test, t(77.8) = 2.35, p < 0.05). Taken together, these data demonstrate that stress leads to an increase in innate fear response and, considering previous evidence of LC’s role in stress response, suggests that LC might be one of the key regulators.

Optostimulation of LC-TH+ Neurons Induced Anxiety-like Behaviors and Accelerated Defensive Responses to Looming

We next tested whether activation of LC tyrosine hydroxylase (TH+) neurons through optogenetic manipulation would be sufficient to elicit accelerated defensive responses to looming without the stress paradigm. Adeno-associated virus (AAV) carrying ChR2-mCherry or mCherry was separately injected into LC of Th-cre transgenic mice. After 4–6 weeks of viral expression, an optic fiber was implanted in LC. Immunohistochemistry (IHC) showed selective targeting of ChR2-mCherry to LC-TH+ neurons (Figures 2B and 2C). Mice were given 3 repetitions of the following: 3 min optostimulation (473 nm, 10 Hz, 10 ms width, 5–10 mW power) in the OF and then a 3 min non-stimulation interval. Optostimulation of LC-TH+ neurons induced anxiety-like behaviors in the OF both during stimulation (“light on” period) and post-stimulation (“light off” period) (Figures S2 and 2D). During stimulation, compared with mCherry controls, the ChR2 optostimulated mice had fewer entries to the OF center (Figure S2B; n = 5 mice for mCherry, n = 6 mice for ChR2; Student’s t test, t(8.69) = 2.80, p < 0.05) and less time in the OF center (Figure S2B; n = 5 mice for mCherry, n = 6 mice for ChR2; Student’s t test, t(4.96) = 2.65, p < 0.05). For post-stimulation, compared with mCherry controls, the ChR2 optostimulated mice had fewer entries to the OF center (Figure 2D; n = 5 mice for mCherry, n = 6 mice for ChR2; Student’s t test, t(8.99) = 2.42, p < 0.05) and less time in...
the open arms of the EPM (Figure 2D; n = 5 mice for mCherry, n = 6 mice for ChR2; Student’s t test, t(7.84) = 2.36, p < 0.05).
The optostimulation of LC TH+ neurons had no effect on the locomotion of the mice as the mCherry and ChR2 groups showed no differences in locomotor activity in the OF (Student’s t test, n = 5 mice for mCherry, n = 6 mice for ChR2: Figure S2B, during stimulation, t(29.5) = 0.700, p = 0.489; Figure S2C, post-stimulation, t(27.5) = 0.766, p = 0.450). One day after optostimulation, mice were presented with the looming stimulus in the looming test apparatus. The ChR2 group exhibited accelerated defensive responses compared with mCherry controls (Figures 2E and 2F). The ChR2 group had a lower flight latency compared with controls (Figure 2F; n = 5 mCherry, n = 6 ChR2; Student’s t test, t(42.3) = 4.49, p < 0.001). Optostimulation at LC TH+ neurons significantly induced more c-fos expression in LC of the ChR2 group compared with mCherry control (Figure S2D; 16 sections of LC from n = 4 mice for mCherry control group and 21 sections from n = 4 mice for ChR2 group; Student’s t test, t(22.0) = 6.72; p < 0.0001). Elevated c-fos expression in SC by optostimulation of LC TH+ neurons was also observed in ChR2 group compared with mCherry controls (Figure S2E; 51 sections of n = 4 mice for mCherry control group and 46 sections from n = 4 mice for ChR2 group; Student’s t test, t(74.5) = 2.41, p < 0.05), suggestive of SC being downstream of LC TH+ neurons activation.
The behavior displayed by these non-stressed mice after optical activation of TH+ LC neurons mimicked that of stressed mice, suggesting the involvement of LC-related circuitry in mediating the stress-induced increased expression of innate fear-related defensive responses.

Chemogenetic Inhibition of LC-TH+ Neurons Rescued Stress-Induced Accelerated Defensive Responses to Looming
To test whether LC-TH+ neuronal activation is necessary for the stress-induced accelerated looming response, AAV virus carrying hM4Di was bilaterally injected into LC. Following the stress paradigm, both mCherry control and hM4Di mice groups were given intraperitoneal (i.p.) injections of clozapine N-oxide (CNO) (0.5 mg/kg body weight [BW]). Considering the recent insights into DREADD activation in the brain by the CNO metabolite clozapine by Gomez et al. [19], CNO was administered to both the mCherry control group and the hM4Di group, and the effect of this relative low dose of CNO (0.5 mg/kg BW) on the basal locomotor activity of the mice was checked. One hour post-CNO administration, mice were presented with a looming test. Following inhibition of LC neural activity, the stress-induced accelerated defensive response of the stressed/hM4Di group was markedly lower than that of the stressed/mCherry control group (Figure 3C; n = 9 mice for mCherry control, n = 10 mice for hM4Di group; Student’s t test, t(48.3) = 6.78, p < 0.001). The hM4Di group had a lower amount of c-fos-activated TH+ neurons than that of the mCherry controls, indicating inhibition of LC TH+ neuronal activity (Figures 3D and 3E; 21 sections of LC from n = 5 mice for mCherry control and 27 sections of LC from n = 5 mice hM4Di group, Student’s t test, t(42.6) = 2.23, p < 0.05). LC neuronal inhibition due to CNO injection into the hM4Di mice did not cause any change in locomotor activity compared to the mCherry controls (Figure 3F; n = 9 mice for mCherry control, n = 10 mice for hM4Di group; Student’s t test, t(17.0) = 0.071, p = 0.944). These results demonstrate the necessity of LC-TH+ neuronal activation for the stress-induced accelerated looming response.

Anatomical Tracing Identified a TH+ LC Input to SC that Is Activated by Stress
Due to the accumulation of evidence of SC involvement in looming-evoked defensive response, we next looked for the probable anatomical connection from LC to SC. Three different approaches were used to characterize the LC inputs to SC (Figures 4 and S4).
For the first experiment, the anatomical LC-SC projection was studied using anterograde labeling (Figures 4A and 4B; n = 4 mice). AAV2/9-DIO-mCherry virus was unilaterally injected into the LC of Th-cre transgenic mice. The LC-TH+ projecting terminals were observed in SC. Immunostaining of TH in SC revealed that the LC-projecting fibers were partially co-labeled with TH (Figure 4B). This experiment showed that LC sends direct TH+ projections to SC.
For the second experiment, the anatomical LC-SC projection was studied using a system of designer variant of AAV, AAV-Retro system developed by Tervo et al. [20] (Figures S4A and S4B; n = 4 mice). In wild-type C57 mice, AAV-Retro-Cre was injected in SC while AAV-DIO-mCherry was injected in LC. After 4 weeks expression, mCherry signal was found in LC. TH immunostaining was carried out to identify the location of LC and revealed that some of the SC-projecting LC neurons were TH-positive (Figure S4B).
For the third experiment, we used Fluor 488 Conjugate Cholera Toxin Subunit B (CTB) tracing together with stress protocol to determine whether the SC-projecting LC TH+ neurons were activated by the stress (Figures 4C–4F). CTB was injected into SC bilaterally in wild-type C57 mice. The SC coordinates for the retrograding tracing was determined by the LC-TH+ fiber localization in SC. Two weeks after CTB injection, the mice were subjected to 4-day repeated stress paradigm. Ruralgrade
**A**

TH-cre LC; hM4Di

AAV-DIO-hM4Di-mCherry

**B**

hM4Di expression in LC^{TH+} neurons

**C**

Post-stress Looming

**D**

Post-stress cfos expression

**E**


dcfos+TH (%)**

**F**

Total Activity (m)

*Please cite this article in press as: Li et al., Stress Accelerates Defensive Responses to Looming in Mice and Involves a Locus Coeruleus-Superior Colliculus Projection, Current Biology (2018), https://doi.org/10.1016/j.cub.2018.02.005*
labeling of the LC neurons from SC-CTB injection was observed, and quantitative analysis of CTB retrograde labeling revealed that 64.8% ± 1.61% of the CTB-labeled SC-projecting LC neurons are TH-positive, suggesting that there are other types of LC neurons projecting to SC (Figure 4E; 70 sections of LC in total from n = 5 mice counted). Also, 48.8% ± 2.56% of the SC-projecting LC TH+ neurons of the stressed mice were c-fos activated, which was much higher than the corresponding percentage of the non-stressed control group, 16.6% ± 3.21% (Figure 4F; 26 sections of LC in total from n = 2 non-stressed mice and 44 sections of LC in total from n = 3 mice were counted, Student’s t test, t(54.4) = 7.77, p < 0.001), indicating that the LCTH+-SC pathway was activated by stress.

**Optostimulation of Th::LC-SC Terminals Induced Anxiety-like Behaviors and Accelerated Defensive Responses to Looming**

We next tested whether TH+-LC-SC projections were sufficient to evoke accelerated defensive responses to looming. The AAV-DIO-ChR2-mCherry or AAV-DIO-mCherry virus was injected into LC of Th::cre mice. After 6–8 weeks virus expression, an optical fiber was implanted in SC (Figures 5A and 5B). The ChR2-mCherry neurons in LC and the ChR2-mCherry terminals in SC co-labeled with TH immunoreactivity (Figure 5C). Mice were given 3 repetitions of the following: 3 min optostimulation (473 nm, 10 Hz, 10 ms width, 15–20 mW power) in the OF and then a 3 min non-stimulation interval. Optostimulation of Th::LC-SC terminals induced anxiety-like behaviors in the OF both during stimulation (“light on” period) and post-stimulation (“light off” period) (Figure 5D, also refer to Figure S5). During stimulation, compared with mCherry controls, the ChR2 optostimulated mice showed fewer entries to the OF center (Figure S5B; n = 5 mice for mCherry, n = 6 mice for ChR2; Student’s t test, t(5.82) = 4.33, p < 0.01). For post-stimulation, compared with mCherry controls, the ChR2 optostimulated mice had fewer entries to the OF center and less time in the OF center (Figure 5D; n = 5 mice for mCherry, n = 6 mice for ChR2; Student’s t test, for entries to center, t(8.93) = 5.56, p < 0.001, for time in the OF center, t(8.52) = 3.29, p < 0.05) and less time in the open arms of the EPM (Figure 5D; n = 5 mCherry, n = 5 ChR2, Student’s t test, t(5.45) = 2.66, p < 0.05). The optostimulation of Th::LC-SC terminals had no effect on the locomotion of the mice as the mCherry and ChR2 groups showed no differences in locomotor activity in the OF (Student’s t test, n = 5 mice for mCherry, n = 5 mice for ChR2; during stimulation, Figure S5B, t(6.09) = 0.704, p = 0.508; post-stimulation, Figure S5C, t(8.98) = 0.85, p = 0.419). One day after optostimulation, the animals underwent a looming stimulus test in the looming test apparatus. The ChR2 group exhibited accelerated defensive responses compared with mCherry controls (Figures 5E and 5F). The ChR2 group showed a lower flight latency compared with controls (Figures 5E and 5F), n = 5 mCherry, n = 6 ChR2; Student’s t test, t(27.9) = 4.43, p < 0.001).

Optostimulation of Th::LC-SC terminals at a lower frequency (473 nm, 5 Hz, 10 ms width, 15–20 mW power) also induced anxiety-like behaviors post-stimulation (Figure S6D; ChR2 group spent less time in the OF center compared with controls, n = 7 mCherry, n = 5 ChR2; Student’s t test, t(7.31) = 2.72, p < 0.05) and accelerated defensive responses to looming (Figure S6E; ChR2 group showed a lower flight latency compared with controls, n = 7 mCherry, n = 5 ChR2; Student’s t test, t(26.5) = 2.90, p < 0.01). These results show that optostimulation of Th::LC-SC terminals induced anxiety-like behaviors and that activation of TH+ LC-SC pathway was sufficient to elicit an accelerated defensive response to looming.

**Adrenergic Receptor Expression Was Modified by Stress, and the Stress-Accelerated Defensive Responses to Looming Were Reduced by Blockade of Adrenergic Receptors in SC**

The LC-NE system has been implicated in diverse behavioral modulation. As such, because we had identified a novel LC-SC TH+ pathway modulating the effect of stress on an innate fear-related defensive behavior, our next step was to determine whether NE and its receptors are the local effectors in SC. To do this, the SC of stressed or non-stressed control mice was collected and used for gene-chip analysis. The gene-chip analysis revealed lower expression of adrenergic receptor, alpha 2a (Adra2a); adrenergic receptor, beta 2 (Adrb2); adrenergic receptor, beta 3 (Adrb3); adrenergic receptor, alpha 1d (Adra1d); and adrenergic receptor, alpha 1a (Adra1a) in SC (Figure 6A; n = 3 mice per group). To validate the results of gene-chip analysis and achieve a quantitative evaluation of SC adrenergic receptor expression, SC from stressed and non-stressed animals was analyzed by qPCR. qPCR analysis revealed lower gene expression of adrenergic receptor, alpha 1b (Adra1b) (t(9.82) = 3.38, p < 0.01), adrenergic receptor, beta 1 (Adrb1) (t(8.12) = 10.56, p < 0.001), and adrenergic receptor, beta 3 (Adrb3) (t(11.5) = 3.54, p < 0.01) in SC of the stressed group (Figure 6C; n = 7 mice for non-stressed controls, n = 8 mice for stressed; Student’s t test). These modified adrenergic receptor expression levels in SC after stress are indicative of involvement of NE and its receptors.
Please cite this article in press as: Li et al., Stress Accelerates Defensive Responses to Looming in Mice and Involves a Locus Coeruleus-Superior Colliculus Projection, Current Biology (2018), https://doi.org/10.1016/j.cub.2018.02.005
To test the functional role(s) of NE and its receptors in stress-induced accelerated defensive responses, cannulas were bilaterally implanted in SC as illustrated in Figure 5D. Following the 4-day stress protocol, either the adrenergic receptor α antagonist idazoxan or the adrenergic receptor β antagonist atenolol was infused into SC bilaterally 15 min before a looming test, using saline as control. The stress-induced accelerated defensive responses to looming were reduced by intra-SC infusion of idazoxan or atenolol, resulting in a recovery of onset of flight latency (Figure 6E for idazoxan, n = 7 saline controls, n = 8 idazoxan; Student’s t test, t(46.8) = 2.87, p < 0.01; Figure 6F for atenolol, n = 7 saline controls, n = 7 atenolol; Student’s t test, t(58.9) = 2.54, p < 0.05).

These results suggest that the stress-induced accelerated defensive response to looming were adrenergic receptor dependent.

DISCUSSION

Generation of appropriate defensive behaviors in response to the external dangerous signals is important for individual survival. In the current study, we report that the accelerated defensive response to looming induced by stress is mediated by LC via direct TH+ projections to SC. Despite numerous studies on LC anatomy and function, the modulatory roles of LC projections in innate defensive responses are not well defined. Optostimulation of TH+ projections from LC to SC was sufficient to induce the acceleration of innate defensive responses caused by stress. Chemogenetic inhibition and receptor antagonist demonstrated the necessity of this pathway and its adrenergic receptor dependence. We also provide evidence for the notion that instinctive and unconditioned innate-behavior-related brain circuits can be modulated by previous experience (i.e., stress) and/or internal emotional states (i.e., anxiety) [21]. These results highlight the important roles neuromodulators can have within neural circuits and the benefit of both systems and molecular levels of analysis when teasing apart the neurobiological underpinnings of specific behaviors [22, 23]. For example, neuromodulators, such as NE, can reconfigure neural circuits and alter behavioral output such that there is an accelerated defensive response to looming.

Anxiety and fear are highly correlated, and this is conserved across species. The brain areas and neuromodulator systems involved in anxiety and fear largely overlap [1, 2]. Nevertheless, the detailed mechanisms of the circuits involved in these emotional states and their relationship with their causal stressors have not been fully teased apart. In our study, the stress-induced LC-SC circuit activation not only resulted in mice displaying anxiety-like behavior but also resulted in accelerated innate-fear-related defensive response to looming. Looming stimuli have been shown to trigger defensive responses that are conserved in rodents [4], monkeys [24], and humans [25, 26]. In human subjects, threatening stimuli (snakes and spiders) are perceived as approaching more rapidly than non-threatening stimuli (butterflies and rabbits). The magnitude of this misperception correlates with self-reported fear [27]. Our accelerated looming-evoked defensive responsive results are in line with this affective modulation of the looming responses in humans. Previous reports have demonstrated the impact of stress in anxiety and paralleled conditioned fear responses [28–33] and the impact of anxiogenic manipulations in ultrasound-evoked defensive behaviors [34]. To our best knowledge, we report here the first neuronal pathway that is involved in regulating the impact of stress on innate visual-cue-induced defensive fear responses.

How stress-activated LC-NE system modulates neuronal activity in distinct brain regions and influences a spectrum of behavioral outputs is intriguing considering how tiny LC is (only around 1,600 cells per nucleus in the rodent) [35, 36]. Possible roles of the LC-NE system in mediating visuomotor function have been put forward, because the spatial and visuomotor-response-related regions (i.e., the tecto-pulvinar-extrastriate visual structures), including SC, were more preferentially innervated by NE fibers in primates [37, 38]. Our data validate the modulatory role of LC-NE system in SC together with its projection-selective mechanism. In respect to looming stimuli, the stress-activated TH+ projections from LC to SC, along with the reported SC-LP and SC-PBGN circuits [5, 7], modulate the defensive responses. Our study begins to illustrate the projection-selective mechanism of the complex LC-NE system regarding to diverse behavioral spectrum, accounting for the behavior output flexibility to external dangerous signals.

However, the detailed mechanism of how LC-SC-projecting neurons regulate SC local neural activity has not yet been established. In our study, the defensive responses of stressed animals were reduced after blocking SC α2 and β1 adrenergic receptors, suggesting that the modulation of the defensive response is adrenergic receptor dependent. Moreover, the expression levels of both α and β adrenergic receptor subtypes were lower in the SC of stressed mice compared to non-stressed mice. This could be an adaption to increased local NE levels after stress [39, 40]. This pharmacological data lead to the implication that LC-SC projections might influence local SC neural activities partially by modulating NE-activated responses.

Figure 4. Anatomical Tracing Identified a TH+ LC Input to the SC that Is Activated by Stress
(A) AAV tracing of TH+/LC-SC projection strategy and experimental timeline, unilateral LC injection.
(B) IHC revealed co-labeling of the TH staining with the mCherry-labeled SC terminals from LC-TH+ neurons in SC (blue, DAPI; green, TH; red, LC-SC projecting terminals-mCherry; arrows, co-localization examples; scale bars, 50 μm; selected from four mice).
(C) Retrograde tracing using CTB strategy and experimental timeline, bilateral SC injection.
(D) Representative figures illustrate the retrograde labeling of LC neurons from injection in SC (blue, TH; green, CTB-Alexa 488; red, c-fos; arrows, co-localization examples; scale bars, 200 μm and 20 μm, respectively; selected from five mice).
(E) Quantitative analysis of CTB retrograde labeling revealed that 64.8% ± 1.61% of the CTB-labeled SC-projecting LC neurons are TH-positive (70 sections of LC in total from n = 5 mice).
(F) In stressed mice, 48.8% ± 2.56% of the SC-projecting LC-TH+ neurons were c-fos activated, which was much higher than the non-stressed control group, which was 16.6% ± 3.21% (70 sections of LC in total from n = 5 mice; Student’s t test, ***p < 0.001). For all graphs, data were presented as mean ± SEM. See also Figure S4,
Figure 5. Optostimulation of TH::LC-SC Terminals Induced Anxiety-like Behaviors and Accelerated Defensive Responses to Looming
(A) Optogenetic strategy and experimental timeline, unilateral SC optical activation.
(B and C) Representative IHC shows position of fiber track in SC (B), selective targeting of ChR2-mCherry to LC-TH+ neurons in LC, and co-labeling of TH immunostaining with the mCherry-labeled SC terminals from LC-TH+ neurons in SC (C; blue, DAPI; green, TH; red, mCherry; arrows, co-localization examples; solid line, fiber track; scale bars, 500 μm, 200 μm, 50 μm, and 20 μm, respectively).
through different patterns of post-synaptic adrenergic receptors. NE and its adrenergic receptors have also been implicated in stress-induced modulation in humans, in which the MRI activity patterns of both cortical and subcortical brain regions after exposure to stressful visual stimuli were distinct from those engaged by neutral stimuli, and this effect was inhibited by the \( \beta \)-adrenergic antagonist [21]. Our mouse model results are consistent with this stress-induced brain modulation in humans, suggesting that the stress-related adaption is conserved across species.

Another possible mechanism by which LC-SC-projecting neurons regulate SC neural activity, aside from varying expression levels of post-synaptic adrenergic receptors, may be local pre-synaptic regulation of NE release. A third possibility is that the LC-SC pathway regulates the co-release of NE and other neurotransmitters in SC, given the recent suggestion that dopamine is involved in the novelty-associated memory enhancement effect of LC TH\(^+\) neuronal activation [41]. Dopamine beta-hydroxylase (DBH) is an enzyme critical for NE synthesis, which catalyzes the chemical reaction of transforming dopamine into NE. We optostimulated the

(D) Optostimulation of Th::LC-SC terminals in OF resulted in fewer entries, less time spent in the OF center, and less time in the open arms of EPM post-stimulation compared with mCherry controls (n = 5 mice for mCherry control, n = 6 mice for ChR2; Student’s t test, \(^{*\*\*}\ p < 0.001, \ ^{*\*}\ p < 0.01\).

(E) Representative speed profiles illustrate quicker flight latency after looming stimulus in the ChR2-mCherry mouse compared with mCherry control.

(F) Following optostimulation of the LC-SC TH\(^+\) terminals, the ChR2 group had lower flight latency compared with controls (n = 5 mice for mCherry control, n = 6 mice for ChR2; each dot represents the result of 1 trial of looming test of the animal; Student’s t test, \(^{***}\ p < 0.001\)).

For all graphs, data were presented as mean ± SEM. See also Figures S5 and S6.
LC-DBH+ neurons, which also resulted in anxiety-like behaviors and accelerated defensive responses to looming (Figure S3). Still, further work is required to test these possible mechanisms.

Dysfunction of the LC-NE system is implicated not only in psychiatric disorders, such as anxiety and depression, but it is also strongly correlated with neurodegenerative disorders, such as Parkinson’s disease and Alzheimer’s disease with selective and early neural degeneration [1, 42]. The development of genetic, viral, optogenetic, and chemogenetic tools have allowed us to determine more precisely the functional significance of the LC-NE system, its specific anatomical and functional connectivity, and molecular mechanisms underlying the fine-tuned regulation of diverse behavioral consequences. Further work identifying and characterizing brain pathways involved in specific behaviors and behavioral modulation may help in the understanding and treatments of these disorders, especially stress-related brain disorders.

**STAR★METHODS**

Detailed methods are provided in the online version of this paper and include the following:

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**SUPPLEMENTAL INFORMATION**

Supplemental Information includes six figures and can be found with this article online at https://doi.org/10.1016/j.cub.2018.02.005.

**ACKNOWLEDGMENTS**

This work was supported by National Natural Science Foundation of China (NSFC) 31630031 (L.W.), NSFC 81425010 (L.W.), NSFC 31471109 (Lei Li), and NSFC 31500861 (Z.Z.); International Partnership Program of Chinese Academy of Sciences 152644KYS820170004 (L.W.); External Cooperation Program of the Chinese Academy of Sciences GJHZ1508 (L.W.); Guangdong Academy of Sciences 172644KYS820170004 (L.W.); External Cooperation and NSFC 31500861 (Z.Z.); International Partnership Program of Chinese (NSFC) 31630031 (L.W.), NSFC 81425010 (L.W.), NSFC 31471109 (Lei Li), and NSFC 31500861 (Z.Z.).

**REFERENCES**


STAR METHODS

KEY RESOURCES TABLE

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CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Liping Wang (lp.wang@siat.ac.cn).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mice

Adult (6-8 weeks) C57BL/6J (Guangdong Medical Laboratory Animal Center, Guangzhou, China) and Th-cre mice (from Dr. Zilong Qiu) were group-housed, given access to food pellets and water ad libitum, and maintained on a 12:12-hr light/dark cycle (lights on at 7:00 a.m.). All husbandry and experimental procedures in this study were approved by Animal Care and Use Committees at the Shenzhen Institute of Advanced Technology (SIAT), Chinese Academy of Sciences (CAS), China.

METHOD DETAILS

Open field test (OFT)

An open field (OF) arena (50 cm x 50 cm x 50 cm) made of white PVC was used to assess anxiety-related behavior and locomotor activity. It is also where the mice received optostimulation. For analyses, we defined the center of the arena as a smaller concentric square inside the arena, covering 25% of the area of the arena floor. (i.e., 25 cm x 25 cm). For the OFT following the stress paradigm (see below), Mice were first allowed to freely explore the OF for 5 min, then a 3-min section were analyzed. The number of entries to
the center, time in the center and the total distance traveled in Open Field (total activity) were recorded and then analyzed by Anymaze software (Stoelting, IL, USA). For the opto-stimulation experiment, the stimulation was given while the mouse was inside the 50 cm x 50 cm x 50 cm open field arena. During optostimulation sessions, animals were first allowed to freely explore the OF for 5 min. Of this, 3 min were analyzed to obtain baselines. Animals were then subjected to 3 min opto-stimulation (473 nm, 10 Hz, 10 ms width, 5-10 mW power for LC soma, 15-20 mW power for SC terminals), then a 3 min non-stimulation interval. Optostimulation alternated off and on for 3 cycles. For the behavioral analysis of the mice in the OF following opto-stimulation, the number of entries to the center, time in the center and the total distance traveled in OF (total activity) were analyzed. The OF was cleaned between mice with 20% ethanol solution. For optogenetic experiments, prior to behavioral testing, the mCherry and ChR2 mice were both handled and habituated to the light fiber being connected to implant.

Elevated plus maze (EPM)
Mice were placed on a four-arm plus maze with two open and two closed arms (white PVC, 30 cm length per arm × 5 cm width) raised 50 cm above the ground for 5 min sessions. The EPM was cleaned between mice with 20% ethanol solution. The number of entries to the open arms and time spent in the open arms were recorded and analyzed by Anymaze software.

Repeated stress paradigm
Mice underwent a four-day repeated stress protocol that included (i) being held in a restraint for 2 hr (ii) a 20 min forced swim and (iii) a foot shock session (0.5 mA, 2 s, 10 s interval) lasting 20 min. The baseline and stressed anxiety states of the animals were evaluated by OFT and EPM. Different groups of stressed animals were presented with a looming test, a chemogenetic inhibition looming test and an adrenergic receptor blockage looming test separately along with non-stressed controls. To map whole brain c-fos activation, another group of mice underwent this stress protocol were anesthetized with an overdose of pentobarbital and perfused with 4% paraformaldehyde in phosphate-buffered saline (PBS) prior to immunohistochemistry (see below) along with non-stressed controls. Sections after IHC staining was subjected to an Olympus VS120 virtual microscopy slide scanning system. The images taken were then overlaid with The Mouse Brain in Stereotaxic Coordinates to locate the related brain regions. Then the c-fos staining within each targeted brain region, including LC, was manually counted by individual experimenter blind to the experiment groups; the location of LC was further checked by immunostaining of tyrosine hydroxylase (TH).

Looming test
The looming test was performed as described previously [4, 5]. It was performed in a closed plexiglass box (40 cm length, 40 cm width and 30 cm height) with a dark shelter nest in the corner. An LCD monitor was placed on the ceiling to present the looming stimulus, which was an expanding black disc expanding from a 2° to 20° visual angle (Figure 1F). Behaviors were recorded using an infrared camera. Animals were handled and habituated for 15 min to the looming box one day before the test. On the day of looming test, each mouse was allowed to first freely explore the looming box for 5 min. Then each mouse was presented with the looming stimulus for 5 trials. The stimulus was triggered by the experimenter when the mouse entered an area at the far end of the arena (away from the nest). The following measures were obtained as indices of looming-evoked defensive behavior: (i) flight latency (ms): the time from looming stimulus presentation to onset of escape to the nest, (ii) time to the nests(s): time taken to escape into the nest and (iii) time spent in nest (% of 3 min bin): time spent in the nest following escape. These data were recorded and analyzed with Anymaze software with additional analyses performed using Adobe Premiere and MATLAB (Mathworks, US). For blinding purposes, all mice used for behavioral experiments were given a unique ear tag numerical identifier. Data obtained from mice with imprecise cannula placement were not used for analyses.

Histology
After completion of experiments, mice were transcardially perfused with 4% paraformaldehyde in PBS. Fixed brains were cryoprotected in 30% sucrose in PBS and cut on a cryostat in 30 µm slices. Immunohistochemistry was performed to map (i) the c-fos activation in the brain after stress, (ii) the co-localization of ChR2-mCherry virus expression at LC and SC with TH immunoreactivity, and (iii) the triple-localization in LC of TH immunoreactivity, c-fos activation and CTB retrograde tracing from SC to LC. Antibody staining was performed on single-well floating tissue sections. Sections were incubated for 48 hr in primary antibodies at 4° C followed by overnight incubation with secondary antibodies at 4° C. Primary antibodies used in this study were: rabbit anti-c-fos (2250, Cell Signaling Technology; 1:500), chicken anti-Tyrosine Hydroxylase (ab76442, abcam; 1:500). Suitable secondary antibodies were chosen to reveal the co-localization with different fluorescent colors. For counterstaining, sections were incubated for 10 min with 4', 6-diamidin-2-phenylindol (DAPI, 0.4 µg/mL, Sigma). All of the images were captured with a Zesis LSM 880 confocal microscope or an Olympus VS120 virtual microscopy slide scanning system. Similarly, somatic and terminal viral expression, virus injection accuracy, optical fiber terminal positions and cannula positions were assessed using sections without antibody staining. Location of LC was confirmed by TH antibody staining.

Stereotaxic surgery and virus injection
Stereotaxic surgeries and virus injections were performed as described previously [5, 43]. Briefly, animals were anesthetized with sodium pentobarbital (80 mg/kg BW) and placed in a stereotaxic apparatus (RWD, China) where anesthesia was maintained with 1% isoflurane. Injections were made using a microsyringe pump (UMP3/Micro4, USA) using a 10 µL syringe connected to a...
33-Ga needle (Neuros; Hamilton, Reno, USA). The syringe was not removed until 10 min after the end of infusion to allow diffusion of the virus.

AAV2/9 viruses encoding Ef1α-DIO-hChR2(H134R)-mCherry (for optogenetic experiments); Ef1α-DIO-hM4Di-mCherry (for chemogenetic experiment); AAV2-Retro-CMV-Cre and AAV2/9-Ef1α-DIO-mCherry (for retrograde tracing) were packaged by BrainVTA, China. Viral vector titers were in the range of 3 to 6 X10¹¹ genome copies/mL. The LC coordinates were AP: -5.3 mm, ML: -0.8 mm and DV: -4.0 mm; The SC coordinates were AP: -3.8 mm, ML: -0.8 mm and DV: -1.8 mm. For chemogenetic experiment, the LC received bilaterally injections.

**Fiber implants and in vivo optogenetic manipulation**
Th-cre mice were used for optogenetic experiments. For in vivo optogenetic manipulation in awake, freely moving mice, an optical fiber cannula (200 µm in diameter, NA: 0.37, RWD, China) was implanted into LC unilaterally (AP: -5.3 mm, ML: -0.8 mm and DV: -3.5 mm) or SC unilaterally (AP: -3.8 mm, ML: -0.8 mm and DV: -1.2 mm) for somatic and terminal optostimulation respectively. The mice were then given 2 weeks recovery time before experiment began. During experiments, the optic fiber was connected to a laser source using an optic fiber sleeve. Control mCherry group mice underwent the same procedure and received the same intensity of laser stimulation (473 nm, 10 Hz, 10 ms width, 5-10 mW power for LC soma, 15-20 mW power for SC terminals; no seizure-like behavior observed for all the stimulation; Th-cre mice, n=5-6/group).

**in vivo chemogenetic manipulation**
Th-cre transgenic mice were used for chemogenetic experiments. Mice injected bilaterally Ef1α-DIO-mCherry and CNO i.p. injection were used as control group and mice injected with Ef1α-DIO-H4Di-mCherry and CNO i.p. injection were used as the LC H4Di inhibition group. After 4-day stress protocol, mice underwent in vivo chemogenetic inhibition of LC prior to the looming test. For this, mice with either Ef1α-DIO-H4Di-mCherry expression or Ef1α-DIO-mCherry expression in LC were i.p. injected with CNO (0.5 mg/kg BW, Clozapine N-oxide, C0832, Sigma) 1 hr before looming test. Possible side effects of CNO injections on locomotor activity were evaluated by comparing performance in an OF 1 hr after CNO injection (Th-cre mice, n=5-7/group) and c-fos expression in LC after 4-day stress protocol was examined to check the effect of chemogenetic inhibition of LC neurons.

**Cannula implants and pharmacological antagonist**
C57BL/6J mice were used for pharmacological experiments. Drug cannulas were bilaterally implanted into the SC (AP: -3.8 mm, ML: +/- 0.8 mm and DV: -1.3 mm). Mice were given 2 weeks to recover after surgery. After undergoing the 4-day stress protocol, either saline, α2-adrenergic receptor antagonist idazoxan hydrochloride (30.0 mg/mL, 200nl; I6138, Sigma; C57 mice, n = 7-8/group) or β1-adrenergic receptor antagonist atenolol (3.3 mg/mL, 300nl; A7655, Sigma; C57 mice, n = 7/group) was infused bilaterally into the drug cannulas 15 min before a looming test to assess the antagonistic effect of adrenergic receptors.

**Anatomical tracing and c-fos and TH co-labeling**
To characterize LC inputs to SC, we used 3 different approaches. Three groups of mice were used. For the first group of mice, AVV2/9-DIO-mCherry virus was injected into LC (Th-cre mice, n = 4) unilaterally. Following 6-8 weeks of viral expression and recovery, animals were anesthetized with an overdose of pentobarbital, perfused with 4% paraformaldehyde in PBS prior to IHC staining of TH. For the second group of mice, AAV2/1-Retro-Cre virus was injected into SC and AAV2/9-DIO-mCherry virus was injected into LC (WT C57, n = 4) both unilaterally. After 4 weeks, the mice were sacrificed for tracing. For the third group of mice, Cholera Toxin Subunit B (Recombinant), Alexa Fluor 488 Conjugate (CTB, C-22841, Invitrogen) was injected into SC bilaterally (WT C57, n = 5). Two weeks later, the mice were subjected to the stress paradigm, then sacrificed as described for CTB retrograde signals at LC and test their co-labeling with c-fos and TH immuno-signals. For virus or neural tracer injections, the SC coordinates used were, AP: -3.8 mm, ML: -0.8 mm and DV: -1.8 mm; LC coordinates were AP: -5.3 mm, ML: -0.8 mm and DV: -4.0 mm; LC coordinates for CTB bilateral injection were AP: -5.3 mm, ML: ± 0.8 mm and DV: -4.0 mm.

**Gene-Chip assay**
Following 4-day stress protocol, the stressed mice and non-stressed controls were anesthetized with an overdose of pentobarbital one day after stress for Gene-chip analysis (C57 mice, n = 3 per group). SC tissues were freshly dissected for RNA isolation with TRIzol Reagent (15596026, Invitrogen). RNA (10 μg) was incubated with DNase I for 30 min, and then cleaned up by RNeasy MinElute Cleanup Kit (74204, QIAGEN). RNA yield and quality was assessed by a micro-spectrophotometer (NanoDrop One, Thermo) and denaturing agarose gel electrophoresis. First Strand cDNA Synthesis was performed using SuperScript III Reverse Transcriptase (Invitrogen) with all-thymine oligo (dT) 18 primer (SO131, Thermo) following the manufacturer’s instruction. The cDNA product was then used for the Gene-Chip analysis using RT² SYBR Green qPCR Mastermix (330522, QIAGEN). Expression of each gene across PCR Arrays was analyzed with ΔΔCt method.

**Quantitative RT-PCR (qPCR)**
To validate the results of gene-chip and achieve a quantitative evaluation of SC adrenergic receptor expression, SC from stressed and non-stressed animals was analyzed by qPCR (C57 mice, n = 7-8 per group). Total RNA was isolated from SC of control and stressed mice with Trizol reagent (15596018, Invitrogen), quantified by a micro-spectrophotometer (NanoDrop One, Thermo). One
microgram total RNA was subject to cDNA synthesis using PrimeScript RT reagent Kit with. Quantitative RT-PCR was performed using SYBR Premix Ex Taq II (Tli RNaseH Plus) (RR820A, Takara), with a LightCycler 480 Real-Time PCR System (Roche). Actin was used to normalize the expression levels of the adrenergic receptor(s).

**QUANTIFICATION AND STATISTICAL ANALYSIS**

All summary data are expressed as mean ± SEM. Statistical significance was taken as *p < 0.05, **p < 0.01, ***p < 0.001. No statistical methods were used to pre-determine sample sizes but our sample sizes are similar to those generally employed in the field. All n values represent the number of mice used in each experiment. Student’s t test was used as appropriate.