Active control of arousal by a locus coeruleus GABAergic circuit

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Arousal responses linked to locus coeruleus noradrenergic (LC-NA) activity affect cognition. However, the mechanisms that control modes of LC-NA activity remain unknown. Here, we reveal a local population of GABAergic neurons (LC-GABA) capable of modulating LC-NA activity and arousal. Retrograde tracing shows that inputs to LC-GABA and LC-NA neurons arise from similar regions, though a few regions provide differential inputs to one subtype over the other. Recordings in the locus coeruleus demonstrate two modes of LC-GABA responses whereby spiking is either correlated or broadly anticorrelated with LC-NA responses, reflecting anatomically similar and functionally coincident inputs, or differential and non-coincident inputs, to LC-NA and LC-GABA neurons. Coincident inputs control the gain of LC-NA-mediated arousal responses, whereas non-coincident inputs, such as from the prefrontal cortex to the locus coeruleus, alter global arousal levels. These findings demonstrate distinct modes by which an inhibitory locus coeruleus circuit regulates arousal in the brain.

Noradrenergic (NA) neurons located in the locus coeruleus (LC) send broad projections to a wide variety of brain regions, and their activity correlates with levels of arousal and cognitive performance

During wakefulness, fluctuations in LC-NA arousal modify plasticity, shift attention, induce anxiety, or affect discrimination and general sensory perception. Even though the effect of NA-mediated arousal on brain processing has become clearer in recent years, we still have a poor understanding of the mechanisms that regulate NA activity.

LC neurons receive inputs from a large number of brain regions, which probably underlies the diverse contexts that drive LC-NA neuronal activity, including sensory stimuli and stressors. In particular, LC neurons are thought to be novelty detectors, as NA release increases in response to novel sensory stimuli and with stimulus saliency. The novelty response also changes with learning, suggesting that distinct mechanisms suppress or promote LC-NA-mediated arousal. Alongside these mechanisms regulating phasic LC responses, the modulation of tonic LC activity can also occur over longer timescales, such as during different levels of vigilance, or during environmental changes or goal-directed behaviors. Tight regulation of the global level of arousal has a key role in brain processing, as unregulated arousal leads to hyperanxiety and detrimental performance. One hypothesis to explain how LC responses are modulated is that local inhibition has an active role in controlling LC-NA tonic activity and phasic responses. Consistent with this idea, previous ex vivo reports using ultrastructural microscopy and slice recordings have shown that LC neurons receive direct GABAergic inputs, and it has been speculated that this inhibitory contribution originates from local GABAergic (LC-GABA) neurons. Recordings across sleep-wake cycles have shown that GABA neurons in or near the LC are modulated during the sleep-wake cycle, similar to LC-NA neurons. However, there has been no study of the location or function of LC-GABA neurons, the inputs they receive, or how they modulate LC-NA activity in the awake animal.

Here, we used a combination of anatomical, electrophysiological, and optogenetic tools to identify the location, inputs, and function of a local population of LC-GABA neurons in mice, and the mode by which they control LC-NA neurons. We found that the pattern of inputs to LC-GABA neurons allows two modes of inhibition of LC-NA activity: coincident inputs to LC-NA and LC-GABA neurons regulate the gain of phasic NA responses, whereas non-coincident inputs, such as preferential inputs from the prefrontal cortex (PFC) to LC-GABA neurons, regulate LC-NA tonic activity. Together, our findings identify a mechanism by which NA-mediated arousal is selectively modulated in the brain.

Results

Location of GABAergic neurons of the LC. Previous electron microscopy ultrastructural studies have proposed the existence of GABAergic neurons surrounding LC. However, a clear map of the location of GABA neurons with respect to LC-NA neurons is lacking. To mark the precise location of LC-NA neurons, we injected dopamine-beta-hydroxylase-Cre (Dbh-Cre) mice with a FlextdTomato virus and examined coronal sections of the entire LC (Fig. 1a). We used immunohistochemical staining against GABA to localize LC-GABA neurons (Fig. 1b) and marked the location of LC-GABA with respect to LC-NA neurons in each mouse (Fig. 1c). Quantification of neuronal density in the LC region revealed a greater density of LC-GABA neurons in the anterior and medial parts of the LC (Fig. 1d,e). Overall, we observed that GABA neurons intermingled with and surround NA neurons in the LC.

We next examined whether these local GABA neurons contact LC-NA neurons. To target LC-GABA neurons, we injected the LC of Gad2-ires-Cre mice with Cre-dependent Flox-mCherry viruses. Quantification of Gad2-Cre-expressing neurons with markers for GABA showed an average of 87 ± 1% and 86 ± 2% of cells coexpressing either GABA or GAD67, respectively (Supplementary Fig. 1a,b,c). In contrast, the expression of Gad2-Cre in tyrosine hydroxylase (TH)-positive somas was 0.4 ± 0.2% and in neuropeptide-S-expressing neurons, which have been shown to be expressed in a subset of LC neurons, 0.8 ± 0.8% (Supplementary Fig. 1c–e). GABAergic processes entering the LC-NA region were apposed to TH-expressing somas, and staining against the vesicular GABAergic

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Fig. 1 | GABAergic neurons surround and contact LC-NA neurons. a, Dbh-Cre mice were injected with AAV-Flex-tdTomato virus in the LC and coronal sections were collected. The boxed area (at +100-μm location) is shown magnified in b. Dbh-tdTom., Dbh-Cre dependent tdTomato expression; M, medial; D, dorsal; L, lateral; V, ventral. b, Locations of GABA-expressing somas were revealed by immunohistochemistry. LC-NA and LC-GABA soma locations derived from a and b. d, Map of LC-GABA and LC-NA neuronal density in a radius of 200 μm around the LC region. The map is projected onto an antero-posterior axis. Bar, Barrington's nucleus; MVe, medial vestibular nucleus; CGPn, central gray of the pons. e, Distributions of LC-GABA and LC-NA neurons in the three axes. Vertical green lines represent the center of the distribution for the GABA+ population. Data are presented as mean ± s.e.m. N = 3 mice used for a–e. f, Methods for infecting LC-GABA neurons with mCherry/ChR2. g, Example of an LC neuron filled with biocytin during whole-cell recordings that expressed TH (arrow). Repeated for eight LC neurons. h, Current-clamp recording of a Gad2-mCherry-expressing neuron. Inset, overlay of multiple trials of the same cell following light activation. i, Distribution of spike delays from light onset of the cell shown in h. j, k, Example traces from the LC-TH+ neurons displayed in g, j. Cell-attached recordings during light activation of Gad2 neurons. k, Raster plot and poststimulus time histogram aligned to light activation. l, Effect of light activation on spike rates for all TH+ neurons recorded (n = 8 neurons from 3 mice, ***P = 0.00015, two-tailed paired t test, t7 = −6.5869). m, Trial average (n = 60 trials) IPSCs recorded in voltage clamp following light activation and the application of strychnine and bicuculline. n, Light-evoked IPSC amplitudes following application of strychnine and bicuculline (n = 10 neurons from 4 mice, one-way repeated measures ANOVA, F2,9 = 8.601, **P = 0.0034, *P = 0.0102 using Tukey post hoc test). Blue bars in h and j–l indicate the timing of blue light activation. BL, baseline; +Stry, strychnine; +Bic, bicuculline. Scale bars represent 200 μm (a, c, and d) and 100 μm (b and g).
transporter (VGAT) revealed the existence of GABAergic contacts (Supplementary Fig. 1f,g).

To directly assess inhibitory synapses between LC-GABA and LC-NA neurons, we performed acute slice electrophysiological recordings in the LC of Gad2-Cre mice injected with Flox-ChR2 virus (Fig. 1f). Patched neurons were filled with biocytin during whole-cell recording and identified as LC-NA neurons by post hoc immunohistochemistry against TH (Fig. 1g). Current-clamp recordings from Gad2-mCherry-ChR2-expressing neurons showed that a 5-ms pulse of blue light was sufficient to elicit a single action potential at short latency (5 of 5 Gad2-Chr2 neurons recorded) (Fig. 1h,i). Activation of Gad2-Chr2 neurons significantly decreased the spontaneous firing activity of TH-expressing LC-NA neurons, recorded in cell-attached mode (Fig. 1j–l). To assess the synaptic basis of this inhibition, we monitored light-evoked inhibitory postsynaptic currents (IPSCs) in TH neurons by performing whole-cell voltage-clamp recordings in these cells. Baseline recording with bath application of D-2-amino-5-phosphonovaleric acid (D-AP5) and 6-cyano-7-nitroquinoxaline-2 (CNQX), to block fast glutamatergic synaptic transmission, showed an average IPSC amplitude of $-50.5 \pm 15.8 \text{ pA}$ (Fig. 1m,n). Blocking glicycnergic transmission with strychnine had no effect on the IPSC, but applying bicuculline, a GABA$_A$ receptor antagonist, abolished the current (Fig. 1m,n). These results thus show that a dense population of GABAergic neurons located in the LC region forms functional synaptic contacts with and inhibits LC-NA neurons.

**LC-GABA neurons reduce LC-NA-mediated arousal.** To test whether LC-GABA neurons affect LC-mediated arousal, we first established the relationship between LC-NA activity and pupil size of awake head-restrained mice (Fig. 2a,b). We examined whether pupil size correlates with NA activity using two-photon imaging of axons filled with the genetically encoded calcium indicator GCaMP6s in the visual cortex and PFC of Dbh-Cre and TH-Cre mice (Supplementary Fig. 2a,b). We found that NA activity correlated positively with pupil size and increased before pupil dilation events (Supplementary Fig. 2c–f). We also confirmed these data with photo-identification of singleunit recording in the LC of Dbh-Cre animals expressing Flox-ChR2 (Supplementary Fig. 2g,h). As an additional indicator of arousal, we examined body movement along with pupil size and found a tight correlation between the two measures (Supplementary Fig. 3). This finding is consistent with previous studies, which have shown that pupil size reflects LC neuronal activity$^{2,5}$. It also confirms the relationship between LC-NA activity, pupil size, and global increase in arousal as previously measured with electrophenologam (EEG) and cortical local field potential (LFP) recordings$^{4,6,12}$. We thus consider pupil size to be a useful measure of NA-mediated arousal.

To demonstrate causality between NA activity and pupil size, we injected a Flox-Chr2-mCherry virus bilaterally in the LC of Ddh-Cre mice (Fig. 2c), implanted optic fibers connected to a solid-state laser light source in the LC of both hemispheres (Fig. 2c,d), and applied pulsed blue light to mimic the observed increase in LC-NA activity preceding pupil dilation, as recorded in photo-tagged LC-NA units (Supplementary Fig. 2g,h). As hypothesized from NA axonal imaging, we observed a robust increase in pupil size after light activation of ChR2-expressing NA neurons (Fig. 2e,h). Pupil dilation was dependent on baseline pupil size, as activating LC-NA neurons in periods of already increased arousal produced smaller increases in pupil size, thus explaining the observed trial-to-trial variability (Supplementary Fig. 4a,b). Even moderate activation of LC-NA neurons (pulse train duration of 0.1 s at a frequency of 5 Hz) was sufficient to dilate the pupil, and this dilation increased with higher intensities of light activation (Supplementary Fig. 4c,d). We did not observe pupil dilation in control experiments using similar patterns of light activation in Dhh-Cre mice expressing only a fluorescent marker (Flex-tdTomato) (Fig. 2h).

Subsequently, we optically silenced LC-NA neurons to examine whether their activity was necessary for pupil dilation. After injection of Flex-ArchT-mCherry virus in Dbh-Cre mice and implantation of optic fibers, we silenced spontaneous LC-NA activity with green light, and observed pupil constriction (Fig. 2f,i). Although all of the Dbh-ArchT mice showed pupil constriction with light, this constriction was not observed in mice expressing the fluorophore alone (Fig. 2i). These results demonstrate that LC-NA activity is sufficient to alter pupil dilation and that a change in NA activity is reliably reflected in pupil size.

Our anatomical and slice electrophysiological results suggest that local LC-GABA neurons are positioned to inhibit LC-NA neurons. We tested this hypothesis by implanting an optic fiber over the LC in Gad2-Cre mice locally injected with Flox-ChR2 or VGAT-YFP-ChR2, in which all inhibitory neurons express ChR2$^{29}$. Light activation produced a decrease in pupil size following focal stimulation, which was not observed in control animals (Fig. 2g,h). Overall, these results demonstrate that local GABA neurons in the LC control the activity of NA neurons and arousal level as reflected in pupil size.

**Inputs to LC-GABA and LC-NA neurons.** To further understand the function of LC-GABA neurons with respect to LC-NA neurons, we targeted a modified rabies virus separately to each subpopulation to examine the anatomical sources of their presynaptic inputs$^{10}$. We injected two Cre-dependent (AAV) ‘helper’ viruses to express the avian-specific retroviral receptor (rhab) and the rabies glycoprotein in Gad2-Cre or Dhh-Cre mice. Three weeks after the first injection, we injected the modified rabies virus (rabies-deleted-glycoprotein-mCherry$^{rEnA}$) that only infected cells expressing rhab and spread only from cells expressing the glycoprotein (Fig. 3a). We waited an additional week and examined coronal sections of the entire brain. Both experiments showed neurons positive for mCherry in a wide range of locations (Fig. 3b,c and Supplementary Table 1). We counted input neurons and assigned them to different brain regions. To visualize the projection patterns to LC-GABA and LC-NA neurons, we created a map of the relative contribution of each brain region to the two subpopulations (Fig. 3d,e, showing regions with >0.5% total inputs). Brain regions contacting LC-NA neurons directly were similar to those reported previously, consisting of diverse structures related to sensory, cognitive, autonomic, and motor functions$^{10,12,14}$ (Fig. 3d). Nearly all of the approximately 50 regions providing input to LC-NA neurons also projected to LC-GABA neurons (Supplementary Table 1 and Fig. 3e). However, there were variations in the extent of projections; mapping the differential contribution of input regions to the two LC neuron subtypes showed seven core regions projecting preferentially to LC-GABA versus two regions to LC-NA neurons (Fig. 3f). Thus, these findings demonstrate that the two neuronal subtypes in the LC receive largely similar inputs, whereas a few regions provide preferential input to one neuronal subtype or another.

**Two types of arousal-related neuronal activity in the LC.** To understand how LC-NA and LC-GABA neurons integrate their inputs to influence arousal levels in the brain, we recorded from Dhh- and Gad2-expressing neurons identified with optogenetics (photo-tagging). Dhh-Cre or Gad2-Cre mice were injected with a Flox-ChR2 virus, and light-activated neurons were recorded with a 16-channel silicon probe equipped with an optic fiber connected to a laser source (Fig. 4a). The probe was coated with 1,1′-dioctadecyl-3,3,3′,3′-tetramethyldifluorobocaine perchlorate (DiI) crystals to verify the recording location, together with immunohistochemistry against TH to identify the LC, after each successful session (Fig. 4b). Using this multichannel probe and automated spike sorting, we isolated single units from our extracellular recordings (Fig. 4c). Brief laser pulses (<5 ms) were applied and units responding to laser pulses with a short delay (<10 ms) were identified as...
expressing either Dbh-ChR2 or Gad2. (Fig. 4d,e). Of 433 units recorded in 21 sessions from 13 mice, 19 Dbh-ChR2 and 18 Gad2-ChR2 units were photo-tagged using this method (Fig. 4f–j). The delay and jitter of light-elicited spikes were similar for the Dbh- and Gad2-ChR2 conditions (Fig. 4f,g). The Gad2 units showed higher levels of spontaneous activity and had shorter spike durations than the Dbh units (Fig. 4h–j).

We next examined the relationship of Dbh- and Gad2-ChR2 photo-tagged units to pupil size (Fig. 5a). As expected from the axonal calcium imaging data from LC-NA neurons (Supplementary Fig. 2a–f) and from responses of photo-tagged LC-NA units (Supplementary Fig. 2g,h), pupil dilation was associated with an increase in activity of photo-tagged Dbh-ChR2 units. In contrast, the activity of Gad2-ChR2 units was more heterogeneous, with individual units showing negative or positive correlation with pupil size (Fig. 5a). The Pearson correlation coefficient between firing rates of LC units and pupil size (Fig. 5b) showed that all Dbh units correlated positively with pupil size (Fig. 5b,c). This matched the correlation recorded with two-photon calcium imaging of LC-NA axons in the cortex (Supplementary Fig. 2c). On the other hand, Gad2 units correlated both positively (Gad2+) and negatively (Gad2−) with pupil size (61% and 33% of Gad2 units, respectively; Fig. 5c). The activity of Gad2+ neurons globally increased during periods of low pupil constriction, whereas Gad2+ and Dbh neurons increased their activity during pupil dilation (Fig. 5d).

We also examined the relationship of 396 units, which were recorded in the same mice, but were not photo-tagged, to pupil dilation (Fig. 5b). We classified these units as fast spiking (FS) or regular spiking based on their spike shape, and extracted two clusters by fitting a Gaussian mixture model to the data (Supplementary Fig. 5a). The FS and regular-spiking clusters overlapped the spikes recorded in Gad2 and Dbh-Cre mice (11 of 18 and 17 of 19 overlap between Gad2 and FS, and Dbh and regular-spiking neurons, respectively). Spike shapes of some Gad2 neurons were not well separated from Dbh neurons, demonstrating the difficulty of cell-type identification based on spike shape alone33. Still, as for phototagged Dbh and Gad2 units, regular-spiking units were positively correlated with pupil size, whereas FS units were both positively (FS+) and negatively (FS−) correlated in similar proportion to Gad2+ and Gad2− neurons (Supplementary Fig. 5b–e), indicating a robust classification of these neuron types based on their pupillary effects.

Fig. 2 | Activating LC-GABA neurons reduces LC-NA-mediated pupil size. a. Methods to measure pupil size in awake head-fixed mice using a complementary metal oxide semiconductor (CMOS) camera and infrared illumination. Right, pupil diameter for an example 20-min session. Boxed area is expanded in b, b. Example images of pupil tracking for constricted (1) and dilated (2) epochs. Scale bar represents 1mm. c. Methods for optogenetic manipulation of LC-NA and LC-GABA neurons using Cre-dependent viruses, Dbh- and Gad2-Cre mice, and fiber optic implantation. d. Coronal section of the LC showing ChR2-mCherry expression and optic fiber tracks in a Dbh-Cre mouse. Scale bar represents 1mm. e–g. Effect of activating (Dbh-ChR2) or silencing (Dbh-ArchT) LC-NA neurons as well as activating LC-GABA neurons (Gad2-ChR2) on pupil size in example mice. Top panels, temporal raster plots of pupil size aligned to optical activation onset (vertical red line). Bottom panels, session averages for trials with and without laser. Data are presented as mean ± s.e.m. h–j. Effect of activating or silencing LC-NA neurons as well as activating LC-GABA neurons on pupil size. Gray lines represent animals where only tdTomato was expressed, but similar optical activation patterns and intensities were used. N = 6, 5, 4, and 5 mice for Dbh-ChR2, ArchT, Gad2-ChR2, and VGAT-ChR2 conditions, respectively. N = 5 mice for tdTomato controls. Paired two-tailed t test with P = 0.822 (t4 = 0.240) and ***P = 0.00001 (t4 = 14.553) for tdTomato and ChR2 conditions in h, P = 0.249 (t4 = 1.426) and **P = 0.0045 (t5 = −5.768) for tdTomato and ArchT conditions in i, P = 0.4747 (t5 = 0.788), **P = 0.0054 (t5 = −5.494), and *P = 0.0153 (t5 = −5.010) for tdTomato, VGAT-ChR2, and Gad2-ChR2 conditions in j.
We examined more closely the effect of the different types of units on pupil dilation by calculating the cross-correlation between spike rate and pupil size (Fig. 5e). The peak in cross-correlation for Dbh+ and Gad2+ units overlapped, whereas the Gad2− units showed a broad trough in cross-correlation (Fig. 5f,g; see also Supplementary Fig. 5f,g). To get a better understanding of the timing of different LC units, we aligned their activity to dilation events (Supplementary Fig. 5f,g). To get a better understanding of the timing of different LC units, we aligned their activity to dilation events (Supplementary Fig. 5f,g). To get a better understanding of the timing of different LC units, we aligned their activity to dilation events (Supplementary Fig. 5f,g). To get a better understanding of the timing of different LC units, we aligned their activity to dilation events (Supplementary Fig. 5f,g). To get a better understanding of the timing of different LC units, we aligned their activity to dilation events (Supplementary Fig. 5f,g). To get a better understanding of the timing of different LC units, we aligned their activity to dilation events (Supplementary Fig. 5f,g). To get a better understanding of the timing of different LC units, we aligned their activity to dilation events (Supplementary Fig. 5f,g).

Coincident inputs to LC-GABA neurons provide gain control of LC-NA neurons. Our anatomical and electrophysiological results imply that a subset of LC-GABA neurons receives coincident inputs with LC-NA neurons. This suggests that feedforward inhibition is present at the level of LC and that local GABA neurons provide gain control to LC-NA neurons. We assessed this hypothesis by presenting auditory tone pips that varied randomly in frequency and amplitude. Tone pips activate LC neurons15,18,19, potentially by direct inputs from the inferior colliculus, the reticular formation of the pons, and the gigantocellular nucleus of the medulla; all of these regions provide similar magnitudes of input to LC-NA and LC-GABA neurons, as revealed by our anatomical tracer experiments (Fig. 3a–e). Coincident recordings from GCaMP6s-expressing NA axons in the cortex confirmed that LC-NA neurons respond to tone pips (Supplementary Fig. 8ab). Unit recordings of Dbh− and Gad2-ChR2 neurons (Fig. 6a,b) showed that both responded to tone pips (Fig. 6c).

Overall, the two types of activity recorded from photo-tagged Gad2-ChR2 neurons are consistent with the findings of tracing experiments demonstrating similar as well as preferential inputs to LC-NA and LC-GABA neurons (Fig. 3), and they lead to specific hypotheses about their roles. In one case, regions providing similar inputs may drive both LC-GABA and LC-NA neurons leading to correlated activity in these neurons, and inhibition could act as a feedforward gain control mechanism for arousal (similar to that seen in the cortex with thalamic inputs34,35). In the other case, LC-GABA neurons that are broadly anticorrelated to arousal may be driven by a different input source than LC-NA neurons, allowing for independent positive and negative control of arousal.

Fig. 3 | LC-NA and LC-GABA neurons receive inputs from similar as well as different sources. a, Schematic for targeting pseudo-rabies virus to LC-NA and LC-GABA subpopulations. b–f, Transynaptically labeled neurons in different brain regions following injection of targeted pseudo-rabies virus in LC of Dbh-Cre or Gad2-Cre mice. Repeated in N = 8 and 4 mice for LC-NA and GABA, respectively. Scale bars represent 200 μm. d, e, Map of brain regions providing the largest fraction of inputs to LC-NA and LC-GABA neurons. Regions providing less than 0.5% of total inputs are not displayed. N = 8 and 4 mice for LC-NA and LC-GABA, respectively. f, Map of the difference in inputs to LC-NA and LC-GABA neurons. Only regions showing significant difference are displayed (P < 0.05 using paired t-test; see Supplementary Table 1). BNST, bed nucleus of the stria terminalis; Cb, cerebellum; CbN, cerebellar nuclei; CnF, cuneiform nucleus; DB, diagonal band; DpMe, deep mesencephalic nucleus; Gi, gigantocellular nucleus; IC, inferior colliculus; IPL, interpeduncular nucleus; LH, lateral hypothalamus; LHb, lateral habenular nucleus; MC, motor cortex; PAG, periaqueductal gray; PaV, paraventricular nucleus; PH, posterior hypothalamus; Pr, prepositus nucleus; PSTh, parasubthalamic nucleus; Rt, reticular nucleus; SC, superior colliculus; SN, substantia nigra; Sp5, spinal trigeminal tract; SPF, subparafascicular thalamic nucleus; SuM, supramammillary nucleus; ZI, zona incerta.
Given that tone pips evoked responses in both types of LC neurons, we used these stimuli to assess the possible modulation of LC-NA responses by LC-GABA neurons. We presented a random and sparse sequence of auditory stimuli of different frequencies and intensities to a group of mice, whereas recording pupil diameter (Fig. 6d). Simultaneous recording of pupil diameter and LC-NA activity showed that increase in pupil size followed the increase in LC-NA activity to auditory stimuli (Supplementary Fig. 8b). Silencing NA neurons prevented this pupil dilation response (Supplementary Fig. 8a,b). Comparison of spontaneous spike rate for all three types of units (one-way ANOVA, \( F_{2,430} = 15.88, P = 10^{-10} \) using Tukey post hoc test). Scatter plot of spike duration and valley FWHM values (see inset for definition) for non-identified, \( \text{Dbh}^- \), and \( \text{Gad2}^- \) units. Comparison of spike duration for all three types of units (one-way ANOVA, \( F_{2,430} = 19.57, P = 10^{-10} \) using Tukey post hoc test). Box plots indicate the median (center line), first quartiles (box edges), minimum/maximum values (whiskers), and outliers (+).

Two-tailed unpaired \( t \) test, \( t_{19} = 1.3272 \). These data suggest that auditory stimuli principally activate coincident inputs to LC-GABA and LC-NA neurons.

Non-coincident inputs to LC-GABA neurons non-specifically suppress LC-NA neurons. The results from our retrograde labeling experiments suggest that, along with similar, coincident activation of LC-NA and LC-GABA neurons, some input regions also provide preferential, potentially non-coincident, drive to LC-GABA neurons. Moreover, our electrophysiological data showed that LC-GABA neurons displayed two types of activity, where the activity of Gad2\(^+\) (and FS\(^+\)) neurons was correlated with LC-NA neurons while the activity of Gad2\(^-\) (and FS\(^-\)) neurons was broadly anticorrelated with LC-NA activity. We thus wished to examine whether activation of non-coincident inputs to LC-GABA neurons would non-specifically suppress LC-NA activity, and hence regulate arousal tone.
We chose to target inputs from the PFC, as our anatomical data supported the existence of a preferential drive to LC-GABA neurons originating from this region (Fig. 3d–f and Supplementary Fig. 11a). We injected a virus expressing ChR2-mCherry, under the CaMKII promoter to target excitatory neurons, in the orbitofrontal part of the PFC and implanted an optic fiber above the LC to activate PFC axons (Fig. 7a and Supplementary Fig. 11b). Coronal sections of the LC demonstrated the existence of PFC axons in the LC, confirming our results with monosynaptic retrograde labeling (Fig. 7b,c). Most of the PFC axons projected to the anterior and medial part of the LC, where the density of GABA neurons peaks (Figs. 1e and 7c). We activated PFC axons to the LC and indeed recorded significant sustained pupil constriction (lasting several seconds) following laser activation (Fig. 7d). This constriction of pupil size was independent of tone intensity (Fig. 7e–g). Along with activating non-coincident inputs by PFC axonal activation, we activated coincident inputs to LC-NA and LC-GABA neurons with randomly varying auditory tone pips and recorded a transient increase in pupil dilation (Fig. 7h). However, analyzing the tone-induced pupil dilation with and without laser activation revealed no effect of PFC activation on the gain of the transient pupil response (Fig. 7i–k). In contrast to the divisive effect of LC-NA activation on pupil responses (Fig. 6m), the major effect of PFC activation was a nonspecific reduction in pupil size (Fig. 7g), and hence a net decrease in arousal. These results demonstrate that non-coincident inputs such as from the PFC control the level or tone of LC-NA-mediated arousal, but not the gain, via their preferential targeting of LC-GABA neurons.

**Discussion**

We demonstrate anatomically and functionally the existence of a local population of GABA neurons in the LC, which inhibits NA activity and thus controls arousal level in the brain, as reflected in pupil size. The pattern of LC afferents is mostly coincident to LC-NA and LC-GABA neurons (Fig. 7l), as shown by tracing experiments and correlated neuronal activity among the two subpopulations. This input pattern supports a role for local LC inhibition in controlling the gain of LC responses. On the other hand, some functionally distinct input regions exert stronger influence on one LC subpopulation over the other, suggesting that the general NA tone and accompanying arousal level can be set by these inputs (Fig. 7l). An example of this second projection type is the PFC, which projects preferentially to the LC-GABA subregion and alters the tone of NA activity without altering its response gain.

Coincident and non-coincident inputs to LC-GABA neurons affect LC-NA modes of activity on many levels. By regulating the response gain of NA activity to novel stimuli, coincident inputs enable phasic LC activity to be maintained in a certain range, potentially restricting NA activity to an optimal level required for

![Fig. 5](https://example.com/figure5.png)

**Fig. 5** | Identified LC-GABA units display two types of activity with respect to pupil size. a, Examples of simultaneous recordings of pupil size and photo-tagged Dbh- (upper panel) or Gad2-ChR2 (middle and lower panels) LC units. Note that the Gad2 unit in the middle panel is positively correlated (Gad2*), whereas that in the lower panel is negatively correlated with pupil size (Gad2–). b, Cumulative probability distribution of the Pearson correlation coefficient of LC unit spike rate with pupil size for all units. The gray area marks non-significant correlations, P < 0.05. c, Percentages of Dbh- and Gad2-ChR2 units that are positively or negatively correlated with pupil size. Proportions were significantly different for the two groups (19 of 19 versus 11 of 18 in the Dbh and Gad2 groups; χ²: 9.1; P = 0.0025). The gray portion indicates non-significant correlations, P < 0.05. d, Average spike rates for different types of units during periods of constricted versus dilated pupil. Each line represents a single unit. Two-tailed paired t test: **P = 0.0005 (t₁₀ = 5.36) for Dbh; **P = 0.0014 (t₁₀ = 4.38) for Gad2*; *P = 0.0208 (t₁₀ = −3.33) for Gad2–. e,f, Normalized cross-correlation and delay of pupil size to LC firing activity for the classes of units sorted in b and c. One-way ANOVA, F₁,₁₃ = 6.33, **P = 0.0088 using Tukey post hoc test. Box plots indicate the median (center line), first quartiles (box edges), minimum/maximum values (whiskers), and outliers (+). Error bars in d and e indicate the s.e.m. In d, f, n = 19, 11, and 6 Dbh, Gad2*, and Gad2– units taken from 13 mice.
Fig. 6 | LC-GABA neurons control the gain of LC-NA-mediated pupil responses. a. Tone pips presented during the recording of LC photo-tagged units in Dbh-ChR2-expressing (right) and Gad2-ChR2-expressing (left) units. Top panels, spike raster plot aligned to tone onset. Bottom panels, session spike rate average.
b. Raster plots of average responses to tone pips sorted between Dbh and Gad2 units. n = 15 and 11 Dbh and Gad2 units, respectively. Scale bar represents 1s.
c. Percentage of responsive neurons for the two types of units. Proportions were not significantly different for the two groups (14 of 15 versus 7 of 11 mice).

F test, df = 14 and 10, P = 0.46.

Comparison of laser to no laser trials for all trials regardless of tone intensity or frequency (***, P = 10⁻⁶⁹ using two-tailed unpaired t test, t_{237} = 11.37). k. Average traces for 77-dB tones with and without laser activation of LC-GABA neurons for one mouse. The dashed box delineates the averaging window used in i, j, h. Pupil size at different tone intensities, with and without laser activation, for the example mouse in g, i. Normalized suppression of tonic pupil response due to activation of LC-GABA neurons (P = 0.763 using one-way ANOVA, F_{1,49} = 0.46). j. Comparison of laser to no laser trials for all trials regardless of tone intensity or frequency (***, P = 10⁻⁹⁹ using two-tailed unpaired t test, t_{237} = 20.7995). For panels j and n, box plots indicate the median (center line), first quartiles (box edges), minimum/maximum values (whiskers), and outliers (+); n = 1,494 and 1,481 laser-off and laser-on trials, respectively. For all other panels, data are displayed as mean ± s.e.m. N = 4 mice in i, j, m, and n.
cognitive processing\textsuperscript{12,22}. By preferentially targeting LC-GABA neurons, non-coincident inputs set thresholds for NA activation and enable modulation of tonic LC activity during different contexts. For example, PFC inputs involved in utility assessment can gate incoming sensory signals to convey the presence or absence of novelty via LC responses\textsuperscript{1,36}. Non-coincident drive to LC-GABA neurons can
also affect attentional shifts by interfering with the ability of LC-NA neurons to respond to novel sensory stimuli. Indeed, recordings in the LC of behaving mice have shown adaptation of LC responses to novelty20,21, and a switch of their response to different components of fear conditioning behavior11. Alterations in inhibition provided by the regions preferentially driving LC-GABA neurons can explain fluctuations in tonic LC activity observed within sessions in animals performing a visual discrimination task12, or during the sleep-wake cycle13,14.

Our results show that LC-GABA neurons are an important source of inhibition for LC-NA neurons. However, we do not exclude other types of inhibitory mechanisms controlling LC activity. First, inhibition in the LC can originate from distal sources. Electrical stimulation of pontine and medullary nuclei, such as the prepositus nucleus, pontine reticular nucleus, and gigantocellular reticular nucleus, yields significant reduction in the firing rate of LC neurons44,45. However, these studies never confirmed whether inhibition arose from direct inhibitory projection from these nuclei or from disynaptic pathways, such as from the preferential activation of local GABA neurons. In addition, retrograde labeling studies have shown that a significant GABAergic neuronal population from the posterior lateral hypothalamic area and the CeA projects directly to the LC region, but we do not know the function of these projections in vivo46. Second, inhibition in the LC can arise from NA-mediated inhibition. A brief period of inhibition following the phasic response of LC neurons to sensory stimuli47 has been attributed, in part, to NA-mediated collateral inhibition, since blocking alpha-2 NA receptors moderately suppresses it48,49. However, other mechanisms, such as feedforward and feedback inhibition from neighboring LC-GABA neurons, could also explain this postactivation inhibition. Finally, inhibition in the LC can originate from presynaptic release modulation. An example of this is κ-opioid receptors that colocalize with glutamate- and corticotropin releasing factor-positive axons in the LC50; activation of these receptors reduces the response of LC neurons to sensory stimuli51.

The effect of PFC activation on LC activity has remained controversial. Pharmacologically silencing the PFC in rats increases LC activity52, whereas direct activation of the PFC tends to also increase LC activity53,54. It has been an open question as to whether the PFC sends direct descending excitatory or inhibitory projections to LC, and whether an inhibitory influence arises from an indirect pathway. Our monosynaptic tracing results (Fig. 3) reconcile these studies, given that both LC-NA and LC-GABA subpopulations receive direct inputs from the PFC, albeit with a significant preference for LC-GABA neurons. In line with the tracing experiments, we found in some animals a brief period of pupil dilation at the onset of laser activation, reflecting direct inputs to LC-NA neurons (Supplementary Fig. 12). However, an extended period of activation of PFC axons in the LC shows a net pupil constriction effect, consistent with a greater influence on LC-GABA than LC-NA neurons.

Early reports using cholera-toxin B retrograde tracing found a limited number of regions that target the LC region13,15, which were mostly concentrated in the region adjacent to LC (for example, parabrachial nuclei and gigantocellular nucleus), mesencephalic areas (periaqueductal gray and deep mesencephalic nucleus), and some nuclei of the hypothalamus (for example, preoptic nucleus and LH). Notably, these studies found almost no retrogradely labeled neurons in the cortex, CeA, bed nucleus of the stria terminalis, superior colliculus, and cerebellum, as reported by us here and in another recent report using similar techniques to trace afferent projections to LC (which used Dhh-Cre mice and Cre-dependent monosynaptic modified rabies virus)14. It should be noted that larger injections of cholera-toxin B in the LC yielded similar results to ours15, showing inputs from the CeA, bed nucleus of the stria terminalis, and from cortical areas. We and others14 found that inputs also exist from the cerebellum and superior colliculus. These findings are consistent with the observation that rabies-mediated trans-synaptic tracing reveals direct synaptic connections14,46, and provides a more sensitive assay of inputs to LC-NA and LC-GABA circuits than what was reported in the literature with earlier tracers.

Our results highlight the causal relationship between LC-NA activity and pupil dilation. The link between neuronal activity in the LC and pupil size had been previously established in different species44,46; however, previous studies had not shown that specifically activating or silencing LC-NA neurons increases or decreases pupil size, respectively. LC-NA neurons could control pupil dilation by direct inputs to parasympathetic and sympathetic preganglionic neurons47,48. Notably, our results demonstrate that LC-GABA neurons have an essential role in regulating LC-NA activity. In addition to maintaining the dynamic range of LC-NA responses via coincidental feedforward inhibition, non-coincident inhibition driven by pathways such as from PFC to LC regulates the global tone of LC activity, and can potentially switch the mode of LC activity in a manner that is dependent on the internal state of the brain.

Online content
Any methods, additional references, Nature Research reporting summaries, source data, statements of data availability and associated accession codes are available at https://doi.org/10.1038/s41593-018-0305-z.

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Author contributions

V.B.-P. and M.S. designed the experiments and wrote the manuscript. V.B.-P. carried out the experiments.

Competing interests

The authors declare no competing interests.

Additional information

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Methods

Animals. All procedures were approved by the Massachusetts Institute of Technology’s Animal Care and Use Committee and conformed to National Institutes of Health guidelines. Adult mice (>2 months old) from a C57BL/6J background were used for this study. Male or female mice were randomly assigned for each experiment. We used the following mouse lines for the specific expression of various viruses in NA, GABAergic, and cholinergic neurons: TH-Cre (B6. Cg.Tg(Th-cre)1Tmd/J, Jackson Laboratory), Dbh-Cre (B6.FVB(Ng)-Tg(Dbh-cre)KH212Gsat(Mmucd, MMRRC), Gad2-ires-Cre (Gad2tm2(cre)Zjh/J, Jackson Laboratory)), and TH-Cre (B6.Cg.Tg(Th-cre)1Tmd/J, Jackson Laboratory). Dhh-Cre (B6.FVB(Cg)-Tg(Dbh-cre)KH212Gsat(Mmucd, MMRRC), Gad2-ires-Cre (Gad2tm2(cre)Zjh/J, Jackson Laboratory)), and TH-Cre (B6.Cg.Tg(Th-cre)1Tmd/J, Jackson Laboratory). The preparation of animals was similar for all surgical procedures. They were performed under isoflurane anesthesia while maintaining body temperature at 37.5 °C using an animal temperature controller (ATC2000, World Precision Instruments). After deep anesthesia was confirmed, mice were placed in a stereotaxic frame (5172SD, Stoelting), scalp hairs were removed with hair-remover cream, the underlying skin was cleaned with 70% alcohol and betadine, and an incision was made in the scalp. The conjunctive tissue was removed by rubbing hydrogen peroxide on the skull. The skull was positioned such that the lambda and bregma marks were aligned on the anteroposterior and dorsoventral axes. For all surgeries, analgesics was given once before and for 3 d following surgery.

For viral injections, a small hole was drilled through the skull at the location of interest. We used the following coordinates (according to bregma, in mm): LC: −5.2 to −5.2 anteroposterior, ±0.9 mediolateral, and 2.9–3.9 dorsoventral; orbitofrontal cortex (PFC): 2.6 anteroposterior, ±1.3 mediolateral, and 2.0 dorsoventral. Viruses were delivered with a thin glass pipette at a rate of 100–200 nl/min by an infusion system (QSI 3331, Stoelting). The following viruses (titer: −10−11 virus molecules per ml) were injected for imaging and optogenetic experiments: Flex-GCaMP6s (AAV1.Syn.Flex.GCaMP6s.WPRE.SV40, Penn Vector Core), Flex-ChR2-mCherry (AAV1.1.Fli1.Glofox.GChR2 (1134R).mCherry.WPRE.Hgh, Penn Vector Core), Flex-ArchT-tdTunato (AAV2-CAG-Flex-Archt-tdTunato, UNC Vector Core), Flex-tdTunato (AAV1-Flex-tdTunato, UNC Vector Core), and (AAV5.CamKKalpha-hChR2 (1134R)-mCherry-WPRE-pA, UNC Vector Core). We delivered a volume of 400–500 nl per injection site in the LC of Dhh-Cre, and in the PFC of wild-type mice. For experiments using TH-Cre and Gad2-Cre mice, 200–300 nl injections were done in the LC to avoid injection of other areas. We confirmed that the virus infection was limited to the LC region for TH-Cre animals injected with Flex-GCaMP6s, and did not spread to the ventral tegmental area. After injection, the site was sutured and we let mice recover for 4–6 weeks after injection of virus for optimal opsin or calcium-indicator expression. For rabies monosynaptic tracing experiments, we injected rAAV1/Syn/DIO1×tPtxpβ helper virus in the LC of Dhh-Cre, Gad2-Cre, or wild-type mice. Then, 3 weeks after the first virus injection, we injected the EnVA-Rag.mCherry (400–500 nl) in the same location.

Optic fibers with 200-µm diameter were implanted with the following procedures. After anesthesia and animal preparation, the skull was removed, the skull was cleared of conjunctive tissue, and the brain tissue were retractors from the interparietal and occipital plates. After careful alignment of the skull, the optic fiber cable was held by a stereotaxic manipulator and directed to different locations (see virus injection for coordinates) slightly on top of (−200 µm dorsoventral) the targeted region. Two-ferrule cannulas (TFC-200/245-0.37_4mm_T2S0_FLT, Doric Lenses) were used for LC injection. A single fiber optic cannula (CFM12L05, Thorlabs) was implanted in LC for activation of LC PFC axons. For LC PFC axon activation, the cannula was implanted using the following coordinates to target the anterior and medial part of LC: −4.5 to −4.8 anteroposterior, ±0.5 mediolateral, and 2.5 dorsoventral. The cannula was attached to the skull with dental cement (Teets Denture Material, or C&B Metabond, Parkell). To avoid light reflection and absorption, dental cement was mixed with black ink pigment (Black Iron Oxide 18727, Schimnich). A custom-designed head plate was also implanted at the end of the surgery for head fixation.

To perform LC single unit recording in awake head-fixed mice, we implanted a head plate 1–2 weeks before recording. We used a custom design stereotactic arm to align the head plate parallel to the median and dorsal line of the skull during implantation. The head plate was attached to the skull using dental cement. The exposed skull was protected using rapidly curing silicone elastomer (Kwik-Cast, WPI) topped with a fine layer of dental cement. Two-photon calcium imaging was done through a cranial window. Following virus injection of GCaMP6s in the LC of TH-Cre or Dhh-Cre mice, we drilled a 3-mm circular window centered over the anterior part of V1 (−3.5 mm posterior and −2 mm lateral to bregma) or the medial PFC (−2 mm anterior to bregma and centered on the midline). A 3-mm centered on a 5-mm coverslip (CS-5R and CS-3R, Warner Instruments), and glued together with ultraviolet adhesive (NOA 61 UV adhesive, Norland Products), was positioned over the craniotomy and attached to the skull using dental cement (C&B Metabond, Parkell). A head plate was also attached to the skull for head fixation. Pupil and body movement monitoring. After fixing the mouse head using a previously implanted head plate, a high-resolution CMOS camera (DCI3145M, Thorlabs) equipped with a 1.0x telecentric lens (58–430, Edmund Optics) was pointed at either the left or right eye depending on the experimental setup. Infrared illumination at 780 nm was provided by a light-emitting diode array light source (LIU780A, Thorlabs). Illumination was done at an angle of −60° for the corneal reflection spot to be cleared of pupil visualization. Video acquisition of eye images (240 × 184 pixels) was performed at 20 Hz by a custom-made MATLAB script. The ambient illumination was controlled by a LED monitor (700Y, Xenarc Direct) placed 8 cm in front of the mouse and displayed a full-field gray stimulus at an illuminance of ≈57lx (40312, Extex Instruments). This level of ambient illumination was sufficient to keep the pupil constricted within the space between the two eyelids. In all experiments, a master computer, controlling the visual and auditory stimuli, triggered pupil camera acquisition, as well as two-photon imaging, optogenetic manipulations, or extracellular electrophysiology recordings via various data acquisition cards depending on the experiment (PCI-DIO2, Measurement Computing; NI USB-6259 or BNC-2110, National Instruments). Time stamps of every pupil frame were saved for further alignment with imaging, electrophysiology, or optogenetic experiments.

We segmented the images of a black pupil on a gray iris background by a sequence of image processing manipulations done with a custom-made MATLAB script. We adjusted the minimum and maximum pixel intensity of images. We then normalized pixel values by the convolution of pupil frames by a 5 × 5-pixel kernel matrix of equal values. The images were binarized by a threshold value that we found after several trials for each experiment. The binary images were filtered to extract the largest components located in the center of the image. The isolated binarized pupil image was then fitted with a least square fit of ellipse. From this fit we estimated the diameter of the pupil for each frame. The pupil segmentation was either done online, during the experiments, or offline on saved images of the pupil. Using this pipeline for pupil segmentation, very few frames had to be dropped due to an unexpected shifting of the pupil. Movement data were synced with other experiments by displaying an indicator on the monitor. The movement metric was calculated by extracting the pixel data in time for regions around the nose, the neck, the paws, and the ears of the animal. The mean difference in pixel value between each frame was calculated for each region and we normalized this value over a region of the image where no movements were expected. Periods of activity or quietness were isolated using a threshold value of 0.5 s.d. on the movement trace.

Optogenetic modulation of LC. We used solid state laser illumination at 473 and 532 nm for activating ChR2 and Arch, respectively (MBL-III-473/1–200 mW or MGL-III-532/1–300 mW, Opto Engine). A 200-µm/0.39 numerical aperture patch cable (FC250/0.2, Thorlabs) was connected to the laser using a custom-made LC source (FC250/0.2, Thorlabs) placed 8 cm in front of the mouse and displayed a full-field gray stimulus on the monitor. The movement metric was calculated by extracting the pixel data in time for regions around the nose, the neck, the paws, and the ears of the animal. The mean difference in pixel value between each frame was calculated for each region and we normalized this value over a region of the image where no movements were expected. Periods of activity or quietness were isolated using a threshold value of 0.5 s.d. on the movement trace.
Two-photon imaging of LC-NA axons.

Four weeks after virus injection, GCaMP6s™ NA axons were imaged in the cortex using a Prairie Ultima IV two-photon microscopy system. Mice were head-fixed and a light shield was attached around the periphery of the microplate, and the O2/C02 (95%:5%) gas mixture and the entire optical equipment (BioRad, Hercules, CA, USA; Olympus, Tokyo, Japan) was lowered on top of their cranial window. The 920-nm excitation of GCaMP6s was provided by a Ti:sapphire tunable laser (Mai-Tai eHP, Spectra-Physics). Power at the objective ranged from 10 to 30 mW depending on GCaMP6s expression levels. After locating axons at 4x optical zoom, their activity was acquired at 5 frames per second for 10 ms blocks while simultaneously imaging the pupil using 8x optical zoom. Axons with significant signal-to-noise ratio were selected for analysis. We imaged 3–8 axons per mouse. The majority of our imaging field of view contained only one axon. After locating one field of view, we moved at least 1 mm away to find new axons. Care was taken to select axons from different branches, even though we cannot exclude the possibility that axons we considered as branches were connected without completely. They were actually branches arising outside our imaging window. Using these criteria, 31 axons were recorded from 6 mice.

After acquisition, time-lapse imaging sequences were corrected for x and y movement using template-matching ImageJ plugins. Multiple circular region of interests (3–7) were taken along each axon to extract the fluorescence intensity. The intensity in time window for each neuron was filtered and normalized to calculate the average by taking the mode of the signal as the reference value (F$_0$).

Sound stimuli.

Tone pips were delivered using a single speaker (HK195, Harman/Kardon) and were presented at a distance of 1 m from the mouse. The duration of each tone pip was 30 ms and the interstimulus interval was set at 30 s. Tone intensity was set to 75 dB and 15 s interstimulus interval for electrophysiological recordings. The duration of each stimulus was fixed at 0.5 s. The speaker frequency range was calibrated using a USB calibrated microphone (Minirig microphone). The light-evoked responses for the ChR2 activation on the spike rate (ill-shaped spikes) and spikes with low amplitudes or low spontaneous amplitudes of evoked IPSCs was measured as the peak current after light activation taken before light activation and right after the onset of the 5-ms pulse. The Gad2-ChR2 activation on the spike rate (ill-shaped spikes) and spikes with low amplitudes or low spontaneous amplitudes of evoked IPSCs was measured as the peak current after light activation taken before light activation and right after the onset of the 5-ms pulse. The engagement of evoked IPSCs was measured as the peak current after light activation for each condition. Access resistance ranged from 10 to 15 MΩ and recordings with variations greater than 15% of the baseline resistance value were excluded.

Single unit recordings of photo-identified LC units in awake mice. One or two days before the experiments, mice were head-fixed for 1 h to habituate to head fixation. On the day of the experiments, the mice were anesthetized with isoflurane and then the center of a silicone elastomer on the skull were removed. The mouse was placed on the stereotaxic frame and a 500-µm-thick) were cut using a vibratome (VT1200S, Leica) and were incubated overnight at 4 °C in PBS and permeabilized with 1% Triton × 100 numerical aperture, 2.0 mm in the ventral axis using a motorized micromanipulator (incubation overnight in PFA overnight. Coronal sections following secondary antibodies at a dilution of 1:500: goat anti-chicken 647 nm (A11034, ThermoFisher), and goat anti-mouse 488 nm (Aves Labs lot no. TYH8727985)., 1:1,000 rabbit anti-VGAT (131002, Synaptic Systems lot no. 131002/34), 1:1,000 rabbit anti-GABA (A2052, Sigma lot no. 126MM491V), 1:500 rabbit anti-neuropeptide S (ab193282 Abcam), and 1:200 streptavidin-488 conjugated antibodies (S32354, ThermoFisher). We used the following secondary antibodies at a dilution of 1:500: goat anti-chicken 647 nm (A21449, ThermoFisher), goat anti-chicken 488 nm (A11039, ThermoFisher), goat anti-rabbit 488 nm (A11034, ThermoFisher), and goat anti-mouse 488 nm (A11035, ThermoFisher). Slides from whole-brain histological recordings, after fixing overnight in 4% PFA, slices were washed 3× 10 min in PBS and permeabilized with 1% Triton + 3% BSA before incubation with primary antibodies. For GAD67 staining, slices were permeabilized for 1 h in 3% BSA and 10% normal goat serum, and then incubated for 4 h at room temperature in 3% BSA, 10% normal goat serum, and 1:200 mouse anti-GAD67 (MAB5406, EMD Millipore lot no. 2923238). (Note that no detergent was used at any steps for the staining). Slices were mounted in Vectashield hard set mounting medium with 4,6-diamidino-2-phenylindole (DAPI) (H-1500, Vector Laboratories). The resulting immunofluorescence was imaged with a confocal system (TCS SP8, Leica) with 10x/0.40 numerical aperture, 20x/0.75 numerical aperture, or 63x/1.4 numerical aperture oil immersion (Leica).

For quantification of Gad2-Cre neurons coexpressing GABA, GAD67, TH, and neuropeptide S, 20× z-stack images were acquired for 2–3 fields of view surrounding the LC of each slice. The percentage of Gad2-Cre cells, as identified by their expression of Fox-mCherry, overlapping with the different markers was calculated for each field of view. For the reconstruction of LC structures and...
surrounding areas, we collected the brain from Flex-tdTomato virus-injected Dbh-Cre mice. We performed staining of GABA by immunohistochemistry on the slices covering a region of 1–1.5 mm on the anteroposterior axis that covered the full extent of the LC. The GABA+ and Dbh+ neurons of the LC were imaged by performing tiling reconstruction with a 20x objective zoomed 2x. We also took low-magnification (10x) images of each slice for later registration across the different slices. The location of GABA neurons located within 200 μm of Dbh+ somas was then marked using ImageJ, and the whole LC was three-dimensionally reconstructed by exporting those values in a custom-made MATLAB program. Due to the different penetrations properties of antibodies, quantification was performed within 10μm of the surface of the slice. All slices were aligned with respect to the center of mass of LC-Dbh neurons. To obtain the neuronal density of each LC-NA and LC-GABA neuron, we counted the number of somas in each bin (bin size: 50 or 100 μm) and normalized to the total number of somas counted.

For analysis of LC-NA versus LC-GABA neurons, we imaged the brain 1 week after injection of EnVA R2G.mCherry. Coronal sections 100 μm thick were produced, and one section every 200 μm was serially mounted on microscope slides. We analyzed sections from the middle of the olfactory bulb (+4.5 mm from bregma) to the end of the brain stem (~8 mm posterior to bregma). Somas positive for mCherry were counted from all selected slices except for regions surrounding LC. The Paxinos and Franklin atlas was used as a reference for identifying brain regions. We also verified our data against the Allen Mouse Brain Atlas and found similar results. Regions adjacent to LC were not considered for analysis due to non-specific expression of virus at the site of injection. The fraction of total inputs for each trial was calculated by dividing the number of each brain region by the total number of somas counted. Animals with too few presynaptic neurons were not considered for analysis. The injection of the same combination of Cre-dependent helper virus and deleted glycoprotein in the LC of 2 wild-type mice showed a small number of neurons positive for mCherry at the injection site, but almost no neurons retrogradely labeled (n=9 and 14 compared with 240 ± 99 and 214 ± 73 neurons for Dbh+ and GABA+ neurons) showing that retrogradely labeled neurons were targeted to Cre-expressing starter cells.

**Data processing.** All data analysis, unless noted, was performed using custom-made MATLAB scripts.

In single unit recordings, we extracted two classes of unit (regular spiking and FS) based on their waveform shape. For each unit, we calculated the full width at half maximum (FWHM) of the valley portions of the average spike and the spike duration defined by the time from peak to valley. A Gaussian mixture distribution model with three components was fitted to the data. The three clusters were then classified as FS for short spike duration or regular spiking for long spike duration, and the third cluster for non-classified units. Valley FWHM and spike duration were used due to the best possible dissociation between FS and regular-spiking units. Spike delay to laser activation for photo-tagged Gad2 and Dbh units was calculated as the average timing for the first peak after the light onset. The jitter was defined as the standard deviation of this peak onset distribution. We calculated the spontaneous firing rate by averaging the number of spikes during a 10-min period, where no sensory stimulus was presented. Instantaneous spiking rate (r(t)) was obtained by using a kernel density estimation using the following equations:

\[
 r(t) = \frac{1}{\sqrt{2\pi}} \exp\left[-\frac{1}{2} \left( \frac{t}{\Delta} \right)^2 \right]
 \]

Where \( t \) is the time the n-th spike and \( N \) is the total number of spikes. \( r(t) \) represents the following exponential kernel:

\[
 r(t) = \frac{1}{\sqrt{2\pi}} \exp\left[-\frac{1}{2} \left( \frac{t}{\Delta} \right)^2 \right]
 \]

Different methods were employed to evaluate how neuronal or behavioral activity correlates with arousal. For measuring the general association between LC single unit activity and pupil size, we first obtained an estimation of the instantaneous spike rate sample at 5 Hz. We then computed Pearson’s linear correlation coefficient between the pupil size and activity of LC. The value of Pearson’s correlation was done at a lag between pupil and neuronal activity corresponding to the peak in cross-correlation. P values for Pearson’s correlation were calculated using a Student’s t-distribution for a transformation of the correlation. An LC unit correlated significantly with pupil size if \( P < 0.05 \). The activity of different classes of units during global pupil constriction or dilation was measured by averaging the spike rate during pupil values lower than the 25th percentile (for constriction) or higher than the 75th percentile (for dilation). To measure the timing of correlation with pupil activity, we computed the instantaneous cross-correlation. Pupil traces and neuronal or behavioral activity metrics were resampled (for extracellular LC recordings: 200 Hz; LC-NA Ca2+ axonal imaging: 5 Hz, and for facial movements video analyses: 10 Hz) and both normalized using z-score before computing cross-correlation. The delay between arousal and NA activity or movement was derived from the average lag value at maximum cross-correlation. To evaluate the increase in activity preceding pupil dilation or constriction, we band-pass filtered the pupil trace between 0.1 and 2 Hz using a second-order Butterworth filter. We then isolated pupil events by locating either the time point of minima (dilation event) or maxima (constriction event) and extracted the pupil and spike rate traces for a window of ~1 to 4 s around those time points. The amplitude of spike rate was evaluated from 0 to 0.5 s after the onset, duration or constriction. Alternatively, we also aligned NA activity (spike rate and calcium imaging) to maxima or minima of the derivative of pupil size (variation of pupil diameter in time).

The effect of activating diverse populations of the LC on arousal was assessed by measuring the alteration in pupil size following light activation or inactivation of neurons expressing ChR2 or Archi7. For each trial, we subtracted the baseline pupil diameter, evaluated for each trial on a period of 2 s before light onset. We then calculated the change in pupil size by averaging for a period of 1.5–8 s following the onset of the stimulus (Dbh-ChR2: 1.5–2 s depending on stimulus duration, Dbh-Archi7: 4 s, and VGAT-ChR2: 8 s). Since there is a delay between increase or decrease in LC-NA activity and pupil dilation, we averaged the response of pupil size for a period of 0.7 s after onset of light activation. The average variation in pupil size was then calculated for each animal. We used trials without laser as control to compensate for spontaneous changes in pupil size.

To evaluate how LC-NA and LC-GABA neurons respond to salient sensory stimuli, we recorded the response of units to tone pips. A unit was considered auditory responsive if there was a significant increase (\( P < 0.05 \) using one-tailed paired t-test) of activity between a baseline period of 500 ms and the amplitude calculated over a 500-ms period following the onset of tone pip. To evaluate the delay between neuronal response and stimulus onset, we calculated the average time for the response to reach a value above half the standard deviation. For two-photon imaging of LC-NA GCaMP6s axons, we compared the response with a 2 s baseline period preceding the auditory stimulus onset.

The effect of LC-GABA neurons on LC-NA-mediated increase in arousal was assessed by recording the pupil diameter while animals were presented auditory stimuli of different intensities. LC-GABA neurons were optically activated by a 25-Hz sine wave (max power ~3 mW) in half of the trials and the order of trials was randomized. Pupil response was calculated by subtracting a baseline pupil size measurement for each trial before the auditory stimulus (calculated over 0.5 s before tone onset). Since the effect of auditory stimulus on pupil dilation is usually delayed by a few milliseconds, we calculated the average amplitude of pupil for a 1.5 s window 0.5 s after the tone onset. This response was then averaged for each trial type to obtain the tone intensity–pupil size increase relationship. The suppression by LC-GABA neuron activation was defined as the difference between the response for trials with and without light activation. This difference was then normalized to the maximum value of suppression for each animal. The same procedure was used for the optical inactivation of LC-NA neurons with ArCh7 during sound stimulation. Optical silencing was performed for a total of 4 s (1 s baseline and 3 s poststimulus) and amplitude of pupil size was calculated for 1.4 and 1.6 s, respectively, surrounding the stimulus.

**Statistics.** Throughout the paper we used paired and unpaired Student’s two-sided t-test for evaluating P values of experiments with two conditions. P values for experiments with multiple conditions were computed using analysis of variance (ANOVA) with Tukey post-hoc test. Significance level were marked as *P <0.05, **P <0.01, and ***P <0.001. P values for Pearson’s correlation were calculated using a Student’s t-distribution for a transformation of the correlation. LC unit correlated significantly with pupil size if \( P < 0.05 \). Proportions of cells positively or negatively correlated for pupil for Dbh+ or Gad2+ units were then computed for significance using a chi-squared \( (\chi^2) \) test. For all experiments, sample sizes were predetermined using a power analysis to provide at least 80% power to detect an effect. Data distribution was assumed to be normal but this was not formally tested. Data collection and analysis were not performed blind to the conditions of the experiments.

**Reporting Summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

The data and code used for analyses that support the findings of this study are available from the corresponding author upon reasonable request.

**References**

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Reporting Summary

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When statistical analyses are reported, confirm that the following items are present in the relevant location (e.g. figure legend, table legend, main text, or Methods section).

- **Sample size** (n) for each experimental group/condition, given as a discrete number and unit of measurement
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- Clearly defined error bars
  - State explicitly what error bars represent (e.g. SD, SE, CI)

Software and code

Policy information about availability of computer code

- **Data collection**: MATLAB (R2015a,R2016a), Psych toolbox (v. 3), Plexon recorder (v.2.3), Bruker’s Prairie software (v.5.3), MultiClamp (v.700b), pClamp (v.10), Leica Application Suite X (v.3.1.5.16308), LifeCam Software Microsoft (v.3.60.253.0)
- **Data analysis**: MATLAB (R2015a,R2016a), Plexon Offline Sorter (v.2.8.8), Mountain Sort Algorithm (v.1.0.0), ImageJ (v.1.48), Clampfit (v.10.4)

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size | No statistical methods were used to pre-determine sample size. We used sample sizes similar to literature in the field. For all experiments, we also used sample sizes to provide at least 80% power to detect an effect. |
|-------------|--------------------------------------------------------------------------------------------------------------------------|
| Data exclusions | For single unit recording in the LC: Upon completion of experiments, we verified that targeting of the locus coeruleus region was successful with immunohistochemical techniques. Experiments where electrodes, fiber optics or viral delivery were mis-targeted were excluded from analysis. Recordings session with no ChR2 responsive units were also excluded from the study. We selected portion of extracellular single unit recordings where no obvious drift was detected. For slice electrophysiology: Recordings with a variation of access resistance greater than 15% were exclude. For pupillometry experiment: we excluded trials where baseline pupil size was hyper-dilated or constricted from analysis (<1st or >99th percentile of pupil size distribution). For histology using monosynaptic rabies tracing: Regions adjacent to LC were not considered for analysis due to non-specific expression of virus at the site of injection. These criteria were not pre-established. |
| Replication | All experiments were reproduced using biological replicates. Attempts at reproduction were successful. We used a range of 3 to 13 mice per conditions for each experiments. |
| Randomization | Auditory stimuli, and timing of optogenetics activation was randomized. Male or female mice were randomly selected for each experiment. |
| Blinding | Data collection and analysis was not performed blind. Sorting of neuronal type was performed after data collection. |

Reporting for specific materials, systems and methods

Materials & experimental systems

| n/a | Involved in the study |
|------|-----------------------|
| ☑ | Unique biological materials |
| ☑ ☑ | Antibodies |
| ☑ | Eukaryotic cell lines |
| ☑ | Palaeontology |
| ☑ | Animals and other organisms |
| ☑ | Human research participants |

Methods

| n/a | Involved in the study |
|------|-----------------------|
| ☑ | ChIP-seq |
| ☑ | Flow cytometry |
| ☑ | MRI-based neuroimaging |

Antibodies

Primary antibodies:
- Chicken anti-tyrosine hydroxylase (TYH, Aves Labs, lot no. TYH8727985), dilution 1:1000
- Rabbit anti-VGAT (131002, Synaptic Systems, lot no. 131002/34), dilution 1:1000
- Rabbit anti-GABA (A2052, Sigma, lot no. 126M4791V), dilution 1:200
- Mouse anti-GAD67 (MAB5406 EMD Millipore, lot no. 2923238), dilution 1:200
- Rabbit anti-neuropeptide S (ab18252 Abcam), dilution 1:500

Secondary antibodies:
- Streptavidin-488 conjugated antibodies (S32354 ThermoFisher Scientific), dilution 1:200
- Goat anti-chicken 647 nm (A21449, ThermoFisher Scientific), dilution 1:500
- Goat anti-chicken 488 nm (A11039, ThermoFisher Scientific), dilution 1:500
- Goat anti-rabbit 488 nm (A11034, ThermoFisher Scientific), dilution 1:500
- Goat anti-mouse 488 nm (A21121, ThermoFisher Scientific), dilution 1:500
### Validation

| Antibody                         | Source and Validation Details                                                                 |
|----------------------------------|-----------------------------------------------------------------------------------------------|
| Chicken anti-tyrosine hydroxylase (TYH, Aves Labs) | Validated in: Carter, M.E., et al. Tuning arousal with optogenetic modulation of locus coeruleus neurons. Nature neuroscience 13, 1526-1533 (2010). |
| Rabbit anti-VGAT (131002, Synaptic Systems) | Validated in: Saunders A, Oldenburg IA, Berezovskii VK, Johnson CA, Kingery ND, Elliott HL, Xie T, Gerfen CR & Sabatini BL (2015). A direct GABAergic output from the basal ganglia to frontal cortex. Nature 521: 85-9. 131 011; |
| Rabbit anti-GABA (A2052, Sigma)   | Validated. R.O. Tasan, A. Bukovac,a Y.N. Peterschmitt, S.B. Sartori, R. Landgraf, N. Singewald, and G. Sperka Altered GABA transmission in a mouse model of increased trait anxiety. Neuroscience. 2011 Jun 2; 183(7): 71–80. |
| Rabbit anti-neuropeptide S (ab18252 Abcam) | Validated in X Liu, J Zeng, A Zhou, E Theodorsson, J Fahrenkrug, RK Reinscheid. Molecular fingerprint of neuropeptide S-producing neurons in the mouse brain. J Comp Neurol. 2011 Jul 1;519(10):1847-66. |
| Mouse anti-GAD67 (MAB5406 EMD Millipore) | Validated in J Dimidschstein, Q Chen, R Tremblay, SL Rogers, GA Saldi, et. al. A viral strategy for targeting and manipulating interneurons across vertebrate species. Nature Neuroscience 2016 Dec; 19(2): 1743-1749. |

### Animals and other organisms

**Policy information about studies involving animals:** ARRIVE guidelines recommended for reporting animal research

**Laboratory animals**

Adult mice (> 2 month old) of either sex were used in this study. We used the following mouse lines for the specific expression of various viruses in noradrenergic, GABAergic: TH-Cre (B6.Cg-Tg(Th-cre)1Tmd/J, Jackson Laboratory), Dbh-Cre (B6.FVB(Cg)-Tg(Dbh-cre)KH212Gsat/Mmucd, MMRRC), , GAD2-Cre (Gad2tm2(cre)2/2J/J, Jackson Laboratory). C57Bl/6 wild-type mice were used for control experiments. Optogenetic activation of LC GABAergic neurons (LC-GABA) was done also on VGAT-YFP-ChR2 (B6.Cg-Tg(Slc32a1-COP4*H134R/EYFP)8Fng/J, Jackson Laboratory).

**Wild animals**

This study does not involve wild animals.

**Field-collected samples**

This study does not include field-collected samples.