

# Heterogeneous pericoerulear neurons tune arousal and exploratory behaviours

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Andrew T. Lusk<sup>1,2,3,12,13</sup>, Li Li<sup>1,2,4,13</sup>, Xiaonan Fu<sup>2,5</sup>, Madison M. Martin<sup>1,2,3</sup>, Kelsey Barcomb<sup>6</sup>, Kasey S. Girven<sup>1,2</sup>, Taylor Blackburn<sup>1,2</sup>, Bailey A. Wells<sup>1,2</sup>, Sarah T. Thai<sup>1,2</sup>, Esther M. Li<sup>1,2,7</sup>, Akshay N. Rana<sup>1,2</sup>, Rhiana C. Simon<sup>1,2,3</sup>, Li Sun<sup>2,5,8</sup>, Lei Gao<sup>5</sup>, Alexandria D. Murry<sup>2,9</sup>, Sam A. Golden<sup>2,9</sup>, Garret D. Stuber<sup>1,2,10</sup>, Christopher P. Ford<sup>6</sup>, Liangcai Gu<sup>2,5</sup> & Michael R. Bruchas<sup>1,2,10,11</sup>✉

As the primary source of noradrenaline in the brain, the locus coeruleus (LC) regulates arousal, avoidance and stress responses<sup>1,2</sup>. However, how local neuromodulatory inputs control LC function remains unresolved. Here we identify a population of transcriptionally, spatially and functionally diverse GABAergic (γ-aminobutyric acid-producing) neurons in the LC dendritic field that receive distant inputs and modulate modes of LC firing to control global arousal levels and arousal-related processing and behaviours. We define peri-LC anatomy using viral tracing and combine single-cell RNA sequencing with spatial transcriptomics to molecularly define both LC noradrenaline-producing and peri-LC cell types. We identify several neuronal cell types that underlie peri-LC functional diversity using a series of complementary neural circuit approaches in behaving mice. Our findings indicate that LC and peri-LC neurons are transcriptionally, functionally and anatomically heterogeneous neuronal populations that modulate arousal and avoidance states. Defining the molecular, cellular and functional diversity of the LC and peri-LC provides a roadmap for understanding the neurobiological basis of arousal, motivation and neuropsychiatric disorders.

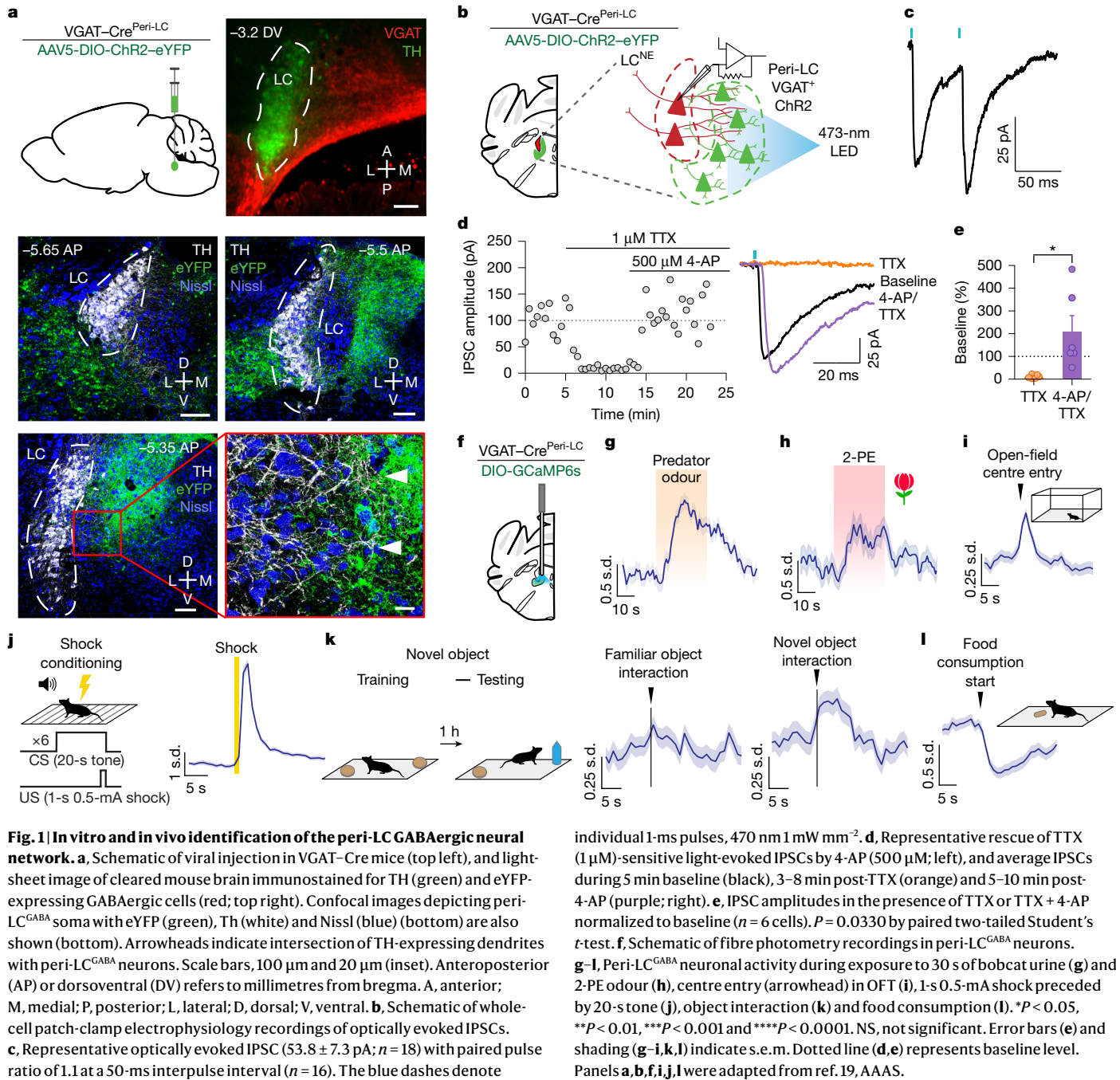
The LC is the primary source of noradrenaline across the central nervous system, and is widely recognized as a critical modulator of arousal states, including global autonomic activity and wakefulness, as well as more granular cognitive processes, such as memory, decision-making and attention<sup>1,2</sup>. The LC has heterogeneous roles in pain, avoidance and reinforcement learning, exploration and feeding, which are modulated by distinct LC subpopulations with diverse morphology, projection patterns and electrophysiological properties<sup>3–9</sup>. However, it remains unclear how LC ensembles and their activity dynamics are controlled locally. Long-range neuromodulatory inputs to the region potentially modulate the LC, but many do not target LC neurons directly<sup>10–14</sup>. LC dendrites extend medially to form a pericoerulear zone (the peri-LC or pre-LC)<sup>15</sup> that is believed to receive inputs from the medial prefrontal cortex (mPFC), dorsomedial and paraventricular hypothalamus, central amygdala, medial preoptic area and dorsal raphe<sup>10–12</sup>. An uncharacterized population of GABAergic neurons in the peri-LC has been suggested to modulate physiological arousal by inhibiting the LC<sup>10,16,17</sup>. We hypothesized that the peri-LC GABAergic (peri-LC<sup>GABA</sup>) population has critical and important roles in coordinating LC activity and can influence arousal-related and avoidance behaviours by modulating firing of noradrenaline-producing LC (LC<sup>NE</sup>) neurons.

Here we report that peri-LC<sup>GABA</sup> neurons directly inhibit the LC and reveal that these neurons increase their activity in behaving mice in response to salient stimuli, during exploration and after feeding. Using a combination of optogenetic and chemogenetic manipulations, we found that stimulating peri-LC<sup>GABA</sup> neurons potentially decreases physiological arousal and exploration, whereas inhibiting them increases avoidance or anxiety-like behaviours. Complementary single-nucleus and spatial RNA transcriptomics uncovered the diverse features of both peri-LC and LC neuronal cell types, and these results informed further experiments, which revealed differential neural responses and a role for neuropeptide-containing peri-LC<sup>GABA</sup> subpopulations in altering arousal-related and avoidance behaviours. Together, our results reveal specialized roles for this group of locally projecting, inhibitory peri-LC neurons in tuning LC<sup>NE</sup> activity to regulate arousal and avoidance states.

## Peri-LC<sup>GABA</sup> neurons directly control LC

We first examined the anatomical properties of peri-LC<sup>GABA</sup> neurons in the pericoerulear zone, the area medial to the LC and spanning the length of the LC dendritic field, from approximately 5.3 mm to 5.8 mm caudal to bregma<sup>15</sup>. Using Cre-inducible viral expression of

<sup>1</sup>Department of Anesthesiology and Pain Medicine, University of Washington, Seattle, WA, USA. <sup>2</sup>Center for Neurobiology of Addiction, Pain, and Emotion, University of Washington, Seattle, WA, USA. <sup>3</sup>Graduate Program in Neuroscience, University of Washington, Seattle, WA, USA. <sup>4</sup>Seattle Children's Research Institute, Seattle, WA, USA. <sup>5</sup>Department of Biochemistry, University of Washington, Seattle, WA, USA. <sup>6</sup>Department of Pharmacology, University of Colorado School of Medicine, Anschutz Medical Campus, Aurora, CO, USA. <sup>7</sup>Department of Psychology, University of Washington, Seattle, WA, USA. <sup>8</sup>TopoGene Inc., Seattle, WA, USA. <sup>9</sup>Department of Biological Structure, University of Washington, Seattle, WA, USA. <sup>10</sup>Department of Pharmacology, University of Washington, Seattle, WA, USA. <sup>11</sup>Department of Bioengineering, University of Washington, Seattle, WA, USA. <sup>12</sup>Present address: Laboratory of Neural Dynamics and Cognition, The Rockefeller University, New York, NY, USA. <sup>13</sup>These authors contributed equally: Andrew T. Lusk, Li Li. ✉e-mail: mbruchas@uw.edu



enhanced yellow fluorescent protein-tagged channelrhodopsin-2 (AAV5-DIO-ChR2-eYFP) and immunostaining of tyrosine hydroxylase (TH) in cleared tissue of vesicular GABA transporter (VGAT)-Cre mice, we found that peri-LC<sup>GABA</sup> neurons form a dense web on the ventromedial face of the LC, where they intertwine with LC<sup>NE</sup> dendrites (Fig. 1a and Supplementary Video 1). Peri-LC<sup>GABA</sup> neurons follow the LC<sup>NE</sup> dendritic field, which is primarily medial in the anterior LC and more ventral in the posterior LC, and extends 300–400  $\mu$ m from the LC, consistent with the length of LC<sup>NE</sup> dendrites measured in previous studies<sup>18</sup>. These GABAergic projections are almost exclusively local; after examining the whole brains of mice, only a single long-range projection was found outside of the LC region, within the lateral interpeduncular nucleus. Using monosynaptic rabies tracing onto peri-LC<sup>GABA</sup> starter cells, we found diverse inputs to peri-LC<sup>GABA</sup> neurons from distant brain regions including the bed nucleus of the stria terminalis, the central amygdala, the lateral hypothalamus and numerous brainstem areas (Extended

Data Fig. 1a and Supplementary Table 1), consistent with previous findings<sup>17</sup>, which increased our confidence in this area as a target of affective-motivational centres and ascending signals from arousing stimuli. We also identified inputs from pontine nuclei including the parabrachial nucleus, which is a hub for sensory information including pain, feeding control and alarm signals<sup>19</sup>, and the pedunculotegmental and laterodorsal tegmental nuclei, implicated in attention, sleep–wake cycles and reward<sup>20</sup>.

Next, we determined whether peri-LC<sup>GABA</sup> neurons directly modulate LC activity using ex vivo patch-clamp electrophysiology. In acute brain slices from VGAT-Cre mice expressing AAV5-DIO-ChR2-eYFP in peri-LC<sup>GABA</sup> neurons (Fig. 1b), we recorded from LC<sup>NE</sup> neurons identified visually and confirmed by the presence of an evoked  $\alpha_2$ -adrenergic receptor-mediated inhibitory current (Extended Data Fig. 1b), a fundamental characteristic of LC<sup>NE</sup> neurons<sup>21</sup>. Photostimulation of peri-LC<sup>GABA</sup> neurons and their terminals evoked fast inhibitory postsynaptic



currents (IPSCs; latency  $2.0 \pm 0.1$  ms) insensitive to tetrodotoxin (TTX) and 4-aminopyridine (4-AP) treatment, demonstrating that peri-LC<sup>GABA</sup> neurons make monosynaptic inhibitory connections onto LC<sup>NE</sup> neurons (Fig. 1c–e). Photostimulation inhibited approximately 78% of LC<sup>NE</sup> neurons examined (Extended Data Fig. 1c). Of note, photostimulation did not affect LC<sup>NE</sup> neurons when ChR2 was expressed laterally to the LC, indicating that the peri-LC<sup>GABA</sup> neurons medial to the LC form a distinct population that directly synapses onto the LC. Subsequent experiments only included mice in which expression was medial to the LC. Photostimulating peri-LC<sup>GABA</sup> neurons at high frequency (20 Hz, 1-s duration) also caused a pause in LC tonic firing, followed by a rapid burst of LC<sup>NE</sup> activity upon ceasing stimulation (Extended Data Fig. 1g). Rebound activity in LC<sup>NE</sup> neurons has been observed in previous studies that directly inhibited the LC<sup>22,23</sup>, which might be mediated by  $\alpha 2$ -receptor activity. Applying DNQX did not affect optically evoked IPSC amplitude in LC<sup>NE</sup> neurons, and picrotoxin reduced IPSC amplitude by more than half, indicating that the IPSCs are primarily GABA<sub>A</sub> receptor mediated (Extended Data Fig. 1h); we hypothesize that glycinergic currents are responsible for the other portion. Together, these results suggest that peri-LC<sup>GABA</sup> neurons directly inhibit LC<sup>NE</sup> activity via GABA release to regulate LC<sup>NE</sup> firing. We also found that peri-LC<sup>GABA</sup> neurons were tonically active at baseline, indicating a level of basal tonic inhibition onto LC<sup>NE</sup> neurons (Extended Data Table 1 and Extended Data Fig. 1d–f).

### Salient stimuli modulate peri-LC<sup>GABA</sup>

To investigate whether peri-LC<sup>GABA</sup> neurons process arousal-related stimuli in freely moving settings, we used fibre photometry to monitor neuronal (calcium) activity in peri-LC<sup>GABA</sup> neurons. We injected AAVDJ-DIO-GCaMP6s and implanted an optical fibre above the peri-LC of VGAT–Cre mice, then used a battery of behavioural tests to determine how various arousing stimuli with different modalities and valence affect peri-LC<sup>GABA</sup> neuron activity (Fig. 1f). First, to determine whether peri-LC<sup>GABA</sup> neurons respond to odourant stimuli, we exposed mice to 30 s of various odours in a chamber with lateral flow. Activity of peri-LC<sup>GABA</sup> neurons significantly increased upon exposure to the aversive odours of predator urine or 2MT, a derivative of predator urine (Fig. 1g and Extended Data Fig. 1i), to appetitive 2-phenylethanol (2-PE; rose oil extract) or the urine of opposite-sex mice (Fig. 1h and Extended Data Fig. 1j), and to peppermint odour (Extended Data Fig. 1k). Air flow without a corresponding odour did not increase peri-LC<sup>GABA</sup> GCaMP activity (Extended Data Fig. 1p). Together, these findings demonstrate that the presentation of salient odours, irrespective of valence, increases peri-LC<sup>GABA</sup> neuronal activity. As salient stimuli generate phasic bursting in LC<sup>NE</sup> neurons<sup>24</sup>, it is plausible that peri-LC<sup>GABA</sup> neurons may suppress certain LC<sup>NE</sup> subpopulations to increase the relative influence of other LC<sup>NE</sup> subpopulations, or that peri-LC<sup>GABA</sup> neurons may receive coincident input to act as a key mechanism for gain control onto LC<sup>NE</sup> neurons while excitatory inputs coordinate phasic bursting<sup>17</sup>. This tuning of excitatory–inhibitory balance has been described in other brain areas, in which distinct subpopulations of neuromodulatory neurons differentially influence behaviour through their influence on local and distal circuit activity<sup>25,26</sup>.

Second, we determined how peri-LC<sup>GABA</sup> neurons respond to threat using aversive conditioning to a 20-s tone that co-terminated with a 1-s 0.5-mA foot shock. Although the foot shock caused a robust increase in peri-LC<sup>GABA</sup> activity (Fig. 1j), the conditioned stimulus alone did not elicit an increase in the GCaMP signal. We also measured peri-LC<sup>GABA</sup> neuronal activity in less hyper-arousing contexts including the elevated-zero maze (EZM) and open-field test (OFT). We found that peri-LC<sup>GABA</sup> neurons increased activity when animals entered the anxiogenic open arm of the EZM or the bright centre of the open field, suggesting that these neurons may be engaged during exploration of threatening contexts (Fig. 1i and Extended Data Fig. 1l–m). Of note, these experiments do not distinguish between bulk versus selective activation of peri-LC<sup>GABA</sup>

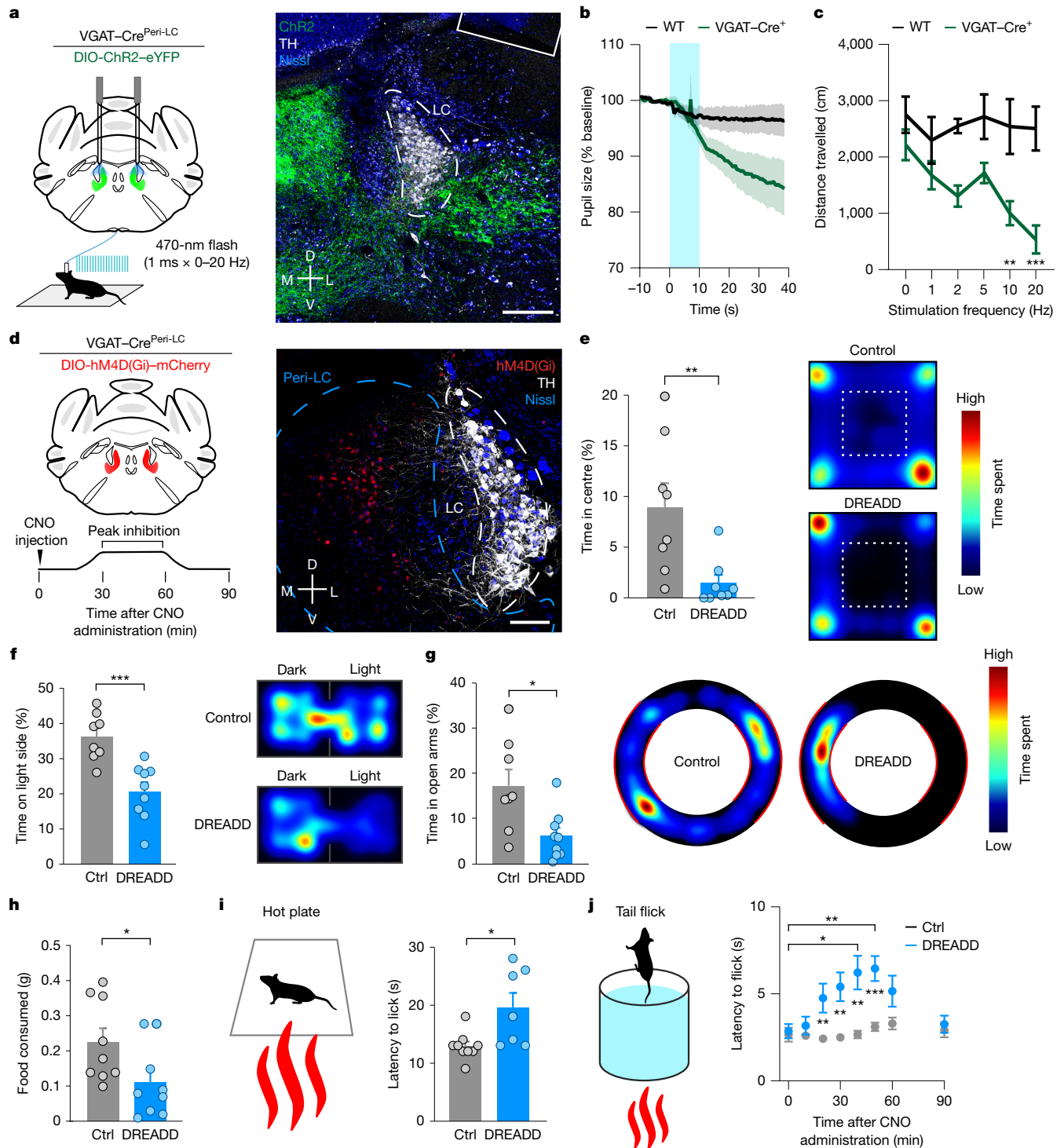
neurons. We hypothesize that only a subset of peri-LC neurons are activated so as to suppress selected LC<sup>NE</sup> subpopulations, whereas other LC<sup>NE</sup> neurons undergo the increase in tonic activity associated with anxiety-like behaviour<sup>14</sup>.

Next, to determine whether peri-LC<sup>GABA</sup> neurons respond to novel salient stimuli, as previously established for LC<sup>NE</sup> neurons<sup>27</sup>, we performed novel object testing<sup>19</sup>. In this experiment, peri-LC<sup>GABA</sup> neurons increased activity during an animal's interaction with a novel object, but did not change their activity when interacting with a familiar object (Fig. 1k). This result indicates that the peri-LC<sup>GABA</sup> neurons respond to novel information, and not to the conditioned memory of a stimulus of an animal. The increased peri-LC<sup>GABA</sup> activity was surprising, but could represent the activation of a subset of these neurons that inactivate a subset of the LC<sup>NE</sup> neurons or adjust the excitatory–inhibitory balance to facilitate coordination of phasic bursting of LC<sup>NE</sup> neurons, similar to the mechanism proposed for local GABAergic control of dopamine activity in the ventral tegmental area<sup>26,28</sup>, as well as in cortical projections from the nucleus basalis<sup>29</sup>.

LC<sup>NE</sup> neurons and a glutamatergic peri-LC neuronal population have been recently implicated in feeding<sup>30</sup>; therefore, we sought to determine whether feeding behaviour affects peri-LC<sup>GABA</sup> activity. In food-deprived animals, peri-LC<sup>GABA</sup> activity strongly decreased during chow consumption and returned to baseline upon the cessation of consumption (Fig. 1l). This pattern of activity was also observed when food-deprived animals attempted to eat a false food pellet, which caused a decrease in activity that increased after stopping the feeding attempt (Extended Data Fig. 1o). When sated mice consumed a highly palatable food item, no significant change in peri-LC<sup>GABA</sup> activity was observed, although these neurons increased activity when mice ceased an eating bout (Extended Data Fig. 1n). This modulation may be associated with switching into and out of a vigilant state<sup>31</sup>. These data show that peri-LC<sup>GABA</sup> neurons are recruited during food consumption behaviour in a deprived state. Together, these behavioural tests and photometry recordings indicate that peri-LC<sup>GABA</sup> neurons respond to salient extrinsic arousing stimuli as well as to learned objects and food based on the homeostatic needs of an animal.

### Peri-LC<sup>GABA</sup> activity alters arousal

Because peri-LC<sup>GABA</sup> stimulation decreases LC activity (Extended Data Fig. 1g), we determined whether physiological and behavioural correlates of arousal and LC<sup>NE</sup> activity<sup>14,32</sup> are reduced with peri-LC<sup>GABA</sup> stimulation. We injected AAV5-DIO-ChR2-eYFP and implanted optical fibres bilaterally in the peri-LC of VGAT–Cre mice (Fig. 2a). As changes in pupil diameter positively correlate with changes in LC<sup>NE</sup> activity and global arousal level<sup>17</sup>, we measured pupil diameter subsequent to peri-LC<sup>GABA</sup> photostimulation in anaesthetized mice (Extended Data Fig. 2a). Photostimulation at 20 Hz for 10 s decreased pupil diameter in anaesthetized mice, consistent with LC<sup>NE</sup> inhibition (Fig. 2b). Stimulation at 20 Hz also caused a decrease in locomotor activity in awake mice in a real-time place preference assay (Fig. 2c and Extended Data Fig. 2b). Similarly, peri-LC<sup>GABA</sup> stimulation did not directly modify anxiogenic behaviours in the EZM, OFT and light–dark box, but it did decrease the mean velocity of animal locomotion (Extended Data Fig. 2c–e). Moreover, stimulating peri-LC<sup>GABA</sup> neurons did not impair aversive memory acquisition when photostimulation was delivered during tone–shock pairing, nor did it affect novel object interaction or food consumption (Extended Data Fig. 2f–h). Peri-LC<sup>GABA</sup> stimulation was also not positively reinforcing when animals were allowed to nosepoke for photostimulation (Extended Data Fig. 2i). Together, these findings suggest that peri-LC<sup>GABA</sup> neurons can modulate sympathetic arousal and locomotor activity, yet the disparity between photometry and activation results suggested that a more nuanced regulation underlies peri-LC<sup>GABA</sup> function. Moreover, the suppression of tonic LC<sup>NE</sup> activity may not eliminate phasic burst firing due to LC<sup>NE</sup> excitatory inputs<sup>33</sup>.



**Fig. 2 | Manipulation of peri-LC GABAergic neurons alters diverse behavioural states.** **a**, Schematic of viral injection and optogenetic stimulation of peri-LC<sup>GABA</sup> neurons (left). The image shows ChR2-eYFP (green), TH (white) and Nissl (blue) expressed in the LC and peri-LC region (right). Scale bar, 100  $\mu$ m. **b**, Pupil size in response to 10 s of 20-Hz stimulation (light blue bar; 2 mW, 5-ms pulse width) in wild-type (WT) control or VGAT-Cre mice starting at 10 s. **c**, Distance travelled in a real-time place preference assay with varying photostimulation frequencies 0–20 Hz (2 mW, 5-ms pulse width).  $P = 0.0081$  (0 versus 10 Hz) and  $P = 0.0001$  (0 versus 20 Hz);  $n = 10$  VGAT-Cre<sup>+</sup> and 10 wild-type participants. **d**, Schematic of viral injection and chemogenetic inhibition of peri-LC<sup>GABA</sup> neurons (left). The image shows hM4D(Gi)-mCherry (red), TH (white) and Nissl (blue) in the LC and peri-LC region (right). Scale bar, 100  $\mu$ m. **e–j**, Chemogenetic inhibition (1 mg kg<sup>-1</sup> CNO) of peri-LC<sup>GABA</sup> neurons in

VGAT-Cre mice versus non-expressing wild-type controls in time spent in the centre of an open field (**e**;  $P = 0.0094$ ), time spent on the light side of a light–dark box test (**f**;  $P = 0.0006$ ), time spent in the open arms of an EZM (**g**;  $P = 0.0117$ ), food consumed in a 20-min period (**h**;  $P = 0.0424$ ), latency to paw lick on a 55 °C hot plate (**i**;  $P = 0.0133$ ) and latency to tail flick in 54 °C water (**j**;  $P = 0.0444$  (0 versus 40 min) and  $P = 0.0014$  (0 versus 50 min)). For VGAT-Cre<sup>+</sup> versus VGAT-Cre<sup>-</sup>:  $P = 0.007206$  (20 min);  $P = 0.001607$  (30 min);  $P = 0.001209$  (40 min); and  $P = 0.00237$  (50 min). Corresponding location heat maps are shown for OFT, light–dark box test and EZM. \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$  by unpaired two-tailed Student's *t*-test (**e–i**) or two-way analysis of variance (ANOVA) with Dunnett's multiple comparison test (**c, j**). The dots represent independent measurements across different animals. Shading (**b**) and error bars (**c, e–j**) indicate s.e.m. Ctrl, control. Drawings in **a, i, j** were adapted from ref. 19, AAAS.

As peri-LC<sup>GABA</sup> neurons tonically inhibit the LC at baseline (Extended Data Table 1), we hypothesized that inhibiting these neurons would release the LC<sup>NE</sup> population from inhibitory restraint, in turn increasing anxiogenic and hyperarousal states<sup>17,34</sup>. We injected AAV5-DIO-hM4D(Gi), an inhibitory DREADD, into the peri-LC of VGAT-Cre mice (Fig. 2d) and measured the behavioural effect of inhibiting peri-LC<sup>GABA</sup> neurons with 1 mg kg<sup>-1</sup> clozapine *N*-oxide (CNO). Control mice were wild-type C57BL/6J that were injected with the identical virus and received the same dose of CNO. This dose of CNO was used because administering 5 mg kg<sup>-1</sup> CNO to DREADD-expressing mice caused behavioural arrest (Extended Data Fig. 2j,k), characterized by a rigid posture and near-zero directed movement, as previously observed when LC<sup>NE</sup> neurons are hyperactivated<sup>35</sup>. As predicted, selective inhibition of peri-LC<sup>GABA</sup> increased anxiogenic behaviour in the OFT, light–dark box and EZM tests (Fig. 2e–g and Extended Data Fig. 2l–m).

A recent study has shown that LC activity decreases during food consumption and that LC<sup>NE</sup> activation attenuates feeding<sup>36</sup>; consistent with this finding, we found that DREADD-mediated inhibition of peri-LC<sup>GABA</sup> neurons significantly decreased food consumption (Fig. 2h). LC activity has differential effects on pain processing based on projection target. Activation of LC projections to the spinal cord reduces nociception and hypersensitivity, whereas the activation of projections to the PFC and basolateral amygdala exacerbate the anxiety and avoidance associated with pain<sup>8,37</sup>. We therefore tested whether inhibiting peri-LC<sup>GABA</sup> neurons could alter nocifensive behavioural responses. DREADD-expressing animals injected with CNO (1 mg kg<sup>-1</sup>), exposed to a noxious thermal stimulus in the hot plate (Fig. 2i) and tail flick (Fig. 2j) assays, indeed showed an increased latency for a nocifensive response relative to non-expressing controls, consistent with an analgesic effect.

Together, these results indicate that peri-LC<sup>GABA</sup> neurons can bidirectionally modulate global arousal level, affective responses and nocifensive behaviour. Both bulk activation and inhibition of peri-LC<sup>GABA</sup> neurons caused similar effects on behaviour, possibly through differential effects on local circuits and distant networks, suggesting that the level of peri-LC inhibition may be more finely regulated. Moreover, the differences that we observed between our circuit manipulation and fibre photometry experiments further support the hypothesis that differential activation of peri-LC<sup>GABA</sup> subpopulations may be a crucial aspect of their function. Together, these findings led us to hypothesize that the peri-LC<sup>GABA</sup> system may be a polymorphic population of neurons with heterogeneous functional roles, which influence multiple types of arousal-like and avoidance behaviours.

## Molecularly diverse LC and peri-LC subpopulations

Having observed diverse and sometimes counterintuitive LC and peri-LC<sup>GABA</sup> neuronal activity responses during different behavioural paradigms, we posited that molecular heterogeneity of LC and peri-LC<sup>GABA</sup> neuronal subpopulations may contribute to these functional variations. To better understand the cellular diversity within the LC and peri-LC, we used single-nucleus RNA sequencing (snRNA-seq) in brain sections containing both the LC and the peri-LC from adult wild-type male or female mice (see Methods; Fig. 3a and Supplementary Table 1 for details)<sup>38</sup>. After filtering low-quality cells and doublets, we identified 30,818 total cells (19,245 male and 11,573 female) across 3 samples, each pooled from 5 to 6 animals, and performed graph-based weighted nearest neighbour clustering analysis. We classified 15,050 cells (48.8%) as neurons, based on their expression of the canonical neuronal markers *Stmn2* and *Thy1* (ref. 38) (Fig. 3b,c and Extended Data Fig. 3a–g). We excluded VGLUT1 neurons in our samples because they were of low quality (Extended Data Fig. 3e–g), were detected in only a single sample and *Slc17a7* (encoding VGLUT1) was not expressed in the medial peri-LC zone in fluorescent in situ hybridization (FISH; Extended Data Fig. 3k).

LC and peri-LC neurons formed clusters based on the glutamate, GABA and monoamine neurotransmitters that they release (Fig. 3d and

Extended Data Fig. 3h). The LC<sup>NE</sup> cluster was highly enriched in genes involved in noradrenaline synthesis and transport, including *Dbh*, *Th*, *Ddc*, *Slc10a4*, *Slc6a2* (encoding NET) and *Slc18a2* (encoding VMAT2), but low in *Slc6a3* (encoding DAT), the gene involved in dopamine transport, suggesting that these are predominantly LC<sup>NE</sup> neurons and not dopamine neurons. Conversely, GABAergic neurons were separated into seven clusters that displayed high expression of *Slc32a1* (encoding VGAT), *Gad1* and *Gad2*, with one cluster co-expressing high levels of *Slc17a6* (encoding VGLUT2). Five additional classes of VGLUT2 neurons were identified, but no cluster highly expressed *Slc17a7* (encoding VGLUT1) or *Slc17a8* (encoding VGLUT3). The last cluster of neurons expressed *Slc10a4*, *Slc18a3*, *Bche*, *Chat*, *Prima1* and *Slc5a7*, which are typically characteristic of cholinergic neurons. We identified LC and peri-LC neurons, which expressed several neuropeptides together with their cognate G protein-coupled receptors (GPCRs), which are likely candidates for modulatory tuning of heterogeneous LC function (Extended Data Fig. 3i,j).

We found differential expression in neuropeptide signalling, neurotransmitter reuptake, endocannabinoid signalling, hormone receptors, LC developmental markers<sup>39</sup> and genes involved in cell morphology and adhesion (Extended Data Fig. 4a–c). Differences in the expression of genes associated with psychiatric diseases (Extended Data Fig. 4d) and dementia, such as genes involved in amyloid and tau interactions (Supplementary Table 2), were also found in our samples. We also report heterogeneous expression of numerous other genes involved in synaptic signalling (Supplementary Table 2).

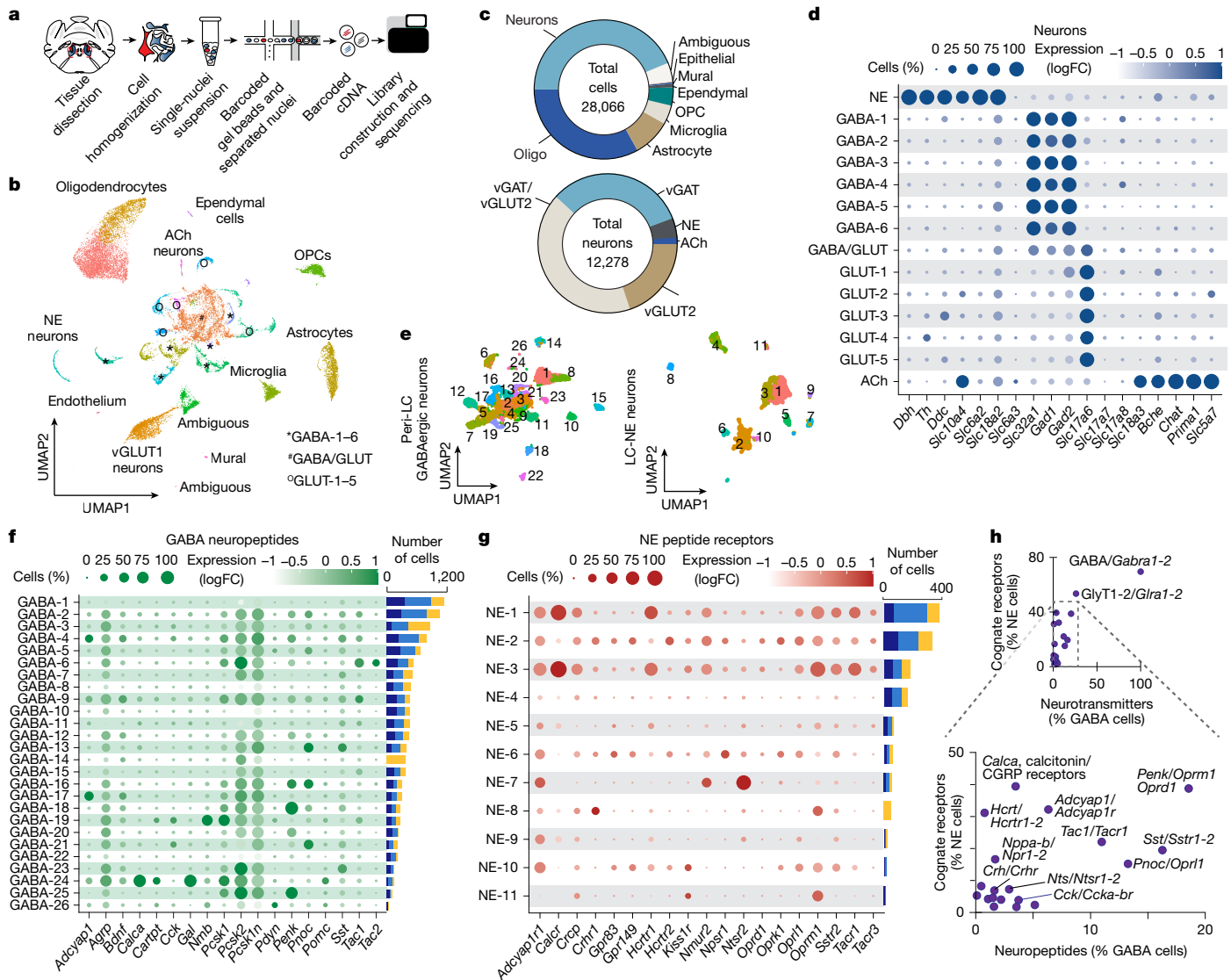
To investigate whether heterogeneity may exist within LC<sup>NE</sup> and peri-LC<sup>GABA</sup> neurons, we next sought to identify subpopulations by performing conservative reclustering only in neurons expressing *Dbh* or *Th* (Fig. 3e,g and Extended Data Fig. 3m), or those expressing *Slc32a1*, *Gad1* or *Gad2* (Fig. 3e,f and Extended Data Fig. 3l). Reclustering parameters were determined by using ChooseR subsampling to identify maximally robust clustering resolution<sup>40</sup> (Extended Data Fig. 3l–m). Given other recent reports describing sexual dimorphism in LC structure and function<sup>41,42</sup>, we also identified sexually dimorphic genes in all cells, the LC<sup>NE</sup> subset and the peri-LC<sup>GABA</sup> subset (Supplementary Table 3). We found surprisingly few sex differences in gene expression, none of which would be expected to have major functional effects. Indeed, all clusters were made up of cells from all samples, with the exception of one GABAergic and one noradrenergic cluster (GABA-14 and NE-8; Fig. 3f,g), which were exclusively female, and probably grouped by the expression of non-coding RNAs, as they show no other functional distinctions.

Striking differences in expression are found in neurotransmitter and gap junction proteins (Extended Data Fig. 5a–c), which may underlie lateral inhibition and some of the previously reported electrotonic coupling and sub-millisecond synchrony between LC<sup>NE</sup> ensembles<sup>18,43,44</sup>. LC<sup>NE</sup> neurons also differ in their receptors for neurotransmitters and neuromodulators, including  $\alpha_2$ -adrenergic receptors, and in ion channel expression (Supplementary Table 2), which may facilitate the heterogeneous, intrinsic, electrophysiological differences in LC neurons<sup>6,45</sup>, rebound activity and responses to synaptic inputs. The peri-LC<sup>GABA</sup> subpopulations exhibit key differences (Fig. 3f), including differential expressions of neuropeptides encoded by *Penk*, prepro-nociceptin (*Pnoc*) and *Tac1* (Fig. 3h). GABA-3, GABA-13 and GABA-21 neurons co-express *Pnoc* and *Slc6a5* (glycine transporter; Extended Data Fig. 6b), suggesting that peri-LC<sup>Pnoc</sup> neurons may be glycinergic. Together, the single-nucleus transcriptional profiles reveal distinct and molecularly heterogeneous subpopulations of both LC and peri-LC neurons.

## Spatial distribution of peri-LC RNA

We next corroborated and extended our snRNA-seq findings by determining the spatial distribution of RNA transcripts using Pixel-seq,



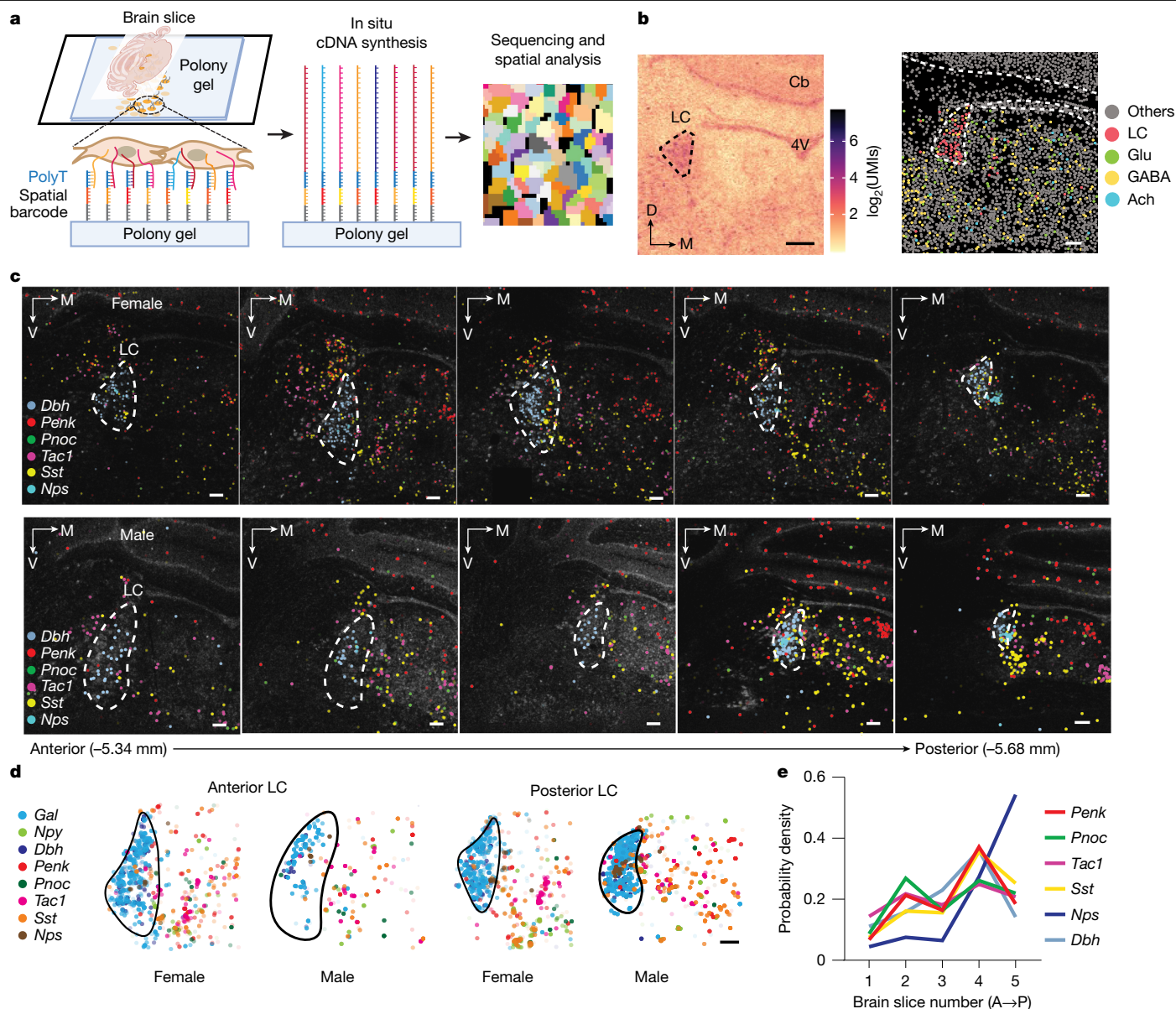


**Fig. 3 | The LC and peri-LC transcriptome.** **a**, Schematic of snRNA-seq experiments. **b**, UMAP visualization based on the transcriptional profile for  $n = 30,818$  cells pooled from 3 different samples. ACh, acetylcholine; NE, noradrenaline; OPC, oligodendrocyte precursor cell. **c**, Distribution of cell types (top;  $n = 28,066$ ) and neuronal classes (bottom;  $n = 12,278$ ) in the snRNA-seq sample after VGLUT1 neurons were removed. **d**, Dot plots of  $n = 12,278$  neurons, with neuronal clusters on the y axis and transcripts on the x axis. The size of circles corresponds to the percent of cells in the cluster expressing a specific transcript, whereas the colour intensity corresponds to the relative expression level of the transcript. Plots correspond to transcripts related to neurotransmission. FC, fold change. **e**, UMAP visualizations of reclustered neurons using *Slc32a1* (VAT), *Gad1* or *Gad2* expression for peri-LC inhibitory

neurons (left;  $n = 11,182$ ), and *Dbh* or *Th* expression for LC<sup>NE</sup> neurons (right;  $n = 1,324$ ). **f, g**, Dot plots of the subset of neurons that express *Slc32a1*, *Gad1* or *Gad2* ( $n = 11,182$ ; **f**) or *Dbh* or *Th* ( $n = 1,324$ ; **g**), with subpopulations on the y axis and neuropeptide (f) or neuropeptide receptor genes (g) on the x axis. The circle size corresponds to the percent of cells in the cluster expressing a specific transcript, whereas the colour intensity corresponds to its relative expression. The cell number in each cluster is graphed on the right, where yellow represents the contribution from the female sample and blue represents the two male samples. **h**, Distribution of neurotransmitter (top) or neuropeptide (bottom) gene expression in GABAergic neurons is shown on the x axis and the gene expression of their cognate receptors in LC<sup>NE</sup> neurons is shown on the y axis.

an approach in which a gel covered by high-density DNA polymerase colonies (polonies) allows capture of tissue mRNA followed by sequencing of RNA transcripts along with spatially identified barcodes<sup>46</sup> (Fig. 4a). Tissue containing the LC and peri-LC was dissected from 10- $\mu$ m-thick sections, its RNA hybridized to the barcoded polony gel, and the cDNA then amplified and sequenced. Coronal sections (six female and five male) of the LC and peri-LC were collected along the anteroposterior axis (Fig. 4b and Extended Data Fig. 7e). Read saturation exceeded more than 70% for both males and females, with similar gene abundance, and approximately 90% of the mapped transcripts were from protein-coding genes (Extended Data Fig. 7b–d). Leveraging cell-type-enriched markers found in our snRNA-seq experiments,

we detected approximately 59,000 cells (Extended Data Fig. 7a) and clustered them into neurons and non-neuronal subtypes (Fig. 4b). Among the neurons, we further identified subpopulations, which, when spatially mapped, showed a dense core of noradrenergic neurons surrounded by a group of GABAergic neurons (Fig. 4c,d), consistent with our anatomical studies in Fig. 1a. Established noradrenergic markers were densely expressed in the LC, in contrast to the various neuropeptides identified by snRNA-seq, which were broadly distributed throughout the peri-LC (Fig. 4c,d and Extended Data Fig. 7f). *Penk*, *Sst* and *Nps* were expressed more highly in the posterior peri-LC (Fig. 4e), with *Sst* showing a distinct cluster ventromedial to the LC.



**Fig. 4 | Spatial transcriptomics using Pixel-seq of the LC and peri-LC region.**

**a**, Schematic depicts spatial transcriptomics via Pixel-seq (left). Barcoded DNA templates were grafted onto polyacrylamide gel, and the spatial coordinates of barcodes were determined by Illumina sequencing by synthesis method (middle). The spatial distribution of unique molecular identifier (UMI) density per cluster (1 pixel =  $0.325 \times 0.325 \mu\text{m}^2$ ; right). The cells were created using BioRender (<https://biorender.com>). **b**, A spatial UMI density image of a representative fresh tissue section containing the LC and neighbouring regions (left). 4V, fourth ventricle; Cb, cerebellum. Scale bar, 200  $\mu\text{m}$ . snRNA-seq-guided annotation of segmented pseudo-cells is shown for neuronal and

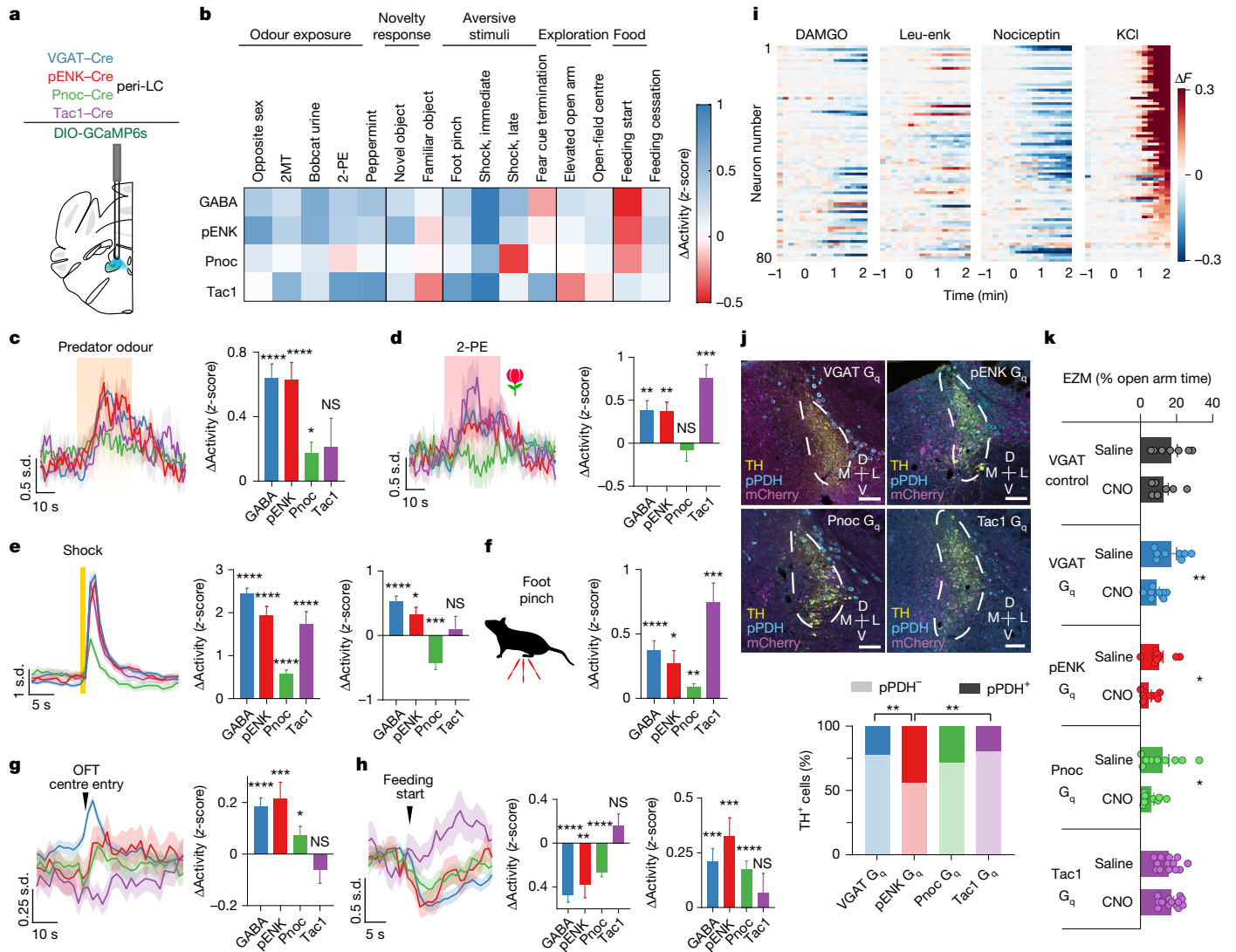
non-neuronal subpopulations (right). Scale bar, 100  $\mu\text{m}$ . **c**, Spatial expression maps of *Dbh*, *Penk*, *Pnoc*, *Tac1*, *Sst* and *Nps* of the LC region along the anterior-posterior axis are shown for a female (top) and male (bottom) mouse. Scale bars, 100  $\mu\text{m}$ . **d**, Summary comparisons of the spatial expressions of *Gal*, *Npy*, *Dbh*, *Penk*, *Pnoc*, *Tac1*, *Sst* and *Nps* in the anterior and posterior LC in male and female mice. Each pseudo-cell has been enlarged 1.5 times to aid with visualization. Scale bar, 100  $\mu\text{m}$ . **e**, Probability density of the expressions of various neuropeptide and *Dbh* transcripts along the anteroposterior axis (bregma -5.34 mm to -5.68 mm) in the LC region.

To better appreciate the functional significance of genetically distinct peri-LC clusters in behaviour, we selected three peptidergic subpopulations to investigate further: pENK, Pnoc and Tac1 (Extended Data Fig. 7g). This was based on their high gene expression in the peri-LC<sup>GABA</sup> population, high gene expression of their cognate receptors within the LC<sup>NE</sup> clusters and their limited overlap in expression within the peri-LC (Fig. 3h and Extended Data Fig. 3n–q). We further established peri-LC expression of peptide mRNAs using FISH (Extended Data Fig. 7h–j) and examined the responses of these subpopulations during behavioural studies using fibre photometry.

### Differential activation within peri-LC

We hypothesized that the peptidergic peri-LC subpopulations have diverse functions, as several previous reports have suggested that different neuropeptide GPCRs regulate LC<sup>NE</sup> function<sup>14,47</sup>. We injected AAVDJ-DIO-GCaMP6s and implanted an optical fibre in the peri-LC of VGAT-Cre, pENK-Cre, Pnoc-Cre and Tac1-Cre mice to determine how different stimuli affect the activity of these peri-LC subpopulations (Fig. 5a,b and Extended Data Fig. 8a).

Exposure to a predator odour significantly increased the activity of peri-LC GABAergic, pENK and Pnoc populations, but not of Tac1



**Fig. 5 | Functional heterogeneity of peri-LC neuronal subpopulations.** **a**, Schematic of fibre photometry recordings in the peri-LC of VGAT-Cre, pENK-Cre, Pnoc-Cre, or Tac1-Cre mice. **b**, Heatmap summary of peri-LC photometry experiments. **c–h**, Photometry traces and change in activity for peri-LC subpopulations (VGAT, pENK, Pnoc and Tac1) for 30-s exposure to bobcat urine odour (**c**;  $P < 0.0001$ ,  $n = 56$  exposures;  $P < 0.0001$ ,  $n = 24$  exposures;  $P = 0.0165$ ,  $n = 20$  exposures; and  $P = 0.2398$ ,  $n = 16$  exposures), 2-PE odour (**d**;  $P = 0.0027$ ,  $n = 44$  exposures;  $P = 0.0032$ ,  $n = 24$  exposures;  $P = 0.5265$ ,  $n = 20$  exposures; and  $P = 0.0002$ ,  $n = 16$  exposures), early (middle; 0–5 s after shock) and late (right; 5–20 s after shock) responses to a 1-s 0.5-mA footshock (**e**;  $P < 0.0001$  and  $P < 0.0001$ ,  $n = 84$  exposures;  $P < 0.0001$  and  $P = 0.0101$ ,  $n = 36$  exposures;  $P < 0.0001$  and  $P = 0.0007$ ,  $n = 30$  exposures; and  $P < 0.0001$  and  $P = 0.6062$ ,  $n = 24$  exposures), foot pinch (**f**;  $P < 0.0001$ ,  $n = 56$  exposures;  $P = 0.0118$ ,  $n = 24$  exposures;  $P = 0.0048$ ,  $n = 30$  exposures; and  $P = 0.0001$ ,  $n = 16$  exposures), entering the centre of an open field (**g**;  $P < 0.0001$ ,  $n = 368$  transitions;  $P = 0.0010$ ,  $n = 56$  transitions;  $P = 0.0384$ ,  $n = 135$  transitions; and  $P = 0.2833$ ,  $n = 90$  transitions), and the start (middle) and end (right) of food consumption (**h**;  $P < 0.0001$  and  $P = 0.0009$ ,  $n = 200$  bouts;  $P = 0.0019$  and  $P = 0.0004$ ,  $n = 59$  bouts;  $P < 0.0001$  and  $P < 0.0001$ ,  $n = 74$  bouts; and  $P = 0.1324$

and  $P = 0.4794$ ,  $n = 36$  bouts). Periods of comparison were 30 s for odour experiments and foot pinch, 5 s for the OFT and 10 s for feeding. Early shock comparison period = 5 s before versus after shock initiation; late shock comparison period = 5–20 s before versus after shock.  $n = 14$  animals for GABA photometry (11 for 2-PE exposure and 12 for OFT), 6 for pENK, 5 for Pnoc and 4 for Tac1. Two-tailed Student's *t*-test; *P* values and *n* are given in order of GABA, pENK, Pnoc and Tac1. Panel **f** was adapted from ref. 19, AAAS. **i**, Heat plots of individual neurons ( $n = 80$  from 5 slices) show GCaMP6s responses to DAMGO, Leu-enk, nociceptin and KCl. **j**, Immunostains for phospho-pyruvate dehydrogenase (pPDH) after activating G<sub>q</sub>-DREADD. The percent pPDH positivity in TH<sup>+</sup> neurons is also shown (bottom).  $P = 0.0081$  (VGAT versus pENK) and  $P = 0.0013$  (pENK versus Tac1) by chi-square test of homogeneity with Bonferroni correction. **k**, Change in the percent time spent in open arms of an EZM after saline or 1 mg kg<sup>-1</sup> CNO treatment in VGAT-Cre fluorophore control ( $n = 7$ ), VGAT G<sub>q</sub> ( $n = 8$ ), pENK G<sub>q</sub> ( $n = 10$ ), Pnoc G<sub>q</sub> ( $n = 9$ ) and Tac1 G<sub>q</sub> ( $n = 13$ ) mice.  $P = 0.0065$  (VGAT),  $P = 0.0290$  (pENK) and  $P = 0.0251$  (Pnoc) by Tukey's multiple comparisons test. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  and \*\*\*\* $P < 0.0001$ . Shading and error bars denote s.e.m.

neurons (Fig. 5c). The same three populations also increased activity when presented with the odour of opposite-sex mice (Extended Data Fig. 8b), demonstrating that the valence of the stimulus did not affect the activity of these populations. Curiously, exposure to 2MT (Extended Data Fig. 8), 2-PE (Fig. 5d and Extended Data Fig. 8m) and peppermint odour (Extended Data Fig. 8d) caused a significant increase in activity in the peri-LC GABA, pENK and Tac1 populations, but not in

Pnoc neurons. Control air flow did not affect the activity of any peri-LC population that we tested (Extended Data Fig. 8e). We also found that peri-LC<sup>GABA</sup> and peri-LC<sup>pENK</sup> neurons showed increased activity when an animal interacted with a novel object but not a familiar object (Extended Data Fig. 8f). By contrast, peri-LC<sup>Tac1</sup> neurons increased activity during novel object interactions, but decreased activity during familiar object interactions (Extended Data Fig. 8f). Together, these results indicate



that peri-LC<sup>GABA</sup>, peri-LC<sup>Pnoc</sup>, peri-LC<sup>Tac1</sup> and peri-LC<sup>pENK</sup> populations probably represent distinct peri-LC nodes, which differentially integrate novel and salient stimuli.

We also examined the activity of peri-LC subpopulations during food consumption. The GABA, pENK and Pnoc populations decreased activity during chow consumption in food-deprived animals, whereas they increased activity after finishing consumption (Fig. 5h). By contrast, Tac1 neurons did not significantly change activity during or immediately after consumption. In addition, no population changed activity during the consumption of highly palatable food when sated, with only the peri-LC<sup>GABA</sup> neurons increasing in activity after an eating bout (Extended Data Fig. 8j), suggesting that an unidentified subpopulation of peri-LC<sup>GABA</sup> neurons may modulate hedonic feeding or contribute to changes in brain state associated with the termination of feeding in favour of observing or exploring their environment.

We next determined whether the peri-LC neurons differentially respond to hyperarousing stimuli, including threat and anxiogenic contexts, using fear conditioning (Fig. 5e), EZM and OFTs. In aversive conditioning, pre-testing showed no inherent response to the tone (Extended Data Fig. 8g) in any of the four peri-LC populations, but the foot shock caused a sharp increase in activity in all cell populations. However, after this initial increase, the peri-LC GABA and pENK neurons remained elevated in activity, whereas Pnoc neurons decreased activity to below pre-shock baseline (Fig. 5e). The next day, none of these peri-LC populations responded to the conditioned tone onset; however, peri-LC<sup>GABA</sup> neurons showed a small but significant decrease in activity at the end of tone presentation, whereas Tac1 neurons sharply increased activity at tone offset (Extended Data Fig. 8h). Another noxious stimulus, a foot pinch, also elicited increased activity in all four populations (Fig. 5f). In experiments involving exploratory contexts, we found that peri-LC<sup>GABA</sup> neurons increased activity when animals entered the open arm of an EZM or the centre of an open field, whereas pENK and Pnoc populations increased activity only in the centre of the open field, but not upon entering the open arm of an EZM (Fig. 5g and Extended Data Fig. 8i). By contrast, peri-LC<sup>Tac1</sup> neurons did not change activity in the centre of an open field, but decreased activity in the open arm of an EZM. Combined, these experiments indicate that peri-LC<sup>pENK</sup> and peri-LC<sup>Pnoc</sup> populations are recruited during exploration of an anxiogenic-exposed environment, whereas Tac1 neurons may decrease activity during exposed and elevated exploratory behaviour. Peri-LC<sup>GABA</sup> neuron activity was correlated with movement in both the EZM and the OFT, consistent with their role in exploratory behaviour, whereas peri-LC<sup>pENK</sup> neurons were active during movement in the OFT and peri-LC<sup>Pnoc</sup> neurons were active during movement in the EZM (Extended Data Fig. 8k–l).

To survey how these neuropeptidergic peri-LC inputs may act to modulate the LC, we imaged brain slices expressing AAVDJ-DIO-GCaMP6f in the LC of Dbh-Cre mice with a two-photon microscope while we washed on the neuropeptide agonists DAMGO, Leu-enkephalin and nociceptin (Extended Data Fig. 9a). We imaged a total of 80 LC<sup>NE</sup> cells that increased fluorescence to 30 mM KCl as a positive control for responsiveness. When all cells were considered, nociceptin exposure significantly decreased neuronal activity across the LC<sup>NE</sup>, but no significant changes were observed in the combined population response to DAMGO, Leu-enk and control artificial cerebrospinal fluid (Extended Data Fig. 9b). Given the literature demonstrating potent  $\mu$ -opioid-mediated hyperpolarization via GIRK activation in LC<sup>NE</sup> neurons<sup>48</sup>, the lack of widespread response to DAMGO and Leu-enk was surprising; however, a subset of DAMGO-treated neurons (shown as Tukey outliers) did exhibit decreased activity, consistent with heterogeneity in neuronal responses. Critically, the three different agonists differentially activated or suppressed neuronal activity at the single-cell level (Fig. 5i), a heterogeneity reflected in the lack of normality and correlation (Extended Data Fig. 9c) in the neuronal responses. These results suggest that the neuropeptides generated by peri-LC GABA neurons may heterogeneously influence the activity of individual LC<sup>NE</sup> neurons.

We then determined whether the activation of these three peri-LC peptidergic subpopulations differentially affect LC<sup>NE</sup> function at the circuit level. The opsin ChrimsonR was selectively expressed in each peri-LC subpopulation using pENK-Cre, Pnoc-Cre and Tac1-Cre mice, and the recently developed fluorescent noradrenaline sensor GRAB<sub>NE2m</sub><sup>49</sup> was expressed in the medial PFC and the piriform cortex (Pir). Optical fibres were implanted in the peri-LC for stimulation and in the PFC and Pir for measuring noradrenaline release dynamics. Although photostimulating the peri-LC pENK, Pnoc and Tac1 subpopulations at 30 Hz for 500 ms did not produce any significant differences in Pir noradrenaline release, PFC noradrenaline release differed significantly in response to activation of the pENK subpopulation compared with the Tac1 subpopulation (Extended Data Fig. 9d and Supplementary Table 1). As a control, we used a tail lift stressor, which is known to robustly increase downstream LC<sup>NE</sup> activity<sup>50</sup> (Extended Data Fig. 9e), and in each genotype, we found equivalent PFC and Pir noradrenaline release dynamics following tail lift stress, further indicative of differences between global and discrete modes of peri-LC activity, which may contribute to polymorphic LC<sup>NE</sup> function<sup>4</sup>. Together, these results indicate that peri-LC neuropeptide subpopulations can have differential effects on LC<sup>NE</sup> system function.

We subsequently determined whether activation of peri-LC subpopulations may differentially affect avoidance behaviour. VGAT-Cre, pENK-Cre, Pnoc-Cre and Tac1-Cre mice were injected with an activating designer receptor (G<sub>q</sub>-DREADD), given either saline or CNO, and then tested in the EZM and OFT. A group of VGAT-Cre mice expressing only a fluorophore was used as a control. As expected, in the control group, time in the open arms of EZM and in the centre of the OFT did not differ (Fig. 5k and Extended Data Fig. 9g). Consistent with previous findings showing that LC<sup>NE</sup> neuron activity is necessary and sufficient for regulation of avoidance and exploration in an aversive environment<sup>34</sup>, DREADD stimulation of peri-LC GABA, pENK and Pnoc neurons decreased time spent in the open arms of the EZM as compared with the saline condition. Stimulation of Tac1 neurons caused no significant difference in either the OFT or the EZM. These groups of mice did not differ in their distance travelled, except for the VGAT G<sub>q</sub>-DREADD group in the EZM (Extended Data Fig. 9f). These results, in combination with our previous results, indicate that the peri-LC subpopulations probably influence LC<sup>NE</sup> function at the behavioural level, although the regulation of distinct behaviours may be more complex.

Finally, to assess whether exciting the different peri-LC populations would lead to differences in LC<sup>NE</sup> neuron inhibition, we used an antibody to phospho-pyruvate dehydrogenase (pPDH) to immunostain putatively inhibited neurons<sup>51</sup>. We observed robust pPDH expression in the LC<sup>NE</sup> cells of mice in which we activated selected peri-LC neurons (Fig. 5j) with CNO injection, and further quantification revealed a significantly higher proportion of pPDH<sup>+</sup>TH<sup>+</sup> neurons in response to the activation of peri-LC<sup>pENK</sup> neurons than to peri-LC<sup>Tac1</sup> or peri-LC<sup>GABA</sup> neurons. This may result from local multisynaptic mechanisms; connectivity within the peri-LC<sup>GABA</sup> population warrants further investigation. Together, these convergent results across numerous experiments support the hypothesis that activation of selected peri-LC subpopulations presents one critical mechanism by which the LC coordinates differential shifts in excitatory–inhibitory balance with LC<sup>NE</sup> neurons, subsequently influencing arousal and exploratory behaviours.

## Discussion

Here we have defined a heterogeneous peri-LC<sup>GABA</sup> neuronal population whose inhibitory inputs modulate LC<sup>NE</sup> firing and arousal, avoidance and related exploratory behaviours. We have identified molecular heterogeneity within LC and peri-LC and provided evidence that suggests non-uniform spatial organization within this region, the functional implications of which require further study.



Increasing the activity of peri-LC<sup>GABA</sup> neurons rapidly but temporarily inhibits tonic activity of LC<sup>NE</sup> neurons, in addition to inhibiting LC phasic bursting, as previously described<sup>52</sup>. In addition, as projections of peri-LC<sup>GABA</sup> neurons are predominantly local (Fig. 1a), and that most, if not all, LC<sup>NE</sup> neurons were subject to modulation by peri-LC<sup>GABA</sup> neurons, peri-LC<sup>GABA</sup> probably controls LC<sup>NE</sup>-mediated arousal states and responses via direct interactions. Consistent with this idea, we found that activating peri-LC<sup>GABA</sup> neurons decreased global arousal and exploratory behaviours (Fig. 2b,c), whereas inhibiting these neurons increased avoidance behaviours (Fig. 2d–g), in alignment with disinhibition of LC activity<sup>53</sup>. To explore any potential functional diversity of these populations, we defined the endogenous activity patterns of peri-LC<sup>GABA</sup> neurons in multiple behavioural domains that the LC<sup>NE</sup> system has been implicated in controlling. These included arousing, aversive and appetitive stimulus processing (Fig. 1g,h and Extended Data Fig. 1i–k), sexual behaviour (Extended Data Fig. 1j), feeding (Fig. 1l), nociceptive response (Fig. 1j) and novelty processing (Fig. 1k). The peri-LC<sup>GABA</sup> response was present across all modalities tested except for the auditory tone (Extended Data Fig. 8g,h), which was surprising in light of previous findings identifying short-lived (less than 100 ms) tone onset responses in a subset of peri-LC<sup>GABA</sup> neurons using electrophysiology<sup>17</sup>. We suspect that a sparse tone onset response may have been washed out by opposing signals in the bulk photometry recordings.

Two complementary transcriptomic approaches revealed substantial molecular and spatial diversity, which translates to functional diversity in various settings. Peri-LC<sup>GABA</sup> neurons expressing a wide repertoire of neuropeptides (Fig. 3f) surround the LC<sup>NE</sup> dendritic field, as revealed by our Pixel-seq spatial transcriptomic results (Fig. 4c,d). As different groups of LC neurons also express diverse sets of cognate receptors (Fig. 3g and Extended Data Fig. 5a), the combinations of neuropeptide–receptor interactions between the peri-LC and LC are likely to generate polymodal LC ensembles that can flexibly alter their dynamics and excitatory–inhibitory balance in response to internal states and behavioural needs. For instance, given that peri-LC<sup>GABA</sup> activity decreased during food consumption (Fig. 1l), we hypothesized that inhibiting these neurons would increase feeding, but chemogenetic inhibition of these neurons instead decreased feeding (Fig. 2h), probably by disinhibiting LC<sup>NE</sup> projections to the lateral hypothalamus involved in feeding<sup>36</sup>. This seemingly paradoxical finding may be due to chemogenetic inhibition affecting a less specific population of neurons than those involved in natural feeding behaviour, which could engage vastly different LC<sup>NE</sup> ensembles and projections (compare ref. 54 with ref. 36). We also found that inhibiting peri-LC<sup>GABA</sup> neurons promoted analgesia (Fig. 2i,j), probably by disinhibiting LC<sup>NE</sup> projections to the dorsal horn of the spinal cord, which act to inhibit noxious afferents<sup>55</sup>, rather than LC<sup>NE</sup> projections to the basolateral amygdala<sup>37</sup> and PFC<sup>8</sup>, which contribute to negative affect in pain response; however, a direct test of this concept requires further investigation. Furthermore, although novel and salient stimuli were generally associated with increased peri-LC GABAergic and peptidergic activity (Fig. 5b,e), Tac1 neurons decreased activity during exploration in contrast to peri-LC<sup>pENK</sup>, peri-LC<sup>pNoc</sup> and the peri-LC<sup>GABA</sup> population as a whole (Fig. 5g and Extended Data Fig. 8i). The number of cells within these subpopulations and their distribution throughout the peri-LC region varied, complicating direct comparison between fibre photometry signals; single-cell imaging studies are likely to facilitate more direct comparisons. The molecular and functional diversity of the peri-LC that we identified here presents unique opportunities for altering noradrenaline function in naturalistic behaviour, cognition, learning, motivated behaviours, exploration, feeding, physiological stress and the many disease-related behavioural states previously associated with the LC. Of note, although peri-LC<sup>GABA</sup> projections outside of the brainstem are sparse, our study does not explore the possible influence of peri-LC populations on other brainstem areas, which may influence the behaviours that we observed.

Our findings identified diverse peri-LC<sup>GABA</sup> neurons at molecular, cellular and systems levels, providing avenues for future studies to dissect how these newly identified peri-LC populations function in a host of ethologically relevant behaviours and neuropsychiatric disease models. We isolated unique peri-LC neuronal groups, which act to integrate ascending and descending LC<sup>NE</sup> control via direct fast neurotransmission and an array of neuropeptide GPCR systems. Further heterogeneity exists in the peri-LC and LC<sup>NE</sup> populations in transcription factors, ion channels and other markers, revealing additional layers of interesting LC system complexity. Subpopulations of peri-LC<sup>GABA</sup> neurons may form distinct modular components of the extended LC<sup>NE</sup> system, impacting noradrenergic tone, physiological arousal, cognition, mood states and avoidance behaviour.

## Online content

Any methods, additional references, Nature Portfolio reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at <https://doi.org/10.1038/s41586-025-08952-w>.

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## Methods

### Animals

Adult wild-type C57BL/6J (Jackson Laboratory #000664), VGAT-IRES-Cre (*Slc32a1<sup>tm2(cre)Low</sup>*/J; Jackson Laboratory #028862)<sup>49,56</sup>, pENK-IRES-Cre (B6;129S-*Penk<sup>tm2(cre)Hze</sup>*/J; Jackson Laboratory #025112), Tac1-IRES-Cre (B6;129S-*Tac1<sup>tm1.1(cre)Hze</sup>*/J; Jackson Laboratory #021877) and *Pnoc*-IRES-Cre (*Pnoc<sup>tm1.1(cre)Mrbr</sup>*/J; Jackson Laboratory #034278)<sup>57</sup> mice were group housed, given access to food and water ad libitum, and maintained on a 12–12 h reverse light–dark cycle (lights off at 09:00 and on at 21:00) with temperature between 18 °C and 23 °C and 30–70% humidity. All animals were kept in an isolated and sound-attenuated holding facility within the laboratory and adjacent to behaviour rooms 1 week before surgery, post-surgery and throughout the duration of the behavioural assays to minimize stress. Male and female mice were used in all experiments, except for slice imaging and rabies tracing. All procedures were approved by the Animal Care and Use Committee of Washington University and the University of Washington and conformed to US National Institutes of Health guidelines.

### Stereotaxic surgeries

After the animals were acclimated to the holding facility for at least 7 days, the mice were anaesthetized in an induction chamber (2% isoflurane) and placed into a stereotaxic frame (Kopf Instruments, model 1900) where they were maintained at 1–2% isoflurane. Mice were then injected using a Nanoject (3000204 and 3000207, Drummond Scientific) with a glass tip of 7–20 µm in diameter. Mice were injected in the peri-LC (anteroposterior (AP) –5.40 mm, mediolateral (ML) +0.75 mm, dorsoventral (DV) –3.70 mm relative to bregma) with three bursts of 9.2 nl each (3 min apart; 27.6 nl total) at a rate of 23 nl s<sup>–1</sup>. Injections were unilateral for tracing and fibre photometry experiments; bilateral for optogenetic or chemogenetic experiments. The needle was slowly removed from the brain 10 min after cessation of injection to allow for diffusion. For fibre photometry and optogenetic experiments, mice were also implanted with a 400-µm fibre optic (MFC\_400/430-0.48\_MF2.5\_FLT, Doric Inc.) or custom-made optical fibres of 70% or more optical efficiency in the same surgery. For mice in the peri-LC photostimulation study to examine the effect on PFC and Pir noradrenaline levels, in addition to the peri-LC injections as before, 500 nl of the virus expressing the fluorescent noradrenaline sensor (GRAB<sub>NE2m</sub>) was injected in the Pir (AP –1.45, ML –3.20 mm, DV –4.75 to –4.50), and 300 nl was injected in the PFC (AP +1.80 mm, ML +0.40 mm and DV –2.40 mm), both at 100 nl min<sup>–1</sup>. Fibres were implanted in the peri-LC (AP –5.40 mm, ML +0.75 mm and DV –3.50 mm), Pir (AP –1.45 mm, ML –3.20 mm and DV –4.50 mm) and PFC (AP +1.80 mm, ML +0.40 mm and DV –2.10 mm), respectively. Mice received carprofen and recovered for at least 6 weeks before behavioural testing, permitting optimal expression of the virus. For optogenetic inhibition, fibre optics were placed bilaterally. Fibre optic implants were secured using Metabond (S380, Parkell). The following viruses were used: AAV5-EF1α-DIO-hChR2-(H134R)-eYFP (WashU HOPE Center Viral Core), AAVDJ-EF1α-DIO-GCaMP6s (Stanford Gene Vector and Viral Core), AAV5-CAG-FLEX-RG (UNC Vector Core), AAV8-EF1α-FLEX-TVA-mCherry (UNC Vector Core), EnvA-G-deleted rabies-eGFP (Salk), AAV5-EF1α-DIO-HA-hM4D(Gi)-mCherry (WashU HOPE Center Viral Core), AAV5-hSyn-DIO-hM3D(G<sub>q</sub>)-mCherry (Addgene), AAV5-EF1α-DIO-HA-hM3D(G<sub>q</sub>)-IRES-mScarlet (WashU HOPE Center Viral Core), AAV5-EF1α-DIO-mScarlet (UNC Vector Core), AAV5-Syn-FLEX-ChrimsonR-TdTomato (UNC Vector Core), AAV1-hsyn-GRAB<sub>NE2m</sub> (Yulong Li<sup>56</sup>) and AAVDJ-EF1α-DIO-GCaMP6f (Stanford Gene Vector and Viral Core).

### Immunohistochemistry

Immunohistochemistry was performed as previously described<sup>19</sup>. In brief, mice were anaesthetized with sodium pentobarbital and transcardially perfused with 4% paraformaldehyde (PFA), post-fixed

overnight in 4% PFA and cryo-protected in 30% sucrose for at least 24 h. Brains were then sectioned (30 µm) and placed in 0.1 M phosphate buffer until immunohistochemistry. Free-floating sections were washed in 0.1 M PBS for 3 × 10-min intervals. Sections were then placed in blocking buffer (0.5% Triton X-100 and 5% natural goat serum in 0.1 M PBS) for 1 h at room temperature. Sections were incubated with primary antibody (chicken anti-TH, 1:2,000, Aves TYH; rabbit anti-pPDH, 1:500, 37115, Cell Signaling Technology)<sup>51</sup> at 4 °C overnight. After 3 × 10 min 0.1 M PBS, sections were incubated in AlexaFluor secondary antibodies (goat anti-chicken 633, 1:1,000; A21103, Invitrogen (Figs. 1a and 2a,d and Extended Data Figs. 1a and 8a); goat anti-chicken 488, 1:1,000, A11039, Invitrogen (Fig. 5n); and goat anti-rabbit 647, 1:1,000, A27040, Invitrogen (Fig. 5n)) and NeuroTrace (1:400, 435/455 blue fluorescent Nissl stain, N21479, Invitrogen) for 1 h, followed by 3 × 10 min 0.1 M PBS, then 3 × 10 min 0.1 M phosphate buffer washes. Sections were then mounted and coverslipped with Vectashield Vibrance HardSet mounting medium (H-1700, Vector Laboratories) and imaged on Olympus Fluoview 3000 confocal microscope. Animals that did not show targeted expression were excluded. For retrograde rabies tracing, areas were registered using the Paxinos and Franklin brain atlas<sup>58</sup>. For pPDH staining, DREADD<sup>+</sup> mice were injected with 1 mg kg<sup>–1</sup> CNO 60 min before perfusion and cell counts were quantified using QuPath<sup>59</sup>.

### FISH

Animals were anaesthetized and rapidly decapitated. Brains were quickly removed and fresh frozen in dry ice, then stored at –80 °C. Sections were cut at 20 µm, mounted on slides and stored at –80 °C. Sections were fixed in 4% PFA for 15 min, dehydrated in serial ethanol concentrations (50%, 70% and 100%) and processed with RNAscope (320293, Advanced Cell Diagnostics). Sections were hybridized with the probes; the following RNAscope probes from Advanced Cell Diagnostics were used: DBH (464621), VGLUT1 (*Slc17a7*; 416631), VGAT (*Slc32a1*; 319191), VGLUT2 (*Slc17a6*; 319171), pENK (318761), Tac1 (410351) and *Pnoc* (437881). Sections were then counterstained with DAPI and coverslips were applied. Confocal images were obtained on an Olympus FV3000RS microscope; counts were specific to the peri-LC region.

### Slice electrophysiology

For electrophysiology experiments, slices of the LC were prepared from injected mice at least 21 days after stereotaxic surgery. Anaesthetized mice underwent cardiac perfusion with ice-cold cutting solution (75 mM NaCl, 2.5 mM KCl, 6 mM MgCl<sub>2</sub>, 0.1 mM CaCl<sub>2</sub>, 1.2 mM NaH<sub>2</sub>PO<sub>4</sub>, 25 mM NaHCO<sub>3</sub>, 2.5 mM D-glucose and 50 mM sucrose, bubbled with 95% O<sub>2</sub> and 5% CO<sub>2</sub>). The brain was then submerged in cutting solution and placed on a VT1200 vibratome (Leica), where 240-µm horizontal LC slices were collected. Slices were transferred to a 32 °C holding chamber containing oxygenated artificial cerebrospinal fluid (ACSF; 126 mM NaCl, 2.5 mM KCl, 1.2 mM MgCl<sub>2</sub>, 2.5 mM CaCl<sub>2</sub>, 1.2 mM NaH<sub>2</sub>PO<sub>4</sub>, 21.4 mM NaHCO<sub>3</sub> and 11.1 mM D-glucose, bubbled with 95% O<sub>2</sub> and 5% CO<sub>2</sub>), where they recovered for at least 1 h.

Whole-cell and cell-attached recordings were made using an Axopatch 200B Amplifier (Molecular Devices). Data were acquired with Axograph X (Axograph Scientific) at 5 kHz and filtered to 2 kHz. Cells were visualized with a BX51WI microscope (Olympus). LC<sup>NE</sup> cells were identified by morphology, location (between the edge of the fourth ventricle and motor neurons of the mesencephalic tract), and the presence of an electrically evoked current (α2-mediated IPSC)<sup>21</sup>. ChR2-expressing peri-LC cells were identified by the presence of a steady-state current during a 150-ms light pulse and were medial to LC<sup>NE</sup> cells. In the recording chamber, slices were continuously perfused at 1.5–2 ml per minute with ACSF warmed to 30 ± 2 °C. Pipettes (1.5–2.0 MΩ) were pulled from borosilicate glass (World Precision Instruments). For whole-cell voltage clamp recordings, cells were held at –60 mV and the internal pipette contained: 135 mM KCl, 0.1 mM CaCl<sub>2</sub>, 2 mM MgCl<sub>2</sub>, 10 mM HEPES, 0.038 mM EGTA, 1 mg ml<sup>–1</sup> ATP,



0.1 mg ml<sup>-1</sup> GTP, 1.5 mg ml<sup>-1</sup> phosphocreatine (pH 7.4, 275 mOsm). Currents were evoked with wide-field stimulation using a 1-ms pulse from an LED (470 nm, approximately 0.5–1.0 mW mm<sup>-2</sup>) through a 40× water immersion objective. Paired pulse ratios were measured using a 50-ms interpulse interval. Glutamatergic and GABAergic synaptic components were blocked using DNQX and picrotoxin, respectively. TTX and 4-AP were used to isolate monosynaptic ChR2-evoked currents. Current clamp recordings were collected (using the same internal solution) to measure action potentials and resting membrane potential of peri-LC cells. For cell-attached recordings, the intracellular pipette solution contained: 126 mM NaCl, 2.5 mM KCl, 1.2 mM MgCl<sub>2</sub>, 2.5 mM CaCl<sub>2</sub> and 1.2 mM NaH<sub>2</sub>PO<sub>4</sub>. Firing rate was measured in 500-ms bins, with two cells that did not recover firing excluded from analysis. In current injection experiments, the duration of current injection was 500 ms.

### Brain clearing and imaging LC and peri-LC

VGAT-Cre mice were injected with 300 nl of AAV5-EF1α-DIO-ChR2-(H134R)-eYFP (1 × 10<sup>13</sup> vg ml<sup>-1</sup>) at -5.40 AP, +0.75 ML and -4.0 to -3.7 DV from bregma with a lateral-facing bevelled syringe. After 5 weeks, mice were euthanized, perfused with PBS and PFA, and the brains were collected. Tissue clearing was modified from iDISCO<sup>+</sup> protocol<sup>60</sup>. In brief, brains were dehydrated with methanol, delipidated with dichloromethane and bleached with hydrogen peroxide solution. After rehydration and blocking, brains were incubated with 1:1,000 chicken anti-GFP antibody (GFP-1020, Aves) and 1:1,000 mouse anti-TH antibody (818001, BioLegend), with boosters over 5 days for a final concentration of 1:200. After washing, secondary antibodies were added: 1:500 goat anti-chicken IgG Alexa Fluor 647 (A21449, Invitrogen) and 1:500 goat anti-mouse Alexa Fluor 488 (A11001, Life Technologies), with boosters over 5 days for a final concentration of 1:100. After washing and dehydration, brain clearing and refraction index matching were performed with dibenzyl ether. Brains were imaged with a light-sheet microscope (UltraMicroscope II, LaVision Biotec), and imaging data were analysed using ImageJ 1.52i and Arivis Vision4D (3.0.0).

### Single-nucleus RNA sequencing

Adult mice were euthanized with 0.01 ml kg<sup>-1</sup> of phenytoin–pentobarbital solution, and perfused transcardially with cold NMDG-ACSF (96 mM NMDG, 2.5 mM KCl, 1.35 mM NaH<sub>2</sub>PO<sub>4</sub>, 30 mM NaHCO<sub>3</sub>, 20 mM HEPES, 25 mM glucose, 2 mM thiourea, 5 mM Na<sup>+</sup> ascorbate, 3 mM Na<sup>+</sup> pyruvate, 2 mM *N*-acetyl-cysteine, 0.5 mM CaCl<sub>2</sub>, 10 mM MgSO<sub>4</sub> and 0.6 mM glutathione-ethyl-ester; pH 7.35–7.40, 300–305 mOsm, oxygenated with 95% O<sub>2</sub> and 5% CO<sub>2</sub>) with the following inhibitors: 500 nM TTX, 10 μM APV, 10 μM DNQX, 5 μM actinomycin and 22.7 μM anisomycin. Extracted brains were sliced using a vibratome (VT1200, Leica), the LC and its neighbouring regions dissected out, and the tissue was flash frozen on dry ice and stored at -80 °C. Tissue from 5–6 mice was pooled for each sample; male and female samples were processed separately. Later, coerulear sections were lysed, homogenized with glass pestle, filtered via a 40-μm strainer, centrifuged initially to remove cellular debris, and again in differing density solvents (50% and 30% OptiPrep) in which the pelleted nuclei were collected. Nuclei were resuspended in PBS as needed for more than 400 nuclei per microlitre.

The cDNA libraries were constructed using the Chromium Next GEM Single Cell 3' Reagents Kits v3.1 Dual Index, 10x Genomics. In brief, approximately 10,000 nuclei were loaded into the chip, single-nuclear mRNAs were captured by barcoded beads, and the transcripts were reverse transcribed. The resulting cDNAs were then PCR amplified, fragmented, ligated with adaptors and added with sample index. To minimize batch effects, experimenters were well-practiced, timing was kept the same and all reagent lots were the same. cDNA libraries were sequenced on Illumina HiSeq 2 × 150 bp and NovaSeq PE150, and sequence alignment to mouse genome done with 10x Genomics Cell Ranger Count (v6.1.2) pipeline.

Integration of datasets, clustering and differential gene expression analysis were performed using Seurat (v4.0)<sup>61</sup>, R 4.0.3 and Jupyter Notebook 6.2.0. Nuclei of potentially low quality (total UMI < 700 or total UMI > 15,000, or percentage of mitochondrial genes > 0.1%) were removed from downstream analysis. Suspected doublet cells were computationally removed using DoubletDecon (1.02)<sup>62</sup>. Cells were clustered by weighted nearest neighbour analysis of normalized gene expression, and these clusters were validated using known markers for cell types in the brain<sup>61</sup>. Dimensions and resolution were determined by elbow plots and ChooseK<sup>40</sup>. Differentially expressed genes between clusters were identified using a non-parametric Wilcoxon rank sum test<sup>63</sup>. Gene lists were acquired and modified from ontology lists at the Mouse Genome Database (MGD), Jackson Laboratory (<https://informatics.jax.org>; December 2021)<sup>64</sup>.

### Pixel-seq

Pixel-seq was performed as previously described in a recent report<sup>46</sup>. In brief, polyacrylamide gels of less than 5-mm thickness were cast and crosslinked on a 40-mm round coverslip (#1.5; 64–1696, Warner Instruments) in an anaerobic chamber. The gels were assembled into a FCS2 flowcell (Bioptechs) and 5' phosphorothioate-modified primers were covalently grafted onto the gel. Next, 368-bp DNA templates (Integrated DNA Technologies) were hybridized onto the gel by washing on 8–12 pm mix (0.6–0.8 million features mm<sup>-2</sup>) and incubated at 75 °C for 2 min, cooling to 40 °C, and washing with 500 μl amplification buffer. Each template contained 24-bp random spatial indices, which serve as spatial barcodes, a 20-bp poly(T) probe, which captures RNAs, and two TaqI restriction sites, which are digested to expose the poly(T) probes for RNA capture. Following this, a 150 μl Taq polymerase mixture was used to synthesize and anchor the first-strand DNAs to the gel by incubating at 74 °C for 5 min.

Polonies were amplified to reduce gaps between the polony templates, then digested with TaqI, and polony sequencing using sequencing-by-synthesis (HiSeq SBS kit v4; FC-401-4002, Illumina) was used to build a spatial index of barcode coordinates. For more details, see ref. 46.

A 10-μm-thick section of fresh-frozen brain containing the LC was dissected using a cryostat (NX70, Thermo Scientific) and placed on the gel in a flat layer. The tissue sections were then gently immersed in 50 μl hybridization buffer (6× SSC (AM9763, Invitrogen), 2 U μl<sup>-1</sup> RNaseOUT (10777019, Thermo Fisher) and incubated at 23 °C for 15 min, hybridizing them to the capture probes adhered to the gel. To create cDNAs, in situ reverse transcription was performed in a reaction chamber by incubating for 42 °C for 1 h in 100 μl reverse transcription mixture (5 μl Maxima H<sup>-</sup> reverse transcriptase (EP0753, Thermo Fisher), 20 μl 5× Maxima reverse transcriptase buffer, 20 μl 20% Ficoll PM-400 (F4375, Sigma-Aldrich), 10 μl 10 mM each dNTPs, 5 μl 50 μM template-switching oligo (339414YCO0076714, Qiagen), 2.5 μl RNaseOUT (40 U μl<sup>-1</sup>) and 37.5 μl H<sub>2</sub>O). After removing the reaction buffer, tissues were digested using proteinase K (10 μl proteinase K (19131, Qiagen) in 90 μl PKD buffer (1034963, Qiagen)) at 55 °C for 30 min and then washed away. To recover the spatially barcoded cDNAs, the chambers were incubated for 15 min at 65 °C with 70 μl second-strand mix (7 μl 10× isothermal amplification buffer (B0537, NEB), 7 μl 10 mM each dNTP mix, 3.5 μl 10 μM TSO primer, 0.5 μl 20 mg ml<sup>-1</sup> BSA (B9000, NEB), 3 μl BST2.0 WarmStart DNA Polymerase (M0538, NEB) and 49 μl H<sub>2</sub>O), then the cDNAs were eluted into a tube. To introduce UMIs, an approximately 35 μl sample was mixed with 65 μl UMI incorporation mix (50 μl 2× Q5 Ultra II master mix (M0544, NEB), 2.5 μl 10 μM UMI primer and 12.5 μl H<sub>2</sub>O), then denatured at 95 °C for 30 s, annealed at 65 °C for 30 s, and extended at 72 °C for 5 min. Twelve cycles of PCR amplification of the cDNA library were performed as previously described<sup>46</sup>. Of DNA, 1 ng was then used for sequencing library construction using a Nextera XT kit (FC-131-1024, Illumina) following the manufacturer's protocol. cDNA libraries were sequenced on NovaSeq PE150.

Transcripts were mapped to the spatial barcode map by processing the FASTQ files. After using Flexbar (3.0)<sup>65</sup> to extract spatial barcode and UMI sequences, Bowtie<sup>66</sup> was used to map index sequences to the spatial barcode map, allowing up to two mismatches. STAR (v2.7.0)<sup>67</sup> was used to align paired-end reads of mapped indices to a reference mouse transcriptome (GRCm38). Sequencing reads with the same transcriptome mapping locus, UMI and spatial barcode were collapsed to unique records for subsequent analysis. The cell segmentation was performed as previously described<sup>46</sup>.

We next performed spatial pattern genes analysis using SpatialPCA<sup>68</sup>. Approximately 1,200 genes were identified. For the female and male LC clustering, SCTransform normalization was applied in Seurat using 2,000 highly variable genes. We chose to combine the spatial pattern genes and highly variable genes as features for the subsequent analysis. After principal component analysis (PCA), 20 principal components were used in the UMAP visualization, and the clustering graph was generated with a resolution of 0.6. Marker genes for each cluster were identified by Seurat using a Wilcoxon test and then compared with the consensus in a published mouse brain cell-type atlas<sup>69</sup>.

Segmented LC cells were annotated with a snRNA-seq dataset (GSE121891)<sup>70</sup> as a reference to predict cell-type compositions using scvi-tools (1.0)<sup>71</sup>. The top 3,000 variable genes were selected for the model training. Raw gene counts for training and testing datasets were scaled to  $10^4$ . The training and prediction were run at max\_epochs = 500. Cell types of segmented cells were determined by the predicted cell types with the highest ratios. For the outcome of Fig. 4b, the threshold of trusted cell type was set as 0.5.

## Fibre photometry

Fibre photometry recordings were performed as previously described<sup>57</sup>. In brief, an optic fibre was attached to the implanted fibre by a ferrule sleeve, then GCaMP6s was stimulated by two LEDs, a 531-Hz sinusoidal light (M470F3, Thorlabs), band pass filtered at  $470 \pm 20$  nm, and a 211-Hz sinusoidal light (M405FP1, Thorlabs), bandpass filtered at  $405 \pm 10$  nm (filter cube: Doric FMC4; LED driver: DC4104). The 470-nm signal evoked  $\text{Ca}^{2+}$ -dependent emission, whereas the 405-nm signal evoked  $\text{Ca}^{2+}$ -independent isosbestic control emission. Before recording, a 180-s period of GCaMP6s excitation with both light channels was used to remove the majority of baseline drift. Laser intensity at the optic fibre tip was adjusted to approximately 50  $\mu\text{W}$  before each day of recording. The GCaMP6s fluorescent signal was isolated by bandpass filtering ( $525 \pm 25$  nm), transduced by a femtowatt silicon photoreceiver (2151, Newport) and recorded by a real-time processor (TDT RZSP). The envelopes of 531-Hz and 211-Hz signals were extracted in real time by the TDT program Synapse at a sampling rate of 1,017.25 Hz. The 565-nm LED was used for peri-LC photostimulation, with light intensity adjusted to approximately 2.5  $\mu\text{W}$  at the optical fibre tip, whereas GRAB<sub>NE</sub> signals were simultaneously recorded from other cables from the PFC and Pir.

Fibre recordings were analysed using custom MATLAB scripts available on GitHub. The isosbestic signal was subtracted from the calcium-dependent signal, then baseline drift due to slow photobleaching artefacts was corrected by fitting a double exponential curve to the raw trace. The baseline-corrected signal was low-pass filtered using a Butterworth filter with cut-off set at 10 Hz. The filtered signal was then normalized by subtracting the median signal value and dividing by the absolute median value of the isosbestic signal. The correct and normalized photometry trace was z-scored relative to the mean and standard deviation of the signal over the entire trial. The mean z-score during comparable periods of time determined by the stimulus duration (when applicable) or the experimentally determined duration of a neural response (Supplementary Table 1) were compared using paired Student's *t*-tests. For determination of neural activity during movement, mice were tracked using Ethovision 10; movement was defined as all samples with movement  $\geq 10$  cm  $\text{s}^{-1}$  (in open field) or movement  $\geq 5$  cm  $\text{s}^{-1}$  (in zero maze) for 0.5 s or more. The average

activity during movement and non-movement per session were then compared using paired Student's *t*-tests. For comparing the evolution of noradrenaline activity across groups (Extended Data Fig. 9d), we used a two-tailed Spearman correlation.

## Two-photon slice calcium imaging

Brains were obtained from Dbh-Cre mice more than 4 weeks after injection of AAVDJ-EF1 $\alpha$ -DIO-GCaMP6f in the LC, and freshly sliced at 250  $\mu\text{m}$  in NMDG solution (92 mM NMDG, 20 mM HEPES, 25 mM D-glucose, 30 mM  $\text{NaHCO}_3$ , 1.45 mM  $\text{NaH}_2\text{PO}_4$ , 2.5 mM KCl, 5 mM sodium ascorbate, 3 mM sodium pyruvate, 2 mM thiourea, 0.5 mM  $\text{CaCl}_2$  and 2 M  $\text{MgSO}_4$ ) using vibratome. Brain slices were placed on an imaging slide and secured with a grating anchor. ACSF (126 mM NaCl, 2.5 mM KCl, 1.2 mM  $\text{NaH}_2\text{PO}_4$ , 1.2 mM  $\text{MgCl}_2$ , 11 mM D-glucose, 18 mM  $\text{NaHCO}_3$  and 2.4 mM  $\text{CaCl}_2$ ) was delivered to the slide by a rotatory pump (Gilson Minipuls) and through an in-line warmer (TC-324C, Warner Instrument) to maintain at  $31\text{--}34^\circ\text{C}$ . Three-minute treatments with ACSF, DAMGO (1171, Tocris), Leu-enk (J61408.MB, Thermo Scientific) and nociceptin (0910, Tocris) were counterbalanced and washed out for 3 min before the subsequent treatment. KCl treatment was for 1 min at the end of experiment. Suite2p was used to detect regions of interest, and Excel for further analysis. The median signal from 1.5 min to 2.5 min after drug perfusion was used for comparison to the 1-min baseline before perfusion.

## Behaviour

**Odour delivery.** Odour delivery was performed in a polyethylene chamber approximately  $12 \times 12 \times 24$  cm, out of which air was continuously vacuumed at a rate of 2 l  $\text{min}^{-1}$ . To minimize odour release into the room, the chamber was placed in a fume hood and the vacuumed air was passed through a carbon filter system. Mice were habituated to the room and chamber three times before odour was delivered and were habituated to the room for 30 min before testing. The chamber was wiped with 70% ethanol between trials, which fully evaporated before the next animal was tested. Odour was delivered for 30-s periods at a rate of 0.5 l  $\text{min}^{-1}$ . Tubing was changed between odourants to minimize cross-contamination. Bobcat urine (<https://predatorpeestore.com>) was used undiluted. Opposite-sex urine was collected from unstressed mice an hour before experiments began. 2-PE (77861, Sigma) was diluted 1:10 in mineral oil. Peppermint oil (77411, Sigma-Aldrich) was diluted 1:100 in mineral oil. 2MT (M0285, TCI) was diluted 1:50 in mineral oil.

**Food consumption.** Feeding assays were performed in a square plexi-glass arena ( $27 \times 27$  cm). Mice were habituated to the room and chamber at least three times before testing and the room 30 min before trials. For food-deprived conditions, mice were food deprived for 24 h before trial. For highly palatable food, mice were habituated to the food item 1 week before trial to eliminate food neophobia. Food items were placed in the corner of the arena and mice were introduced to the area for 30 min. To record the amount of food consumed, food was measured before and after the assay; the bottom of the chamber was scraped after each trial to collect all crumbs resulting from chewing without consumption. When multiple stimulation or inhibition conditions were tested, they were performed in a counterbalanced manner across mice. For DREADD experiments, CNO (1 mg  $\text{kg}^{-1}$ ; BML-NS105, Enzo Life Sciences) was administered to both control and DREADD<sup>+</sup> animals 30 min before their individual test time.

**Threat conditioning.** Pavlovian threat conditioning was performed in Med Associates fear-conditioning chambers (NIR-022MD). This equipment consisted of a conductive grid floor inside a 29.53 cm (length)  $\times$  23.5 cm (width)  $\times$  20.96 cm (height) chamber inside of a soundproof box lit by an infrared light. Stimuli occurred at random timing, at least 110–140 s apart. On day 1, mice were exposed to six 20-s tones without an associated shock. On day 2, mice were conditioned

to six 20-s tones co-terminating with 1-s 0.5-mA shocks. On day 3, mice were presented with six 20-s tones in the absence of shock.

**False food.** False food testing was performed as previously described<sup>72</sup>. Mice were food deprived for 24 h before testing and were habituated to the room for 30 min before testing. Mice were then placed in a dimly lit (25 lux) plexiglass arena (27 × 27 cm) with a foam plug shaped like a piece of chow placed in the middle. Videos were scored for attempts to eat the false food.

**Novel object.** Novel-object testing was performed as previously described<sup>19</sup>. In brief, mice were habituated to the room for 30 min before testing. Mice were then placed in a dimly lit (25 lux) plexiglass arena (27 × 27 cm) with two identical objects (test tubes or petri dishes; counterbalanced) fixed at the corners. Mice were given 10 min to explore the objects, returned to the homecage for 1 h, and then were placed in the arena with one familiar object and one novel object. Objects were thoroughly cleaned with ethanol and allowed to dry between trials. Videos were scored for interactions with each object.

**EZM.** EZM testing was performed as previously described<sup>19</sup>. The EZM (Harvard Apparatus) was made of grey plastic, 200 cm in circumference, comprising four 50-cm sections (two opened and two closed). The maze was elevated 50 cm above the floor and had a path width of 4 cm with a 0.5-cm lip on each open section. Mice were connected to fibre optic cables, positioned head-first into a closed arm, and allowed to roam freely for 20 min. Open arm time was the primary measure of anxiety-like behaviour. For optogenetic experiments, animals received 4 Hz (5-ms pulse width) constant photostimulation (2 mW light power). For DREADD experiments, 1 mg kg<sup>-1</sup> CNO was administered to both control and DREADD<sup>+</sup> animals 30 min before their individual test time.

**OFTs.** OFT was performed as previously described<sup>19</sup> in a plexiglass arena (50 × 50 cm). The centre zone was defined as the middle 50% of the arena size. Mice were connected to fibre optic cables, placed in the outside of the arena, and allowed to roam freely for 20 min. Two VGAT-Cre mice were removed from fibre photometry analysis because they made no transitions into the centre. For optogenetic experiments, animals received 4 Hz (5-ms pulse width) constant photostimulation (2 mW light power). For DREADD experiments, 1 mg kg<sup>-1</sup> CNO was administered to both control and DREADD<sup>+</sup> animals 30 min before their individual test time. The centre time was the primary measure of anxiety-like behaviour.

**Real-time place preference.** Mice were placed into a custom-made unbiased, balanced two-compartment conditioning apparatus (52.5 × 25.5 cm × 25.5 cm) as previously described<sup>19</sup> and allowed to freely roam the entire apparatus for 20 min. Entry into one compartment triggered constant photostimulation (0–20 Hz; 2 mW light power) while the animal remained in the light-paired chamber. Entry into the other chamber ended the photostimulation. The side paired with photostimulation was counterbalanced across mice and between days. Time spent in each chamber and total distance travelled was measured using Ethovision 10 (Noldus).

**Light–dark box.** The light–dark box was performed in a real-time place preference chamber where one side was brightly lit (1,000 lux) and the other side was dimly lit (5 lux). Animals were allowed to freely move throughout the chamber for 20 min. Time spent in the light side was the primary measure of anxiety-like behaviour. For optogenetic experiments, animals received 4 Hz (5-ms pulse width) constant photostimulation (2 mW light power). For DREADD experiments, CNO was administered to both control and DREADD<sup>+</sup> animals 30 min before their individual test time.

**Foot pinch.** Animals were lightly anaesthetized at 1.5% isoflurane and placed in a stereotax. Their pupil size was monitored under infrared lighting<sup>73</sup>. Mice were pinched on their contralateral paw for 10 s.

**Tail flick.** The tail-flick assay was performed as previously described<sup>74</sup>. Of the mouse's tail, 2–4 cm was immersed into 54 °C water and the time between immersion and withdrawal was recorded, up to a maximum of 10 s to prevent tissue damage. For DREADD experiments, DREADD<sup>+</sup> and control mice were injected with 1 mg kg<sup>-1</sup> CNO after the initial test (time 0) and tested every 10 min for 1 h, then again at 90 min.

**Hot plate.** The hot-plate assay was performed as previously described<sup>75</sup>. Mice were placed on a 55 °C hot plate and the time for a nocifensive response (paw withdrawal or licking, stamping, leaning or jumping) was recorded, up to a maximum of 30 s to prevent tissue damage. All mice were injected with 1 mg kg<sup>-1</sup> CNO 30 min before their individual test time.

### Statistics and reproducibility

All summary data are expressed as mean ± s.e.m. Statistical significance was taken as \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001 and \*\*\*\**P* < 0.0001, as determined by two-tailed Student's *t*-test (paired and unpaired), two-way repeated measure analysis of variance (ANOVA) followed by Tukey's multiple comparisons test, two-tailed Wilcoxon matched-pairs signed rank test, and Friedman repeated measures ANOVA followed by Dunn's multiple comparison test. Statistical analyses were performed in GraphPad Prism 9. For sequencing experiments, analyses were performed as described in the corresponding Methods section. Summary data are available in Supplementary Table 1. Representative images were chosen from similar images collected from 2 mice (Fig. 1a, top), 10 mice (Fig. 1a, bottom, and Fig. 2a), 9 mice (Fig. 2d), 2 mice (Fig. 4c), 1 mouse (Extended Data Fig. 7h–j), 2 mice (Extended Data Fig. 9a), 1 mouse (Extended Data Fig. 1a), 2 mice (Extended Data Fig. 3k), 2 mice (Extended Data Fig. 7f) and, from left to right, 14, 6, 5 and 4 mice (Extended Data Fig. 8a).

### Reporting summary

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

### Data availability

All data are available in the article or the Supplementary Information. snRNA-seq data have been deposited in the NCBI Gene Expression Omnibus (GEO) database with the accession number GSE201998; the sample GSM6086497 (LC\_FEMALE\_01) has been included for the sake of completeness, but it is poor quality and we do not recommend using it. Pixel-seq data have been deposited in the GEO database with the accession number GSE244659. The reference mouse transcriptome GRCm38 is available at [https://www.ncbi.nlm.nih.gov/datasets/genome/GCF\\_000001635.20/](https://www.ncbi.nlm.nih.gov/datasets/genome/GCF_000001635.20/). The full behavioural dataset has been uploaded as source data. Source data are provided with this paper.

### Code availability

The custom MATLAB analysis code was created to appropriately organize, process and combine photometry recording data with associated behavioural data. Custom R scripts were written to analyse snRNA-seq datasets. The custom code is available on GitHub (<https://github.com/Bruchas-Lab>).

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**Author contributions** A.T.L., L.L. and M.R.B. conceptualized and designed the entire study. A.T.L., L.L. and M.M.M. performed the stereotaxic surgeries. A.T.L., M.M.M., T.B., B.A.W., S.T.T., E.M.L. and A.N.R. performed the behavioural experiments. A.T.L., L.L., M.M.M., T.B., B.A.W., S.T.T., E.M.L. and A.N.R. performed the histology. A.T.L., L.L., X.F., L.S. and L. Gao performed the Pixel-seq experiments. K.B. and K.S.G. performed the electrophysiological experiments. A.T.L., L.L. and T.B. performed the fibre photometry experiments. A.T.L., L.L. and R.C.S. performed the snRNA-seq experiments and analysis. A.T.L., L.L., M.M.M. and K.B. performed the statistical analysis. L.L. and A.D.M. performed tissue clearing and imaging. A.T.L., L.L., X.F., M.M.M., K.B. and M.R.B. made the figures. A.T.L., L.L. and M.R.B. wrote the manuscript with collective input from all authors. S.A.G. supervised tissue clearing and imaging. G.D.S. supervised the single-nucleus sequencing. C.P.F. supervised the electrophysiological experiments. L. Gu supervised the Pixel-seq experiments. M.R.B. oversaw, provided resources and supervised the entire study.

**Competing interests** Two patents on polony gel stamping and Pixel-seq have been filed by the University of Washington and TopoGene Inc. L. Gu and L.S. are co-founders of TopoGene, which manufactures polony gels used in our approaches herein. The other authors declare no competing interests.

## Additional information

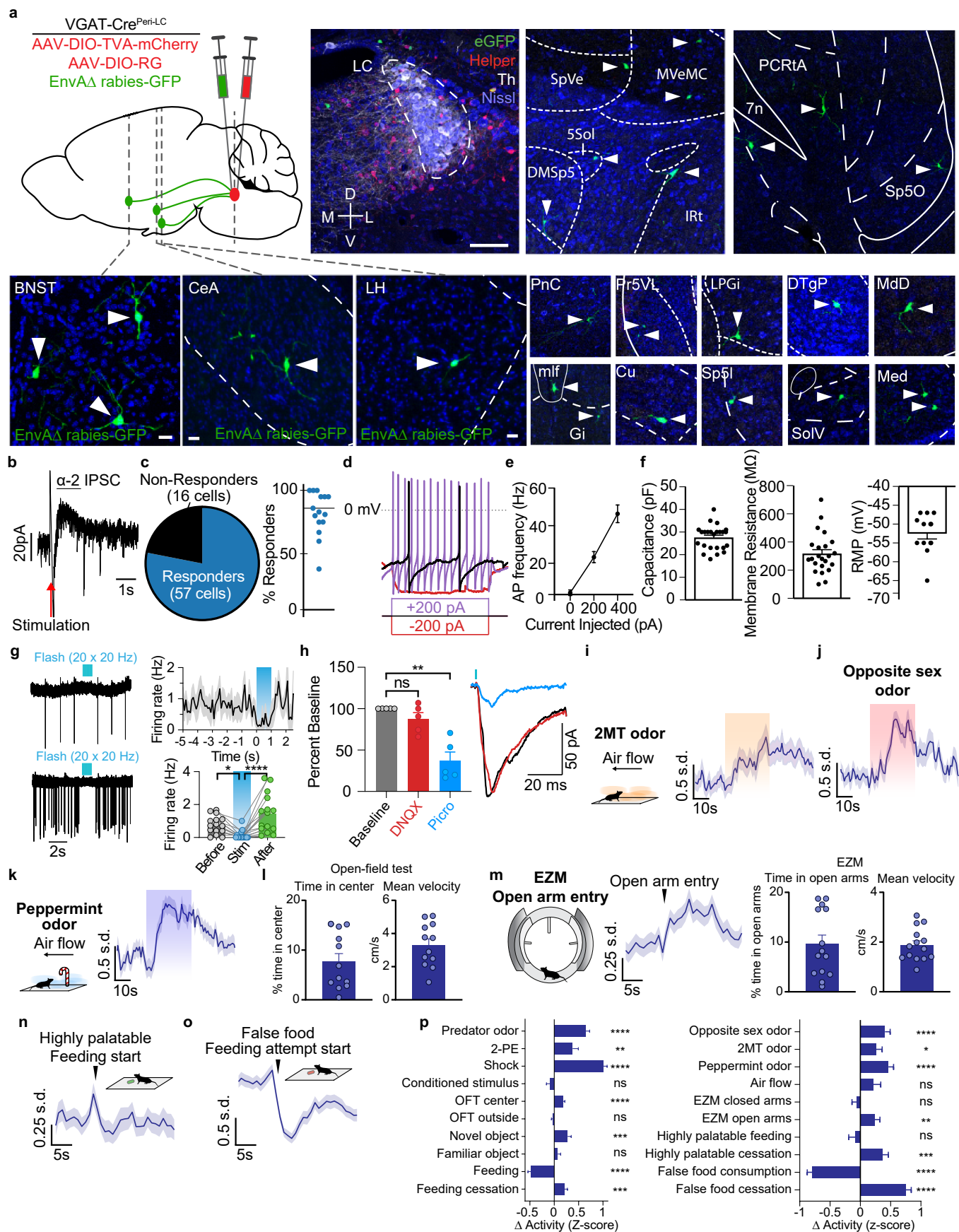
**Supplementary information** The online version contains supplementary material available at <https://doi.org/10.1038/s41586-025-08952-w>.

**Correspondence and requests for materials** should be addressed to Michael R. Bruchas.

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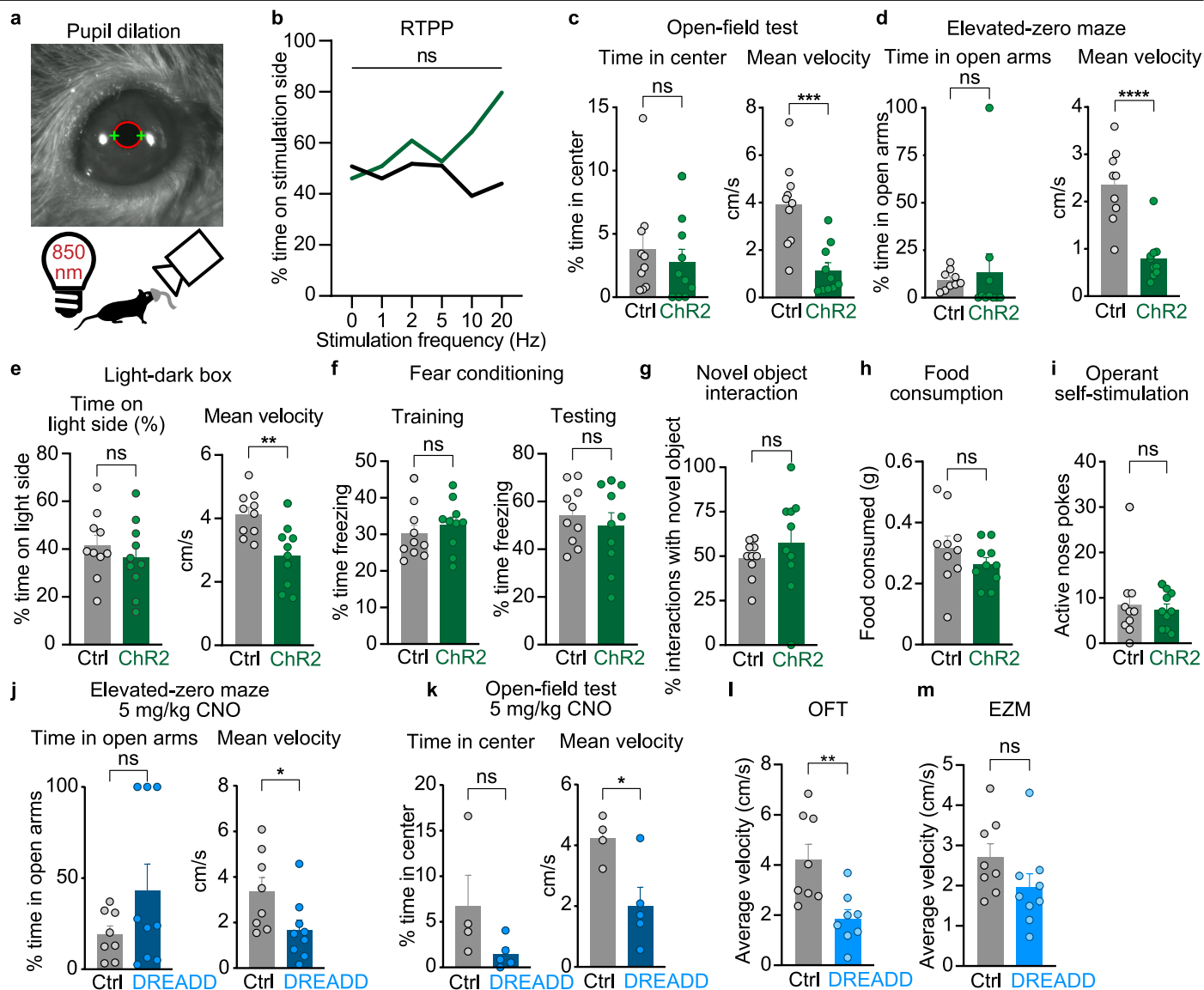


**Extended Data Fig. 1** | See next page for caption.

# Article

**Extended Data Fig. 1 | In vitro and in vivo identification and characterization of pericoerulear GABAergic neurons.** (a) Viral schematic for rabies tracing of monosynaptic inputs to peri-LC<sup>GABA</sup> neurons. Starter cells in peri-LC express TVA-mCherry and rabies-GFP (scale bar: 100  $\mu$ m in LC; 20  $\mu$ m in input regions). One mouse out of four injected showed starter cell expression. (b) Electrically evoked  $\alpha$ 2-mediated outward current recorded from LC<sup>NE</sup> neuron. (c) 78% of LC<sup>NE</sup> neurons displayed measurable fast IPSCs, though the success rate appeared dependent on injection specificity as 6 of 14 injection sites had 100% response rate (each data point represents one hemisphere, line shown is median; injections that were completely off target and/or had 0% response rate were excluded). (d) Properties of peri-LC GABA neurons. ChR2-expressing peri-LC neurons were identified by the presence of eYFP expression and a steady state response to a 150 ms light pulse (not shown). Representative trace of a current clamp recording with steps of 200 pA current injection. (e) Peri-LC<sup>GABA</sup> neuron firing rate upon 500 ms current injection (mean values  $\pm$  SEM). n = 11 cells. (f) Peri-LC<sup>GABA</sup> neuron capacitance, membrane resistance, and resting membrane potential (RMP) (mean values  $\pm$  SEM). n = 24 cells (capacitance), 23 cells (membrane resistance), and 13 cells (RMP). (g) Representative LC<sup>NE</sup> cell-attached trace of a single sweep (top) and an overlay of 10 sweeps (bottom). Blue hash marks indicate light stimulation (1 ms pulses, 20 Hz, 1 s). (Right top) Time course of action potential firing rate for an average of 6 cell-attached recordings with a pulse train for 1 s (20 Hz, 1 ms pulses). (Right bottom) Firing rate before (mean = 0.77 Hz), during (0.13 Hz), and after (1.45 Hz) photostimulation. Periods of comparison were 500 ms before, middle-to-end, and after stimulation. p = 0.0266 for pre-stimulation vs. post-stimulation periods by Wilcoxon signed-rank test; p = .0185 for baseline vs. stimulation period and p < 0.0001 for stimulation vs. rebound period by Dunn's multiple comparison test. (h) (Left) Average amplitudes of optically evoked IPSCs normalized to baseline (n = 5 cells) during the wash on of DNQX (10  $\mu$ M) followed by picrotoxin (100  $\mu$ M).

(Right) Representative traces showing PSCs during 5 min of baseline (black), 5-10 min post DNQX (red), and 5-10 min post picrotoxin (green). p = 0.5721 for baseline vs. DNQX, p = 0.0017 for baseline vs. picro, and p = 0.0064 for DNQX vs. picro by Tukey's multiple comparisons test. (i) Peri-LC<sup>GABA</sup> neurons increase activity during 30 s exposure to 2MT. (j) Peri-LC<sup>GABA</sup> neurons increase activity during 30 s exposure to odor of opposite-sex mice. (k) Peri-LC<sup>GABA</sup> neurons increase activity during 30 s exposure to peppermint oil odor. (l) Summary of exploration in open-field test (OFT). n = 12 trials; 2 mice that did not enter center were excluded from analysis. (m) Peri-LC<sup>GABA</sup> neuronal activity increases upon open arm entry in an elevated zero maze (EZM). n = 14 trials. (n) Peri-LC<sup>GABA</sup> neurons are not affected by the consumption of highly-palatable food. (o) Peri-LC<sup>GABA</sup> neurons decrease activity during attempts to eat a false food pellet. (p) Summary of peri-LC<sup>GABA</sup> fiber photometry experiments. Bars represent change in activity before vs. after events. Left, in order from top to bottom, p < 0.0001 (n = 56 exposures), p = 0.0027 (n = 44 exposures), p < 0.0001 (n = 84 exposures), p = 0.2682 (n = 84 exposures), p < 0.0001 (n = 368 transitions), p = 0.4499 (n = 368 transitions), p = 0.0002 (n = 133 interactions), p = 0.2539 (n = 141 interactions), p < 0.0001 (n = 200 bouts), and p = 0.0009 (n = 198 bouts). Right, in order from top to bottom, p < 0.0001 (n = 44 exposures), p = 0.0168 (n = 56 exposures), p < 0.0001 (n = 56 exposures), p = 0.0912 (n = 44 bouts), p = 0.4514 (n = 86 transitions), p = 0.0059 (n = 81 transitions), p = 0.4234 (n = 65 bouts), p = 0.0006 (n = 66 bouts), p < 0.0001 (n = 62 bouts), and p < 0.0001 (n = 57 bouts), by paired two-tailed t-test. Periods of comparison were 30 s for odor experiments and air flow; 20 s for shock and conditioned sound; 5 s for OFT, EZM, and novel/familiar object interaction; and 10 s for feeding. N = 14 animals for photometry experiments, 12 for OFT, and 11 for 2-PE, air flow and opposite-sex odor. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001, \*\*\*\*p < 0.0001 by paired two-tailed t-test. Error bars and shading indicate SEM. Panels a, i, k, m–o were adapted from ref. 19, AAAS.



**Extended Data Fig. 2 | Selective manipulation of pericoerulear GABA neurons modulates physiological arousal and exploratory behaviors.**

(a) Schematic and image of pupil dilation experiment. Panel a was adapted from ref. 19, AAAS. (b) Photostimulation did not cause a real-time place preference. (c-e) Photostimulation (2 mW, 4 Hz, 5 ms pulse width) affected movement, but not anxiety-like behavior, in open-field test (OFT) (c) ( $p = 0.5403$  (time in center) and  $p = 0.0004$  (velocity)), elevated zero maze (EZM) (d) ( $p = 0.7184$  (time in open) and  $p < 0.0001$  (velocity)), and light-dark box (e) ( $p = 0.4567$  (time in light) and  $p = 0.0028$  (velocity)). (f) Photostimulation (2 mW, 4 Hz, 5 ms pulse width) during 20 s presentation of cue during fear conditioning) did not affect acquisition of fear response. (g) Photostimulation (2 mW, 4 Hz, 5 ms pulse width) did not affect novel object interaction. (h) Photostimulation (2 mW,

4 Hz, 5 ms pulse width) did not affect food consumption in food-deprived state. (i) Animals did not preferentially nose-poke to receive photostimulation (2 mW, 4 Hz, 5 ms pulse width). (j, k) Chemogenetic inhibition (5 mg/kg CNO) of peri-LC<sup>GABA</sup> neurons caused behavioral arrest in EZM (j) ( $p = 0.1555$  (time in open) and  $p = 0.0378$  (velocity)) and open-field test (k) ( $p = 0.1248$  (time in center) and  $p = .0231$  (velocity)). (l) Chemogenetic inhibition (1 mg/kg CNO) of peri-LC<sup>GABA</sup> neurons decreased movement in OFT ( $p = 0.0231$ ). (m) Chemogenetic inhibition (1 mg/kg CNO) of peri-LC<sup>GABA</sup> neurons did not affect movement in EZM. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < .001$ , \*\*\*\* $p < 0.0001$  by unpaired two-tailed  $t$ -test. Error bars indicate SEM. Dots represent independent measurements across different animals.

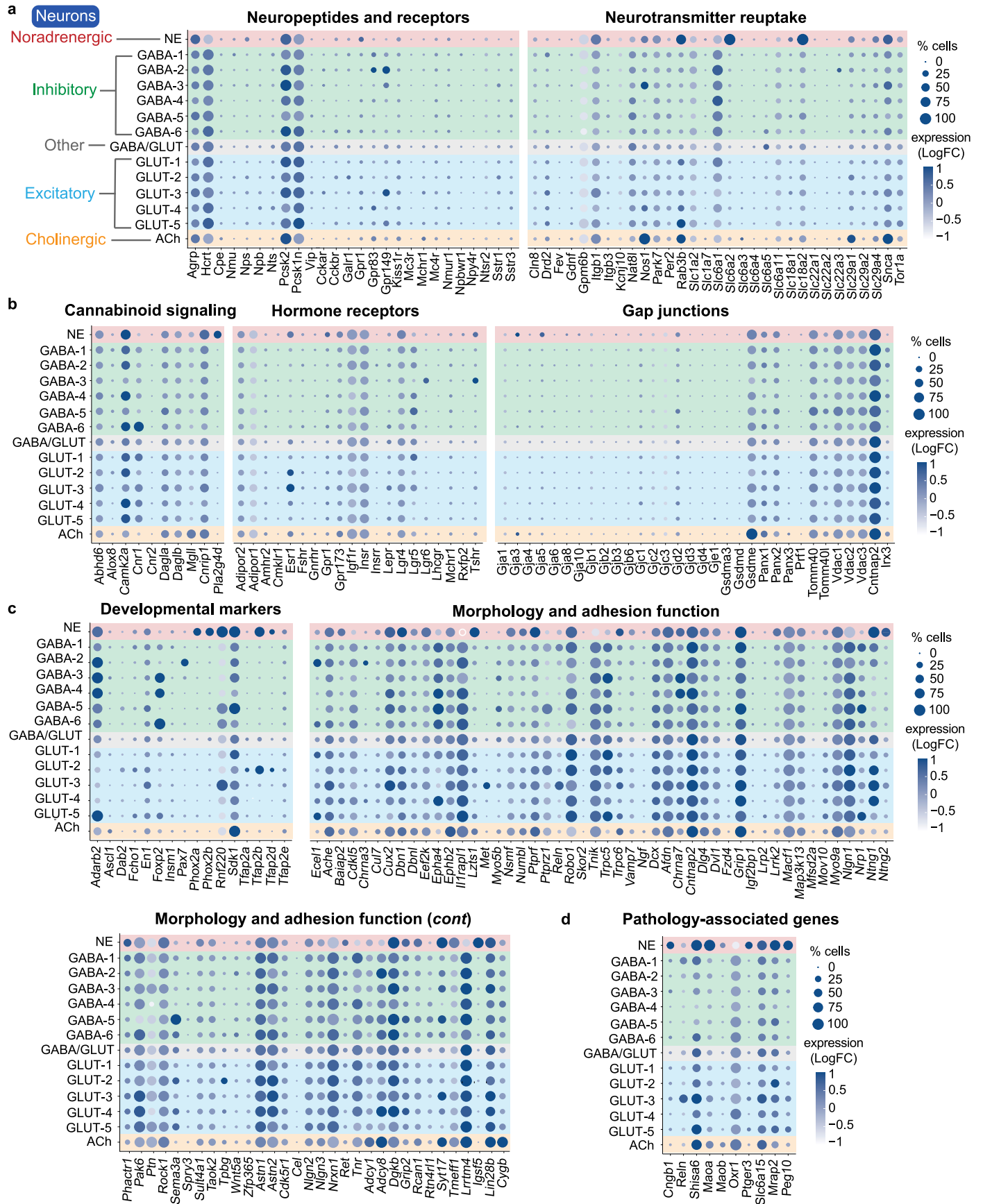


**Extended Data Fig. 3 | Single-cell RNA-sequencing of locus coeruleus and pericoerulear region.** (a) Violin plots show expression of canonical marker genes in cells clustered by principal component analysis (PCA) based on transcriptional profile. The 15 neuronal clusters are shown in orange. (b) Genes detected per cell type. (c) Unique molecular identifiers (UMIs) per cell type. (d) Percent mitochondrial genes per cell type. (e) Genes detected per neuronal cell type. (f) Unique molecular identifiers (UMIs) per neuronal cell type. (g) Percent mitochondrial genes per neuronal cell type. (h) Cluster tree shows relationships between clustered cell types. (i,j) Dot plots of  $n = 12,278$  neurons are shown, with neuronal clusters on the y-axis and gene transcripts on the x-axis. Size of circles corresponds to percent of cells in the cluster expressing a specific transcript, while color intensity corresponds to the relative expression

level of the transcript. Plots correspond to transcripts related to neuropeptides (i) and neuropeptide receptors (j). (k) Representative image for in situ hybridization of *Dbh* and VGLUT1 (*Slc17a7*) mRNA. Scale bar is 100  $\mu\text{m}$ . (l,m) (Top) Elbow plots show the number of principal components (PC) versus standard deviation and (bottom) silhouette scores show co-clustering performance for (l) peri-LC inhibitory neurons (*Slc32a1*, *Gad1*, *Gad2*) and (m) LC<sup>NE</sup> neurons (*Dbh*, *Th*). Dots represent clusters at each resolution; boxes represent median scores with 95% confidence interval. (n) Graph shows the frequency of neuropeptide-expressing peri-LC inhibitory neurons. (o) Graph shows the frequency of neuropeptide receptor-expressing LC<sup>NE</sup> neurons. (p-q) Venn diagrams show the overlap of *Penk*-, *Pnoc*-, and *Tac1*-expressing cells among cells that express (p) or do not express (q) GABAergic markers.



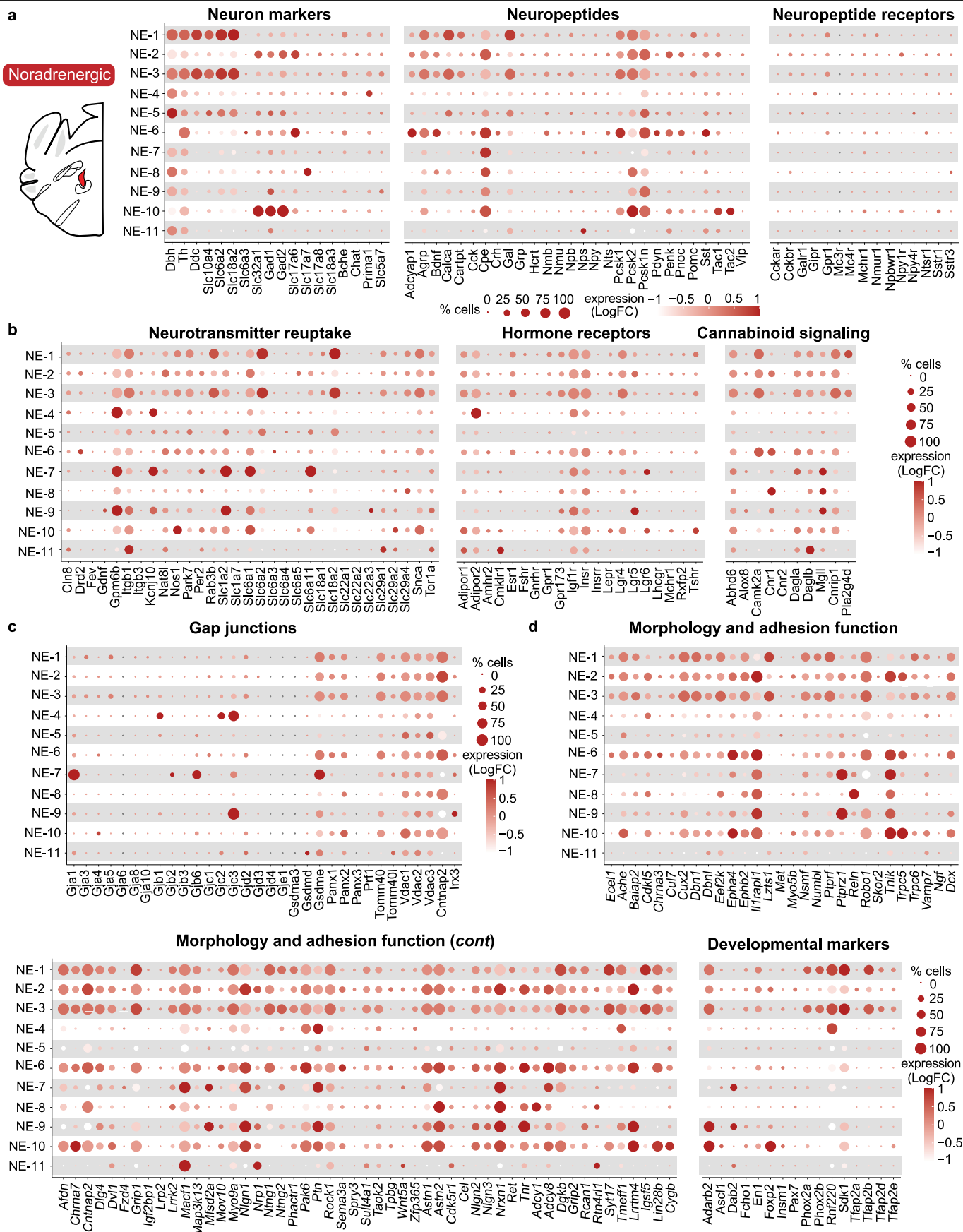
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**Extended Data Fig. 4** | See next page for caption.

**Extended Data Fig. 4 | Neuromodulatory, signaling, structural, and disease-related genes in single-cell RNA sequencing of all peri-LC neurons (n = 12,278).** (a) Dot plot of neuropeptide and receptor (left), and neurotransmitter reuptake (right) genes across all neuron clusters. Circle size corresponds to percent of cells in the cluster expressing the specific transcript, while color intensity corresponds to its relative expression. (b) Dot plot of genes associated with

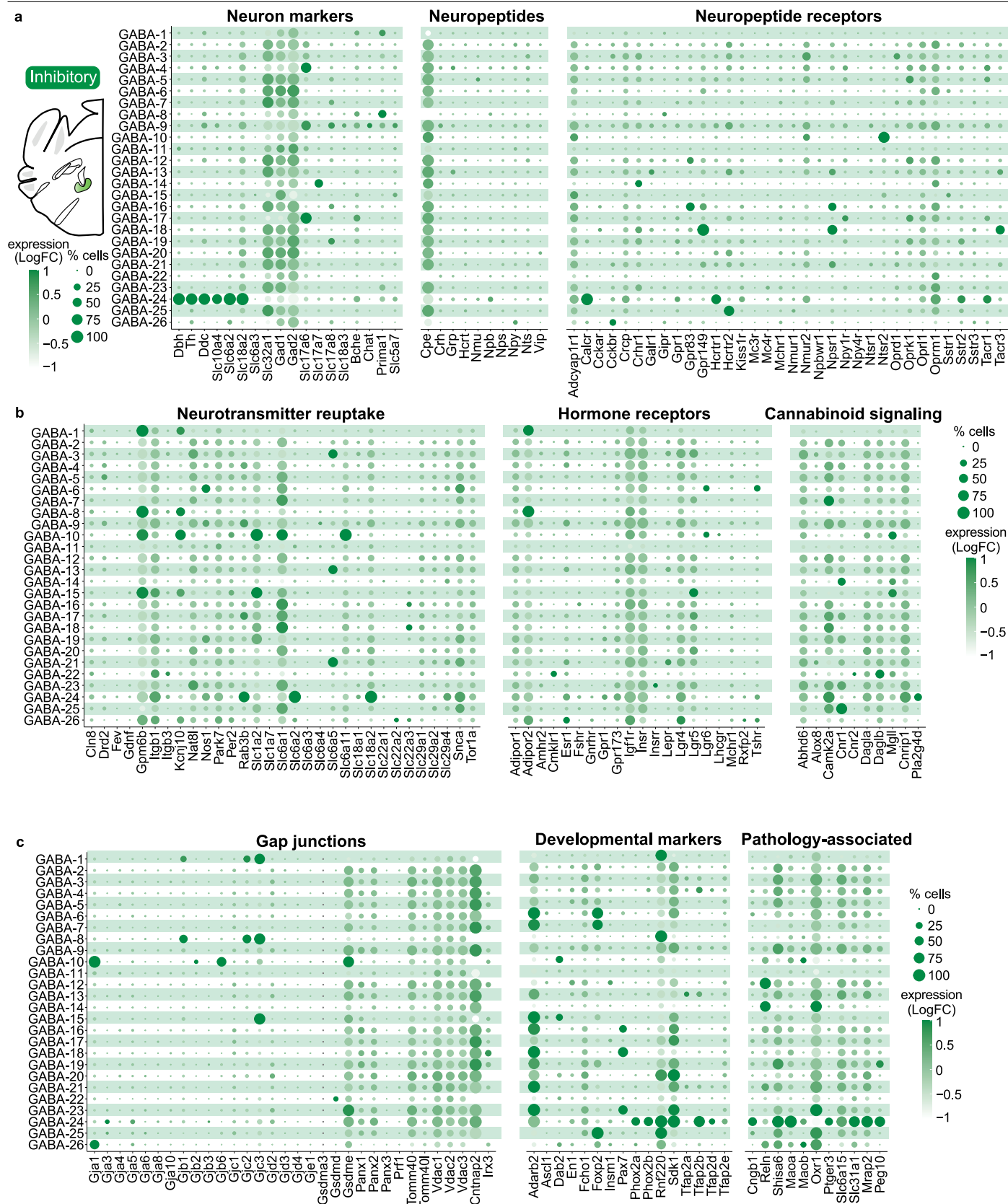
cannabinoid signaling (left), hormone signaling (middle), and gap junctions (right) across all neuron clusters. (c) Dot plot of genes associated with development (left), and morphology and adhesion functions (right) across all neuron clusters. (d) Dot plot of genes associated with pathology across all neuron clusters.



Extended Data Fig. 5 | See next page for caption.

**Extended Data Fig. 5 | Neuromodulatory, signaling, and structural genes in single-cell RNA sequencing of LC<sup>NE</sup> neurons (n = 1,324).** (a) Cartoon of the sampled locus coeruleus noradrenergic neurons (left). Dot plot of neuronal subtype (left), neuropeptide (middle), and receptor (right) genes across LC<sup>NE</sup> neuron clusters. Circle size corresponds to percent of cells in the cluster expressing the specific transcript, while color intensity corresponds to its

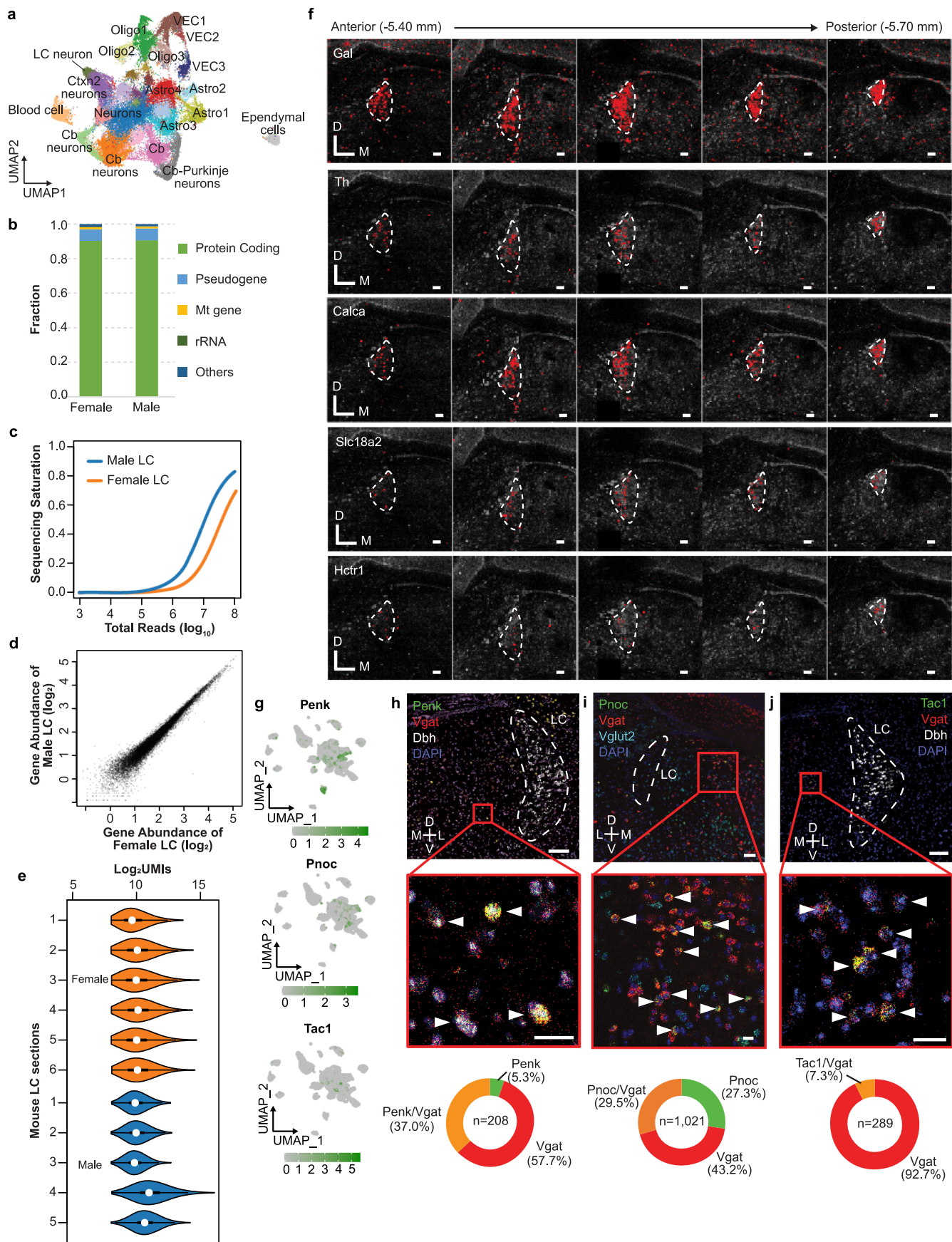
relative expression. (b) Dot plot of genes associated with neurotransmitter reuptake (left), hormone signaling (middle), and cannabinoid signaling (right) across LC<sup>NE</sup> neuron clusters. (c) Dot plot of genes associated with gap junctions across LC<sup>NE</sup> neuron clusters. (d) Dot plot of genes associated with morphology and adhesion functions and development across LC<sup>NE</sup> neuron clusters.



**Extended Data Fig. 6 | Neuromodulatory, signaling, and structural genes in single-cell RNA sequencing of peri-LC<sup>GABA</sup> neurons (n = 11,182).** (a) Cartoon of the sampled peri-LC inhibitory neurons (left). Dot plot of neuronal subtype (left), neuropeptide (middle), and receptor (right) genes across peri-LC<sup>GABA</sup> neuron clusters. Circle size corresponds to percent of cells in the cluster expressing the

specific transcript, while color intensity corresponds to its relative expression. (b) Dot plot of genes associated with neurotransmitter reuptake (left), hormone signaling (middle), and cannabinoid signaling (right) across peri-LC<sup>GABA</sup> neuron clusters. (c) Dot plot of genes associated with gap junctions, development, and pathology across peri-LC<sup>GABA</sup> neuron clusters.

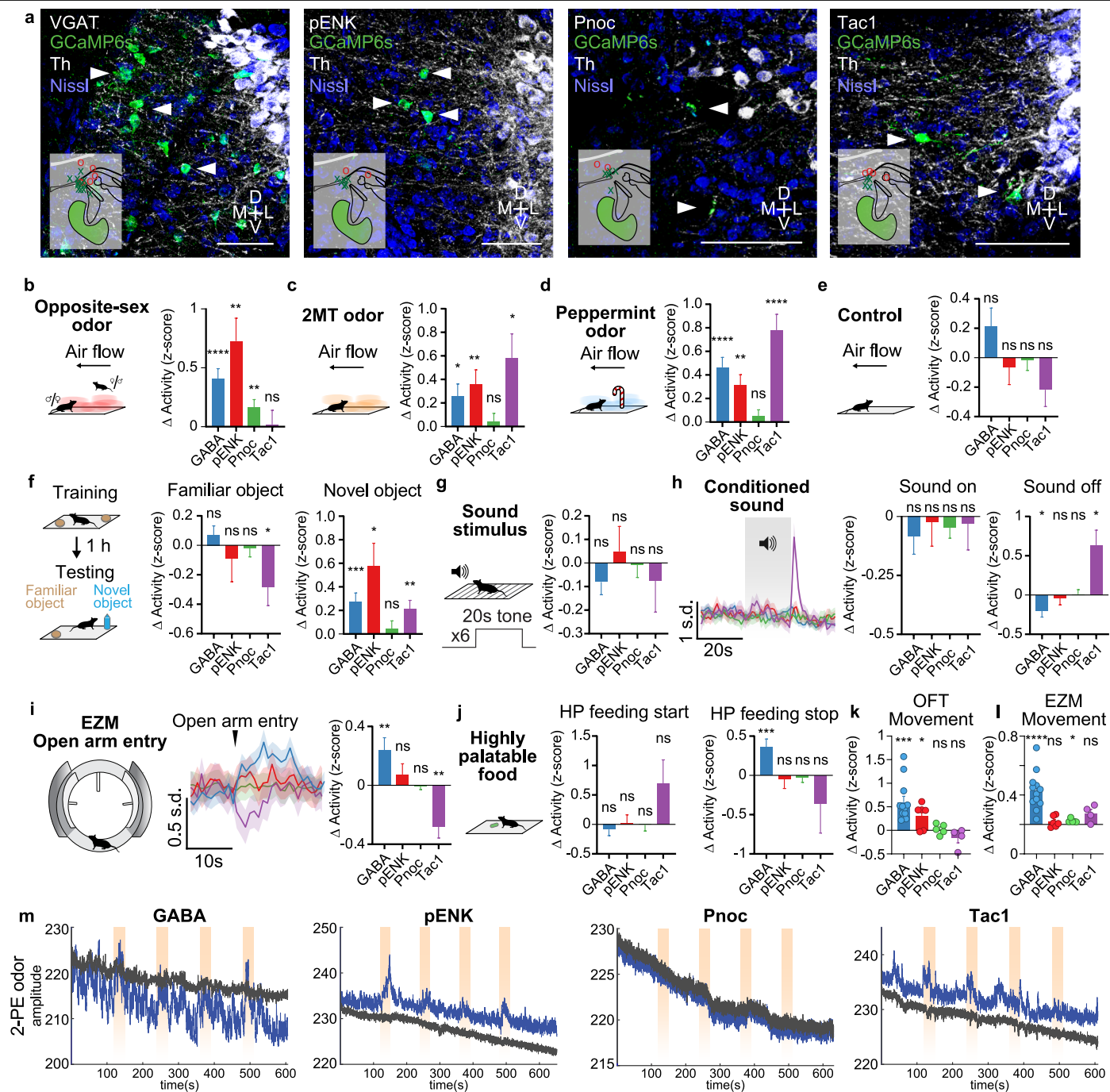




**Extended Data Fig. 7** | See next page for caption.

**Extended Data Fig. 7 | Pixel-seq quality control.** (a) Uniform Manifold Approximation and Projection (UMAP) embedding of ~59,000 cells passing quality control metrics, colored by clustering analysis. (b) Proportion of the RNA type in the brain slices obtained from male and female mice used for Pixel-seq. (c) Sequencing reads and saturation for 11 brain slices (6 female, 5 male) containing the LC use for Pixel-seq. (d) Comparison of transcripts found in brain slices of male and female mice used for Pixel-seq. (e) Unique molecular identifier (UMI) counts for the 11 brain slices used for Pixel-seq. The box plot parameters (min, Q1, median, Q3, max) for the slices are FLC1: 8.022, 9.043, 9.652, 10.396, 13.644; FLC2: 8.011, 9.301, 10.065, 10.857, 14.434; FLC3: 8.044, 9.219, 9.974, 10.805, 14.898; FLC4: 8.033, 9.342, 10.118, 10.92, 14.375; FLC5: 8.022, 9.257, 10.013, 10.777, 14.706; FLC6: 8.066, 9.342, 10.077, 10.883, 14.569;

MLC1: 8.05, 9.444, 9.892, 10.456, 12.66; MCL2: 8.017, 9.501, 9.964, 10.596, 12.761; MLC3: 8.017, 9.408, 9.843, 10.332, 12.704; MLC4: 8.066, 10.312, 10.983, 11.797, 16.096; MLC5: 8.011, 10.087, 10.641, 11.238, 14.207. (f) Spatial expressions of *Gal*, *Th*, *Calca*, *Slc18a2* and *Hctr1* in the LC region are shown along the anterior-posterior axis (Bregma -5.40 mm to -5.70 mm). Scale bars are 100  $\mu$ m. (g) Feature plots show the peri-LC inhibitory neuron clusters expressing *Penk* (top), *Pnoc* (middle), and *Tac1* (bottom). (h-j) Representative images for in situ hybridization of VGAT (*Slc32a1*) (h-j), *Dbh* (h,j), and VGLUT2 (*Slc17a6*) (i) with (h) *Penk*, (i) *Pnoc*, (j) *Tac1* mRNA. Scale bars are 100  $\mu$ m (top) and 20  $\mu$ m (bottom). The respective quantification of the relevant in situ cell populations within the peri-LC are shown in the bottom row.

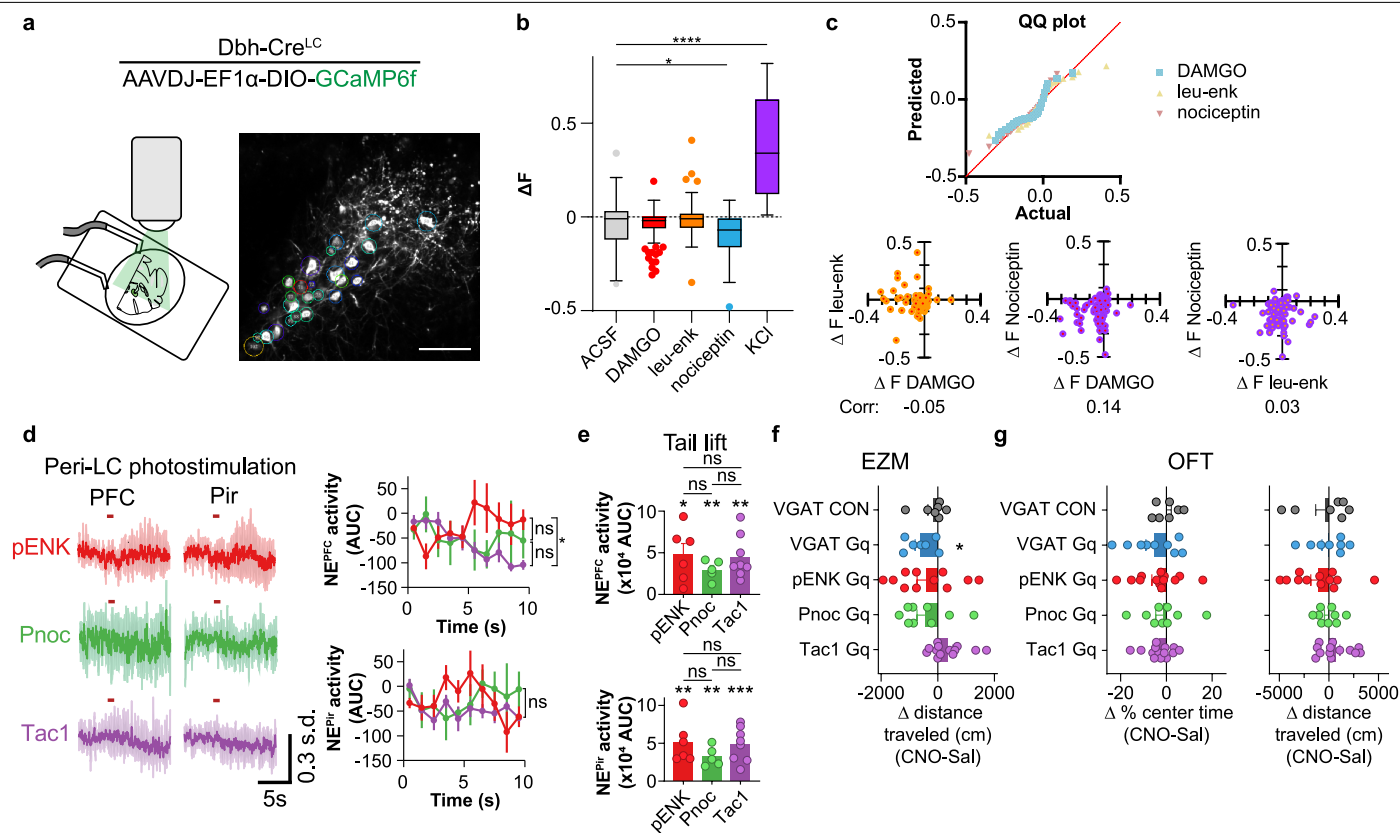


Extended Data Fig. 8 | See next page for caption.

**Extended Data Fig. 8 | Peri-LC neuronal subpopulations differentially respond to arousing and stress stimuli.** (a) Example images of GCaMP6s expression in peri-LC subpopulations. Insets show placements of fibers, with 'x' representing on-target fibers and expression and 'o' representing no or off-target expression. Scale bars are 50  $\mu$ m. (b) Peri-LC neurons are modulated by 30 s exposure to odor of opposite-sex mice ( $p < 0.0001$ ,  $n = 44$  exposures;  $p = 0.0010$ ,  $n = 24$ ;  $p = 0.0232$ ,  $n = 20$ ;  $p = 0.8766$ ,  $n = 16$ ). (c) Peri-LC neurons are modulated by 30 s exposure to 2MT ( $p = 0.0168$ ,  $n = 56$  exposures;  $p = 0.0067$ ,  $n = 24$ ;  $p = 0.5479$ ,  $n = 20$ ;  $p = 0.0122$ ,  $n = 16$ ). (d) Peri-LC neurons are modulated by 30 s exposure to peppermint oil odor ( $p < 0.0001$ ,  $n = 56$  exposures;  $p = 0.0020$ ,  $n = 24$ ;  $p = 0.3059$ ,  $n = 20$ ;  $p < 0.0001$ ,  $n = 16$ ). (e) Peri-LC neurons are not affected by 30 s of blowing air through chamber ( $p = 0.0912$ ,  $n = 44$  exposures;  $p = 0.5947$ ,  $n = 24$ ;  $p = 0.8010$ ,  $n = 20$ ;  $p = 0.0763$ ,  $n = 16$ ). (f) Peri-LC neurons are modulated by interactions with a novel object or a familiar object ( $p = 0.2539$ ,  $n = 141$  interactions, and  $p = 0.0002$ ,  $n = 133$ ;  $p = 0.5814$ ,  $n = 26$ , and  $p = 0.0106$ ,  $n = 16$ ;  $p = 0.7377$ ,  $n = 28$  and  $p = 0.5585$ ,  $n = 27$ ;  $p = 0.0404$ ,  $n = 22$  and  $p = 0.0075$ ,  $n = 22$ ). (g) Peri-LC neurons are unaffected by exposure to a tone that had not yet been paired with a shock ( $p = 0.1423$ ,  $n = 84$  exposures;  $p = 0.6458$ ,  $n = 36$ ;  $p = 0.8857$ ,  $n = 30$ ;  $p = 0.5828$ ,  $n = 24$ ). (h) Peri-LC neurons do not respond to a conditioned tone, but GABA neurons decrease activity and Tac1 neurons increase activity after the tone stops ( $p = 0.2682$  and  $p = 0.0166$ ,  $n = 84$  exposures;  $p = 0.8360$  and  $p = 0.6586$ ,  $n = 36$ ;  $p = 0.3400$  and  $p = 0.8482$ ,  $n = 30$ ;  $p = 0.7967$  and  $p = 0.0029$ ,  $n = 24$ ). (i) Peri-LC GABA neurons increase

activity and Tac1 neurons decrease activity when an animal enters the open arm of an elevated zero maze ( $p = 0.0059$ ,  $n = 81$  transitions;  $p = 0.3220$ ,  $n = 40$ ;  $p = 0.7189$ ,  $n = 54$ ;  $p = 0.0011$ ,  $n = 32$ ). (j) Peri-LC GABA neurons increase activity after they stop eating highly palatable food; no other conditions are affected ( $p = 0.4234$  and  $p = 0.0006$ ,  $n = 66$  bouts;  $p = 0.8965$  and  $p = 0.7484$ ,  $n = 31$ ;  $p = 0.9600$  and  $p = 0.6597$ ,  $n = 24$ ;  $p = 0.1080$  and  $p = 0.3578$ ,  $n = 14$ ). Bars represent change in activity before vs. after events. Periods of comparison were 30 s for odor experiments and air flow, 20 s for sound stimuli, 10 s for feeding, and 5 s for novel/familiar object interaction and maze exploration. (k) Activity of peri-LC neurons during movement in the open field test. Bars represent difference in activity during movement vs. non-movement for each trial.  $p = 0.0005$  ( $n = 12$  trials);  $p = 0.0348$  ( $n = 6$ );  $p = 0.6327$  ( $n = 5$ );  $p = 0.2310$  ( $n = 4$ ). (l) Activity of peri-LC neurons during movement in the elevated zero maze. Bars represent difference in activity during movement vs. non-movement for each trial.  $p < 0.0001$  ( $n = 12$  trials);  $p = 0.2752$  ( $n = 6$ );  $p = 0.0153$  ( $n = 5$ );  $p = 0.0776$  ( $n = 4$ ). (m) Representative signal in 470 nm (blue) and 405 nm (gray) channels for peri-LC GABA, pENK, Pnoc, and Tac1 recordings of 2-PE odor. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$  by paired two-tailed  $t$ -test;  $p$ -values and  $n$  given in order of GABA, pENK, Pnoc, and Tac1. Error bars and shading indicate SEM.  $N = 14$  animals for GABA photometry (11 for opposite-sex odor and air flow test), 6 for pENK, 5 for Pnoc, and 4 for Tac1. Panels b–g, i, j were adapted from ref. 19, AAAS.





**Extended Data Fig. 9 | Differential responses to peri-LC stimulation.** (a) Left, 2-photon setup for slice imaging and solution exchanges. Right, example brain slice with GCaMP6f-expressing LC<sup>NE</sup> neurons of Dbh-Cre mice. Scale bar is 100  $\mu$ m. (b) Box plots show fluorescent changes of GCaMP6f-expressing LC<sup>NE</sup> cells after perfusing with either ACSF (control), 1-4  $\mu$ M DAMGO, 1  $\mu$ M leu-enk, 1  $\mu$ M nociceptin, or 30 mM KCl.  $p = 0.0280$  (ACSF vs. nociceptin) and  $p < 0.0001$  (ACSF vs. KCl) by two-stage linear step-up procedure after one-way ANOVA ( $p < 0.0001$ ). Error bars indicate SEM. The box plot parameters (min, Q1, median, Q3, max) are ACSF: -0.36, -0.12, -0.01, 0.025, 0.34; DAMGO: -0.31, -0.06, -0.02, 0, 0.19; Leu-enk: -0.35, -0.0525, -0.01, 0.0125, 0.41; Nociceptin: -0.48, -0.16, -0.07, -0.01, 0.09; and KCl: 0.01, 0.1275, 0.34, 0.615, 0.82.  $n = 80$  neurons from 5 slices. (c) Top, QQ plot shows divergence from test normality of LC<sup>NE</sup> neuronal responses for combinations of peptides, with the Pearson's correlational coefficient shown on the bottom. (d) Left, NE activity measured by photometry traces of GRAB<sub>NEZm</sub> sensor, aligned to peri-LC photostimulation (30 Hz 500 ms every 20 s repeated 50 times), averaged across all events and across mice for

pENK-Cre ( $n = 6$ ), Pnoc-Cre ( $n = 5$ ), and Tac1-Cre ( $n = 8$ ) mice in the PFC (top) and Pir (bottom). Right, plots show 1-s binning of NE changes in response to photostimulation at time 0. pENK vs. Tac1:  $p = 0.031$  by two-tailed Spearman correlation. (e) Area under curve over 10 s of photometry recordings of NE activity (as measured by GRAB<sub>NEZm</sub>) in the PFC (top) and Pir (bottom) during tail lifts in the pENK-Cre ( $n = 6$ ), Pnoc-Cre ( $n = 5$ ), and Tac1-Cre ( $n = 8$ ) mice used for the peri-LC photostimulation experiments. PFC:  $p = 0.0152$  (pENK),  $p = 0.0047$  (Pnoc),  $p = 0.0017$  (Tac1); Pir:  $p = 0.0068$  (pENK),  $p = 0.0050$  (Pnoc),  $p = 0.0005$  (Tac1) by paired two-tailed t-test. (f) The difference in total distance traveled in the elevated zero maze (full 20 min of assay 30 min after saline or 1 mg/kg CNO treatment) for VGAT fluorophore control ( $n = 7$ ), VGAT Gq ( $n = 7$ ), pENK Gq ( $n = 10$ ), Pnoc Gq ( $n = 9$ ), and Tac1 Gq ( $n = 13$ ) mice. \* $p = 0.0411$  by Tukey's multiple comparisons test. (g) The difference in time spent in the center and total distance traveled in the open field test (full 20 min of assay 30 min after saline or 1 mg/kg CNO treatment) for VGAT fluorophore control ( $n = 7$ ), VGAT Gq ( $n = 7$ ), pENK Gq ( $n = 10$ ), Pnoc Gq ( $n = 9$ ), and Tac1 Gq ( $n = 13$ ) mice.



Extended Data Table 1 | Electrophysiological properties of recorded LC and peri-LC GABAergic neurons

|                                |                 |             |  |
|--------------------------------|-----------------|-------------|--|
| LC <sup>NE</sup> neurons       |                 |             |  |
| IPSC amplitude                 | 53.8 ± 7.3 pA   | n/N = 18/9  |  |
| Paired pulse ratio (50 ms IPI) | 1.13 ± 0.11     | n/N = 16/9  |  |
| Rise time                      | 3.15 ± 0.47 ms  | n/N = 18/9  |  |
| Width                          | 16.1 ± 1.7 ms   | n/N = 18/9  |  |
| Decay constant                 | 17.2 ± 1.2 ms   | n/N = 18/9  |  |
| Peri-LC GABA neurons           |                 |             |  |
| Capacitance                    | 27.5 ± 1.1 pF   | n/N = 24/12 |  |
| Membrane resistance            | 316.3 ± 29.8 mΩ | n/N = 23/12 |  |
| Resting membrane potential     | -53.5 ± 1.4 mV  | n/N = 13/7  |  |
| Basal firing rate              | 4.4 ± 1.5 Hz    | n/N = 11/7  |  |
| Firing rate +200 pA            | 23.3 ± 2.9 Hz   | n/N = 11/7  |  |
| Firing rate +400 pA            | 46.4 ± 4.7 Hz   | n/N = 11/7  |  |

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| <input type="checkbox"/>            | <input checked="" type="checkbox"/> A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons                                                                                                                                        |
| <input type="checkbox"/>            | <input checked="" type="checkbox"/> A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals) |
| <input type="checkbox"/>            | <input checked="" type="checkbox"/> For null hypothesis testing, the test statistic (e.g. <i>F</i> , <i>t</i> , <i>r</i> ) with confidence intervals, effect sizes, degrees of freedom and <i>P</i> value noted<br><i>Give P values as exact values whenever suitable.</i>                     |
| <input checked="" type="checkbox"/> | <input type="checkbox"/> For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings                                                                                                                                                                      |
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Software and code

Policy information about [availability of computer code](#)

|                 |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       |
|-----------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Data collection | The following software was used to collect data, as described in the methods: Olympus Fluoview FV3000, QuPath, Apograph X, Synapse v95, Ethovision 10, Spinnaker SDK, Med-PC IV v4.2.                                                                                                                                                                                                                                                                                                                                                                 |
| Data analysis   | The following software was used to analyze data, as described in the methods: ImageJ 1.52i, Arivis Vision4D (3.0.0), 10x Genomics Cell Ranger Count v6.1.2, Seurat v4.0, R 4.0.3, DoubletDecon 1.02, ChooseR 1.0, Flexbar 3.0, Bowtie 1.3.1, STARv2.7.0, SpatialPCA1.3.0, MATLAB R2021b, Ethovision 10, GraphPad Prism 9, Excel v2402, Suite2p v0.14.0, Jupyter Notebook 6.2.0, scvi-tools 1.0. Custom code used in snRNAseq and photometry experiments is available at <a href="https://github.com/Bruchas-Lab">https://github.com/Bruchas-Lab</a> . |

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Portfolio [guidelines for submitting code & software](#) for further information.

## Data

Policy information about [availability of data](#)

All manuscripts must include a [data availability statement](#). This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A description of any restrictions on data availability
- For clinical datasets or third party data, please ensure that the statement adheres to our [policy](#)

All data are available in the manuscript or the supplementary materials. snRNAseq data is deposited in the NCBI Gene Expression Omnibus (GEO) database with accession number GSE201998; sample GSM6086497 – LC\_FEMALE\_01 is included for the sake of completeness but it is poor quality and we do not recommend using it. PIXEL-seq data is deposited in GEO database with accession numbers GSE244659. Reference mouse transcriptome GRCm38 is available at [https://www.ncbi.nlm.nih.gov/datasets/genome/GCF\\_000001635.20/](https://www.ncbi.nlm.nih.gov/datasets/genome/GCF_000001635.20/). The full behavioral dataset has been uploaded as a supplement.

## Research involving human participants, their data, or biological material

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Recruitment

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Note that full information on the approval of the study protocol must also be provided in the manuscript.

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## Life sciences study design

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

**Sample size** For behavioral (optogenetics and DREADD), imaging/photometry, and electrophysiological experiments, sample sizes were determined based on power analyses indicating the minimum to achieve 99% power to detect a difference in means, accounting for losses in case of poor acclimation or other issues, in line with previous experience in the lab. The pPDH quantification experiments used samples from the behavioral cohort. Sample sizes for sequencing experiments were determined based on a goal of collecting 10,000 nuclei per sequenced sample, as recommended by our sequencing protocol (see, for example, Hashikawa et al., 2020, and 10x Next GEM sequencing guidelines) and standard within the field for studying a specific brain region (Armand et al., 2021). For PIXEL-seq experiments, we included as many samples as was necessary to capture the full extent of the peri-LC field, which exceeded the number of cells commonly used in spatial sequencing experiments (Moses and Pachter, 2022). For rabies tracing experiments, since rabies is primarily useful as a qualitative method to identify possible circuit inputs (Luo, Callaway, and Svoboda, 2018), we used the minimum number necessary to get starter cell expression. Because the goal of the in situ hybridization experiments was to validate overlap and spatial distribution between the GABA population and the neuropeptides, we used the minimum number necessary to qualitatively determine this, in accordance with NIH principles of animal use reduction.

**Data exclusions** As described in the manuscript, sample GSM6086497 was excluded from the snRNAseq dataset due to a loss of single-cell behavior, as identified by CellRanger software. Histology was used to validate placement of fibers and viral expression.

**Replication** Results were reproduced over an adequate number of samples to meet confidence intervals. These replications are reported in the results, and null results have also been reported. Experiments included male and female subjects in order to ensure robust and unbiased results. Experiments were designed to produce convergent evidence. Methods have been rigorously described to allow other researchers to verify these results, and relevant data has been made available. Independent experiments were performed for each animal in each experiment, except for snRNAseq, in which 5–6 animals were pooled for each sample; 4 independent samples were run. Information on representative images is available in "Statistics and Reproducibility" section of manuscript. All attempts at replication were successful.

**Randomization** Sequencing, descriptive electrophysiology, and observations of cell/transcript localization are provided as observational data, and since they

## Randomization

are not comparative, they do not require randomization - there is no treatment to randomize across. In comparative electrophysiology, fiber photometry, single-cell imaging, and experiments where optogenetic/DREADD comparisons are made to non-stimulation/saline conditions, the statistical comparisons are made between treatment conditions or between recordings of the same population before and after an event or treatment, and this within-subject comparison is used to control for covariation. In experiments where different viruses were injected into Cre+ animals, mice were randomly assigned to experimental groups before any behavioral tests were undertaken. In experiments where comparisons were made between groups based on genotypes (wherein different Cre driver lines were compared to each other or to wild-type controls), randomization into groups was not possible. Covariation was controlled by counter-balancing experiment order, matching housing conditions and experimental timing, and reporting other relevant behavioral metrics (such as overall movement) that might affect the experimental interpretation. In fiber photometry experiments, activity was z-scored to reduce effects of intrinsic variability in signal strength. Additionally, all Cre mice were backcrossed into the same C57BL/6J mouse line that was used for wild-type controls, limiting genetic variability.

## Blinding

For observational and descriptive studies (sequencing, electrophysiology, cell/transcript location), there was no condition to blind across. Comparisons between male and female sequencing data were completely automated. In comparative electrophysiology, fiber photometry, and single-cell imaging, comparisons were made to before and after a defined time point (the wash-on of a compound or an automated behavioral stimulus), and human subjectivity was not involved in signal collection or processing. For optogenetic and chemogenetic measurements of behavior, experimenters were blinded to animal condition while running the assays. In optogenetic, chemogenetic, and free-behavior fiber photometry experiments, automated analyses were used to track behavior.

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### Methods

- |                                     |                                                 |
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| n/a                                 | Involved in the study                           |
| <input checked="" type="checkbox"/> | <input type="checkbox"/> ChIP-seq               |
| <input checked="" type="checkbox"/> | <input type="checkbox"/> Flow cytometry         |
| <input checked="" type="checkbox"/> | <input type="checkbox"/> MRI-based neuroimaging |

## Antibodies

## Antibodies used

Rabbit anti-phospho-pyruvate dehydrogenase (Cell Signaling Technology 37115S Lot 3); Chicken anti-tyrosine hydroxylase (Aves TYH Lot TYH88967984), Chicken anti-GFP (Aves GFP-1020), mouse anti-tyrosine hydroxylase (BioLegend 818001), goat anti-chicken IgG Alexa Fluor 647 (Invitrogen A21449 Lot 2186435), goat anti-mouse Alexa Fluor 488 (Life Technologies A11001 Lot 1397999), goat anti-chicken AlexaFluor 633 (Invitrogen A21103 Lot 2015314), goat anti-rabbit 647 (Invitrogen A27040 Lot 2477087), Goat anti-chicken AlexaFluor 488 (Invitrogen A11039 Lot 1812246).

## Validation

Each antibody used has been certified by the manufacturer for specificity, and each has been used in the same application and species (mouse immunohistochemistry) in previous studies (for example, CST 37115S - Yang et al. 2024; Aves TYH - Seo et al. 2021; Aves GFP - Erickson and Motta et al. 2024; BioLegend 818001 - Marfull-Oromi et al. 2021; Invitrogen A21449 - Ambrosi and Lerner 2022; Life Technologies A11001 - Rajani et al. 2024; Invitrogen A21103 - Sandhu et al. 2018; Invitrogen A27040 - Campelo et al. 2020; Invitrogen A11039 - Chung et al. 2024). Further validation of specificity is provided by low levels of background in the desired channel as well as restricted expression to cells of interest, which is apparent in the images provided in the manuscript.

## Animals and other research organisms

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## Laboratory animals

This study used adult (8-32 week old, 18-35g) Mus musculus on a C57BL6/J background. Strains included wild-type C57BL/6J (Jackson Laboratory #000664), vGAT-IRES-Cre (Slc32a1tm2(cre)Low/J; Jackson Laboratory #028862), pENK-IRES-Cre (B6;129S-Penktm2(cre)Hze/J; Jackson Laboratory #025112), Tac1-IRES-Cre (B6;129S-Tac1tm1.1(cre)Hze/J; Jackson Laboratory #021877) and prepronociceptin (Pnoc)-IRES-Cre (Pnocm1.1(cre)Mrbr/J; Jackson Laboratory #034278). Mice were group housed, given access to food and water ad libitum, and maintained on a 12:12 hr reverse light:dark cycle with temperature between 18-23 degrees Celsius and 30-70% humidity.

## Wild animals

None

## Reporting on sex

Behavior and physiology experiments were performed in male and female mice, identified by visual inspection at weaning (17-21 days). Genetic data (Fig. 3, Fig. 4, Extended Data Fig. 3-7) was collected with separate male and female samples; the raw data from these samples has been despoited in disaggregated files as described in the manuscript (GSE201998 and GSE244659). The quality

and composition of these samples was similar (Fig. 3f-g, Extended Data Fig. 7b-e). Differentially-expressed genes between males and females is available in Supplementary Table S3. All studies were performed with both male and female mice, except as listed here. We did not detect differences between male and female mice, and therefore have presented all data as aggregated, except as described above. Total numbers included in the study: 12 male and 5 female mice (electrophysiology), 4 male and 6 female (Chr2+), 5 male and 5 female (Chr2 control), 1 female (rabies tracing), 4 male and 5 female (VGAT Gi DREADD+), 4 male and 5 female (VGAT Gi DREADD control), 11 male and 5 female (snRNAseq), 1 male and 1 female (PIXEL-seq), 8 male and 6 female (VGAT GCaMP photometry), 3 male and 3 female (pENK GCaMP photometry), 3 male and 2 female (Pnoc GCaMP photometry), 1 male and 3 female (Tac1 GCaMP photometry), 2 female (slice imaging), 4 male and 2 female (pENK GRAB-NE photometry), 2 male and 3 female (Pnoc GRAB-NE photometry), 2 male and 6 female (Tac1 GRAB-NE photometry), 4 male and 1 female (VGAT Gq DREADD control), 5 male and 4 female (VGAT Gq DREADD), 9 male and 5 female (pENK Gq DREADD), 4 male and 5 female (Pnoc Gq DREADD), and 7 male and 7 female (Tac1 Gq DREADD).

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| Field-collected samples | None                                                                                                                                                                                      |
| Ethics oversight        | All procedures were approved by the Animal Care and Use Committee of Washington University and the University of Washington and conformed to US National Institutes of Health guidelines. |

Note that full information on the approval of the study protocol must also be provided in the manuscript.

## Plants

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|-----------------------|------------------------------------|
| Seed stocks           | This study did not involve plants. |
| Novel plant genotypes | This study did not involve plants. |
| Authentication        | This study did not involve plants. |