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A septo-hypothalamic-medullary circuit directs stress-induced analgesia

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Abstract

Stress is a potent modulator of pain. Specifically, acute stress due to physical restraint induces stress-induced analgesia (SIA). However, where and how acute stress and pain pathways interface in the brain are poorly understood. Here, we describe how the dorsal lateral septum (dLS), a forebrain limbic nucleus, facilitates SIA through its downstream targets in the lateral hypothalamic area (LHA) of mice. Taking advantage of transsynaptic viral-genetic, optogenetic, and chemogenetic techniques, we show that the dLS→LHA circuitry is sufficient to drive analgesia and is required for SIA. Further, our results reveal that the dLS→LHA pathway is opioid-dependent and modulates pain through the pro-nociceptive neurons in the rostral ventromedial medulla (RVM). Remarkably, we found that the inhibitory dLS neurons are recruited specifically when the mice struggle to escape under restraint and, in turn, inhibit excitatory LHA neurons. As a result, the RVM neurons downstream of LHA are disengaged, thus suppressing nociception. Together, we delineate a poly-synaptic pathway that can transform escape behavior in mice under restraint to acute stress into analgesia.

eLife Assessment

This **important** work explores the modulation of pain by intense stress. The authors employed a series of cutting-edge techniques and provided **convincing** evidence suggesting that the dorsal lateral septum-> lateral hypothalamus-> rostral ventromedial medulla circuit is responsible for mediating stress-induced analgesia. This work will be of interest to neuroscientists interested in the neural circuits of behavior, and scientists interested in stress or pain.

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Introduction

Descending modulation of pain is the mechanism through which the brain imparts control over somatosensory information processing in the spinal cord. Forebrain and midbrain regions encoding internal states such as stress and hunger can harness the brainstem circuits that project to the spinal cord neurons to alleviate pain (Tracey and Mantyh, 2007). Such analgesic

mechanisms are critical for survival since they prepare animals and humans to cope with the stressors or enable them to meet their physiological needs on time. Moreover, harnessing the underlying modulatory mechanisms may lead us to novel therapeutic approaches for chronic pain. Stress Induced Analgesia (SIA) is one such incidence where an acute stressor, such as physical restraint, can result in analgesia. However, the neural mechanisms that facilitate SIA remain poorly understood.

Experiments involving human subjects in the 1950s and 1960s have shown that direct electrical stimulation of the dorsal lateral septum (dLS) has anti-nociceptive (Gol, 1967; Olds and Milner, 1954) effects. In rats, dLS activation suppressed behavioral responses to foot-shocks (Vincent Breglio et al., 1970) or sustained noxious stimuli such as intraplantar formalin injections (Abbott and Melzack, 1978). Further, septum inactivation or lesion renders rats hypersensitive to sensory stimuli, and as a result, they display exaggerated stimulus-driven defensive responses (Albert and Wong, 1978; Köhler, 1976). dLS neurons receive nociceptive inputs from the thalamus and somatosensory cortices, as well as anxiogenic information from the hypothalamus (Chen et al. 2021; Menon et al. 2022). Neurotensin expressing dLS neurons with projections to the lateral hypothalamic area (LHA) mediate acute stress mediated suppression of food consumption (Azevedo et al., 2020). Despite these convincing leads indicating the involvement of dLS neurons in the processing of noxious somatosensory stimuli, mechanistic investigation of the role of dLS in nociception remains scarce. Classical studies involving lesions to lateral parts of the septal nucleus resulted in a phenomenon known as septal rage, which leads to heightened defensive responses against non-threatening stimuli, indicative of increased levels of stress and anxiety (Brady and Nauta, 1953); (Brady and Nauta, 1955). Interestingly, dLS is essential to sensing acute stress and instrumental in stress-induced fear and anxiety (Anthony et al., 2014; Rizzi-Wise and Wang, 2021; Sheehan et al., 2004); (Terrill et al., 2018). This involvement of dLS in acute stress-induced anxiety is reflected in behavioral phenotypes and the elevated blood corticosterone levels (Singewald et al., 2011). Taken together, dLS neurons were shown independently to mediate the effects of both nocifensive behaviors and acute stress. However, the role of dLS neurons in pain modulation in the event of an ongoing stressful stimulus remains unexplored. Hence, we hypothesized that dLS might play a facilitatory role in SIA.

The dLS neurons communicate primarily through their inhibitory neurotransmitters and have prominent projections to brain areas involved in nociception and pain processing (Rizzi-Wise and Wang, 2021). Of note are the projections to the lateral habenula (LHb) and the LHA (Dafny et al., 1996; Shelton et al., 2012). The midbrain LHb can modulate the affective aspects of pain via its projections to the brainstem periaqueductal gray (PAG) and dorsal raphe (Shelton et al., 2012). LHA neurons have strong connections to the pain-modulatory nuclei in the brainstem, such as the rostral ventromedial medulla (RVM) and the lateral parabrachial nuclei (LPBN). In addition, recent studies have reported that LHA neurons can sense noxious stimuli and suppress pain (Siemian et al., 2021; Carr and Uysal, 1985), and specifically, the projections to the RVM were shown to have anti-nociceptive effects (Behbehani et al., 1988; Dafny et al., 1996; Franco and Prado, 1996; Fuchs and Melzack, 1995; Holden and Pizzi, 2008). Notably, LPBN neurons are one of the primary targets of the ascending nociceptive projection neurons from the dorsal horn of the spinal cord (Todd, 2010), and are instrumental in determining nociceptive thresholds (Chiang et al., 2020) and mediating nocifensive behaviors (Barik et al., 2018; Arthurs et al., 2023; Han et al., 2015). Thus, we reasoned that the dLS→LHA circuitry, through downstream circuits, may mediate SIA.

Here, we have explored the mechanisms through which acute restraint stress (RS)-responsive dLS neurons recruit downstream circuits to provide pain relief. Taking advantage of the anterograde trans-synaptic and retrogradely transporting viral genetic tools for anatomic circuit mapping, we have traced a pathway that originates in the dLS, and routes through LHA while finally terminating onto the spinally-projecting RVM neurons. Optogenetic and chemogenetic manipulations of the activity of each node of this pathway informed us on how they play

interdependent roles in transforming restrain-induced stress into the suppression of acute thermal pain. Fiber photometry recordings revealed how the inhibitory dLS neurons may suppress the excitatory LHA neurons upon stress, which can disengage pro-nociceptive RVM cells, resulting in analgesia. Taken together, our data propose a mechanism that can explain how RS can suppress pain and lead to understanding how the circuitries dedicated to detecting stress can interface with the ones built for modulating pain.

Results

dLS neurons drive acute stress and SIA

The dLS neurons have been known to be engaged by stressful stimuli such as physical restraint (Azevedo et al., 2020). To confirm this, we compared the expression of c-Fos, a molecular proxy for neural activation, in the dLS of control mice with the mice subjected to the RS assay (see methods). Mice that underwent RS showed increased cFos expression in the dLS as compared to the control unstressed mice (**Figures 1A-C**, **Figure 1-figure supplement 1A**). This suggests that dLS neurons may be involved in mediating acute stress, in agreement with previous studies (Azevedo et al., 2020; Kubo et al., 2002). Notably, internal states, such as stress, can determine nociceptive thresholds (Amit and Galina, 1986; Butler and Finn, 2009). Despite extensive investigation into the dLS's role in stress, not much is known about the involvement of these neurons in pain modulation. Thus, we tested if artificial stimulation of the dLS neurons in CD1 strain of mice can affect the animal's latency to response on the tail-flick test and nocifensive behaviors such as licks on the hot-plate test. Tail-flick and hot-plate tests can reveal the involvement of spinal and supraspinal circuitries in behavioral responses to noxious thermal stimuli, respectively. Since most of the dLS neurons are inhibitory (**Figure 1D**), we expressed the Gq-coupled excitatory Designer Receptors Exclusively Activated by Designer Drugs (DREADD) hM3Dq (Alexander et al., 2009) under the inhibitory neuron-specific *Gad1* promoter ($dLS^{Gad1\text{-}hM3Dq\text{-}mCherry}$) (**Figures 1E**, **1F**). Upon intraperitoneal (i.p.) administration of the ligands CNO or DCZ, hM3Dq enables neuronal firing. When dLS neurons were activated by administration of i.p. DCZ (Alexander et al., 2009; Nagai et al., 2020), the mice exhibited stress and stress-induced anxiety-like behaviors as confirmed by blood corticosterone levels (**Figure 1-figure supplement 1B**), the light-dark box test (**Figure 1-figure supplement 1C, D**), and the open field test (**Figure 1-figure supplement 1E, F**). Nocifensive behaviors like licks on the hotplate were suppressed post-DCZ administration in $dLS^{Gad1\text{-}hM3Dq\text{-}mCherry}$ mice, with no changes in the jumping behavior (**Figure 1-figure supplement 1H, I**). The tail flick latency in these mice was significantly increased, number of licks on the hotplate reduced and the latency to lick initiation increased (**Figures 1H, I**). Mice with activated dLS neurons showed higher thresholds for mechanical pain (**Figure 1-figure supplement 1G**). Similar changes in mice behavior were observed when these tests were combined with optogenetic activation of the dLS neurons ($dLS^{Gad1\text{-}ChR2\text{-}GFP}$) (**Figure 1-figure supplement 1K-M**). Further, it was seen that the analgesic effects of dLS neurons lasted for up to 30 minutes post optogenetic activation (**Figure 1-figure supplement 2A**), and this timeline was like the analgesic effects induced by RS (**Figure 1-figure supplement 2B**). Concluding, that the RS induced thermal analgesia and pain-modulation attained by artificial activation of dLS neurons are comparable. This phenomenon, known as SIA, has previously not been associated with the septal neurons. Further, to investigate whether these dLS neurons are necessary for bringing about SIA, we blocked the spike-evoked neurotransmitter release of dLS neurons by expressing the tetanus toxin light chain protein fused with GFP (TetTox-GFP) ($dLS^{Gad1\text{-}TetTox\text{-}GFP}$) (Campos et al., 2018; Xu et al., 2012) (**Figures 1I**, **1J**). The efficacy of the tetanus toxin virus was confirmed by testing its effect on mouse behavior in pairing with the excitatory DREADD hM3Dq (**Figure 1-figure supplement 2C, D**). Post-silencing of the dLS neurons, we observed that the stress caused by RS was unable to cause analgesia that was

previously seen on both the tail flick assay and the hotplate test (**Figures 1K**, **1L**). Again, no difference was seen in the jumping behavior of the mice (**Figure 1-figure supplement 2E-G**). Concluding, that the dLS neurons are both sufficient and necessary for SIA.

Next, we probed if the dLS neurons are engaged by RS and/ or noxious stimuli. To that end, we expressed the genetically encoded calcium sensor GCaMP6s (Chen et al., 2013) in the dLS neurons ($dLS^{Gad1\text{-}GCaMP6s}$) (**Figures 2A**, **2B**), and performed fiber photometry recordings (**Figure 2C**). Fiber photometry enables activity monitoring from the genetically and anatomically defined neuronal population in behaving mice and can provide critical insights into how neural activity corresponds to animal behavior in response to a particular stimulus (Lerner et al., 2015). As expected, we observed spontaneous transients in the dLS neurons of mice expressing GCaMP6s but not in the neurons with GFP (**Figure 2D**). Notably, in mice under RS, $dLS^{Gad1\text{-}GCaMP6s}$ neurons were active only when the animals in the RS assay apparatus (see methods) struggled and not when they were freely moving in their home cages (**Figures 2E**, **2F**, **2G**, S3, **Figure 2-figure supplement 1B**). This suggests that the increased neural activity during the struggle bouts in mice under RS is not simply due to the increased physical activity but due to the need and inability to escape the restraint (**Figure 2-figure supplement 1A, B**). Similarly, we restrained the mice acutely by hand, and $dLS^{Gad1\text{-}GCaMP6s}$ neurons were active during the initial immobilization phase while the mice struggled, after which the activity was reduced when they were unable to move due to the restraint (**Figure 2-figure supplement 1C, D**). The neurons were active again when the mice were released from the hand restraint and were free to run away (**Figure 2-figure supplement 1C, D**). Thus, in agreement with the previous result, the $dLS^{Gad1\text{-}GCaMP6s}$ neurons were active when the mice actively struggled to escape any physical restraint. Another acute stress assay that engaged the $dLS^{Gad1\text{-}GCaMP6s}$ neurons was the tail hanging assay, wherein mice were suspended in air by holding their tails for 10 seconds (**Figure 2-figure supplement 1E, F**). Since artificial stimulation or blocking dLS^{Gad1} neuronal activity had suppressive effects on pain thresholds, we next tested if the $dLS^{Gad1\text{-}GCaMP6s}$ neurons respond to noxious thermal stimuli (52 degrees for one minute on the hot plate). The $dLS^{Gad1\text{-}GCaMP6s}$ neurons were found to be inactive during the hot-plate test at innocuous (**Figures 2H**, **2I**), as well as at a noxious temperature when the mice exhibited nocifensive behaviors such as licks and shakes (**Figures 2J**, **2K**). Thus, from our fiber photometry data, we concluded that the $dLS^{Gad1\text{-}GCaMP6s}$ neurons are preferentially tuned to stress caused by physical restraint and not to noxious thermal stimuli.

dLS neurons facilitate SIA through downstream LHA neurons

In the following experiments, we sought to determine the postsynaptic targets through which dLS neurons signal and facilitate RS-induced analgesia. To address this, we mapped the axonal targets of dLSGad1 neurons by labeling these neurons with cell-filing GFP ($dLS^{Gad1\text{-}GFP}$) (**Figures 3A**, **3B**). In agreement with previous reports, we observed $dLS^{Gad1\text{-}GFP}$ axon terminals in the LHA, the habenula (Hb), and the hippocampus (**Figures 3C**) (Amit and Galina, 1986; Kubo et al., 2002). To confirm that the axonal terminals of the dLS neurons at the targets form synapses with the neurons in the LHA, Hb, and hippocampal formation we labeled the axon terminals of dLS^{Gad1} neurons with synaptophysin fused with the red fluorescent protein ruby ($dLS^{Gad1\text{-}SynRuby}$) (**Figure 3-figure supplement 1A**) and found SynRuby puncta in all aforementioned dLS^{Gad1} axonal targets (**Figure 3-figure supplement 1B**), suggesting that dLS neurons synapse onto its downstream LHA (**Figure 3-figure supplement 1C**), Hb, and hippocampal neurons. We hypothesized that the dLS neurons may exert their analgesic effects through downstream LHA and/ or Hb connections for following reasons: firstly, both LHA and Hb neurons have been implicated in determining nociceptive thresholds (Singewald et al., 2011); (Butler and Finn, 2009); second, LHA and Hb have direct connections to pain modulatory regions in the brain stem such as the PAG, the RVM, and the dorsal raphe nucleus (DRN) (Singewald et al., 2011); (Ma

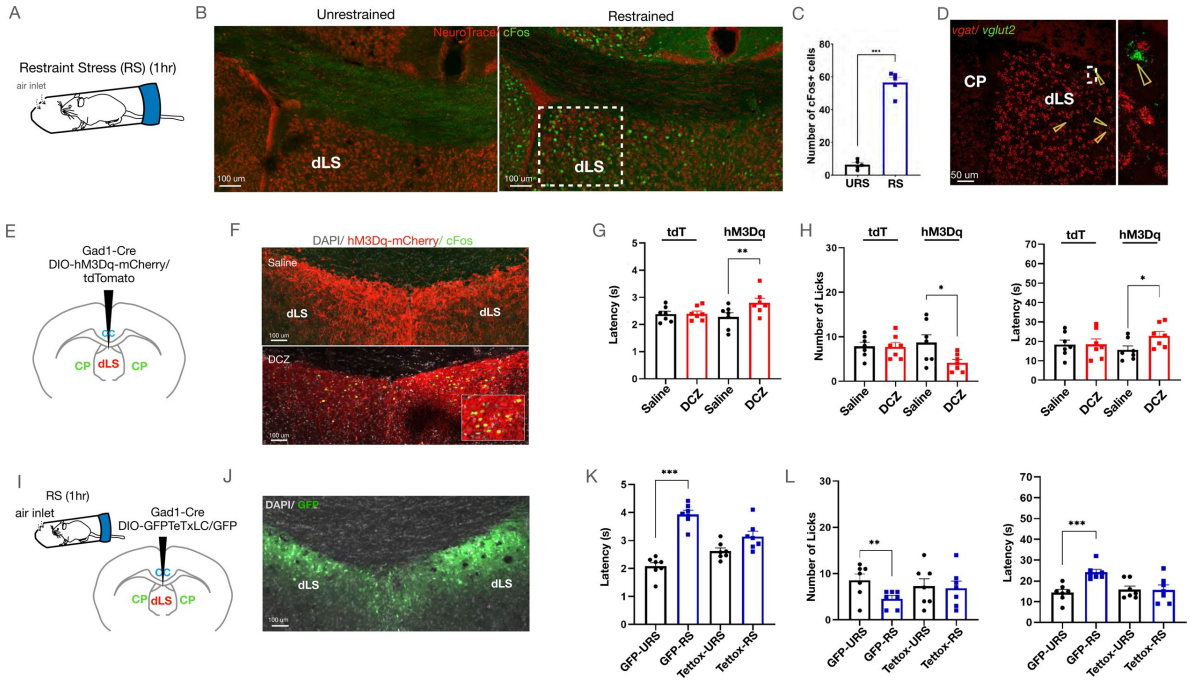


Figure 1.

dLS neurons are both sufficient and necessary for causing acute restraint-induced analgesia.

(A) Schematic representing the Restraint Stress (RS) assay used to induce stress in mice. (B) cFos expression in the dLS of restrained mice compared to unrestrained control mice (Green-cFos, Red-neurotrace (nuclear dye specific to neuronal nuclei)). (C) Total number of cFos+ cells in the dLS (area marked by dotted lines) in restrained vs unrestrained mice (6.40±1.21 compared to 56.40±3.09, respectively; t-test, ***P=0.0001, n=5). (D) Multiplex In Situ hybridization with *vglut2* and *vgat* (red-*vgat*, green-*vglut2*), highlighted by arrowheads (left), with zoom-in view of a rare *vglut2*+ (green) cell (right). (E) Gad1-Cre and DIO-hM3Dq-mCherry/DIO-tdTomato were injected in the dLS of wild-type mice. (F) i.p. DCZ and not Saline evoked cFos expression (green) in the mCherry-positive cells (red) in the dLS$^{Gad1\text{-}hM3Dq}$ neurons. (G) Tail-flick latency (seconds) (2.28±0.16 compared to 2.8±0.16, respectively; t-test, **P=0.0056, n=7) post-saline or DCZ administration in dLS$^{Gad1\text{-}hM3Dq}$ mice, with no significant difference in dLS$^{Gad1\text{-}tdTomato}$ mice. (H) Licks (8.71±1.76 compared to 4.14±0.74, respectively; t-test, *P=0.0335, n=7), and latency to lick (seconds) (15.57±2.03 compared to 22.86±2.26, respectively; t-test, *P=0.0338, n=7) on the hot-plate test of dLS$^{Gad1\text{-}tdTomato}$ mice, administered with either i.p. Saline or DCZ, with no significant difference in dLS$^{Gad67\text{-}tdTomato}$ mice. (I) Gad1-Cre and DIO-Tettox-GFP or DIO-GFP were co-injected in the dLS of wild-type mice. (J) GFP-positive neurons (green) seen in the dLS. (K) Tail-flick latency (seconds) (2.08±0.14 compared to 3.9±0.15, respectively; t-test, ***P=0.0001, n=8) with and without restraint for 1 hour using the RS assay in dLS$^{Gad1\text{-}GFP}$ mice, with no significant difference in dLS$^{Gad1\text{-}Tettox\text{-}GFP}$ mice. (L) Number of licks (8.57±1.34 compared to 4.57±0.69, respectively; t-test, **P=0.0089, n=7), and latency to lick (seconds) (14.29±1.54 compared to 24.14±1.40, respectively; t-test, ***P=0.0001, n=7) with and without restraint in dLS$^{Gad1\text{-}GFP}$ mice, with no significant difference in dLS$^{Gad1\text{-}Tettox\text{-}GFP}$ mice.

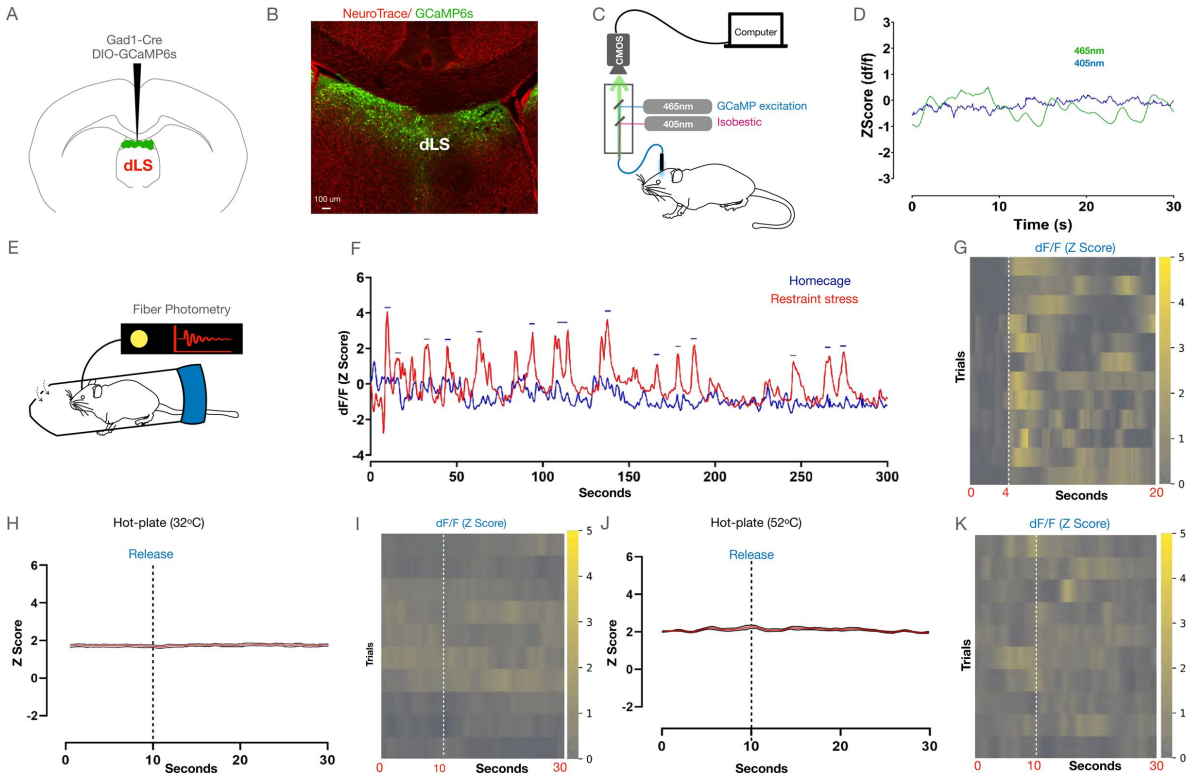


Figure 2.

dLS neurons are engaged by acute stress due to physical restraint.

(A) AAVs encoding Gad1-Cre and DIO-GCaMP6s were co-injected in the dLS, and fiber optic cannulaes were implanted above for recording neural activity. (B) Confirmation of the expression of GCaMP6s (Green) in the dLS neurons. (C) Schematic representation of the fiber photometry system. (D) Representative trace or neural activity from GCaMP6s (green) vs control GFP (blue) mice in the homecage. (E) Neural activity from the dLS while mice were under restraint. (F) Traces of neural activity when mice were allowed to move freely in the homecage (blue), and when they were under restraint (red). Peaks corresponding to neural activity (blue dashes) were seen when mice struggled in the tube. (G) Heatmap depicting neural activity during individual instances of struggles (initiation of struggle indicated by white dotted line). (H and I) Average plots and heatmaps for dLS responses on the hot plate at 32 deg (5 mice, 20 trials; dotted line indicating time point when mice were placed on the hotplate). (J and K) Average plots and heatmaps for dLS engagement on the hot plate at a noxious temperature of 52 deg (5 mice, 20 trials; dotted line indicating time point when mice were placed on the hotplate).

et al., 1992); third, LHA and Hb contain pro-nociceptive neurons, inhibition of which can cause analgesia (Benabid and Jeaugey, 1989; Mahieux and Benabid, 1987); and fourth, the role of hippocampal neurons in pain modulation is less clear.

To test if dLS modulates nociceptive thresholds through its projections to the LHA, we selectively stimulated the axon terminals of the dLSGad1 neurons in the LHA by expressing ChR2 in the dLS ($dLS^{Gad1\text{-}ChR2}$) and shining blue light on the terminals at LHA through fiber optic cannulae (**Figure 3D**). Selective activation of the $dLS^{Gad1\text{-}ChR2}$ terminals in the LHA resulted in increased latency on the tail-flick assay (**Figure 3E**) and a reduced number of licks with an increased threshold on the hot-plate assay (**Figure 3F**), suggestive of analgesia. These observations were like the results from the experiments where the cell bodies of $dLS^{Gad1\text{-}ChR2}$ neurons were stimulated (**Figure 1-figure supplement 1I-L**). Taken together, these results suggest that stimulating dLS^{Gad1} cell bodies or their axon terminals in the LHA is sufficient to cause analgesia. In complementary experiments, we chemogenetically activated dLS^{Gad1} terminals in the LHA. To that end, we devised a novel microparticle (MP)-based delivery system (Jain, 2000; Sharma et al., 2022) for CNO (for the MP-based delivery system, hM3Dq agonist CNO was used due to greater hydrophobicity, which is essential for MP packaging, compared to DCZ), which can be injected specifically in the LHA where dLS neurons terminate (**Figure 3-figure supplement 2A-C**). Compared to the widely-used method for in-vivo drug delivery at deep brain nuclei through cannulae (Campbell and Marchant, 2018; Stachniak et al., 2014), our poly-DL-lactic-co-glycolic (PLGA) microparticle (MP-CNO) based system (see methods) can serve as a stable, cost-effective, non-invasive, site-specific, and sustained method for CNO delivery. To demonstrate the efficacy of the MP-CNO, we stereotaxically injected MP-CNO in the dLS of $dLS^{Gad1\text{-}hM3Dq}$ and recorded cFos expression in the dLS to confirm that the beads were able to activate the neurons. We observed peak cFos expression at Day 4 (**Figure 3-figure supplement 2D**), which is in agreement with our observations of analgesic behavior in the mice. In the $dLS^{Gad1\text{-}hM3Dq}$ mice, MP-CNO was analgesic, and the effects were pronounced between 48-96 hours of delivery (**Figure 3-figure supplement 2E, F**). Remarkably, the MP-CNO-induced analgesia was comparable to that of i.p. CNO administration (**Figure 3-figure supplement 2H, I**). The control mice ($dLS^{Gad1\text{-}tdTomato}$) did not demonstrate any difference in behavior over days (**Figure 3-figure supplement 2G-I**). Finally, we delivered MP-CNO in the LHA of $dLS^{Gad1\text{-}hM3Dq}$ mice for terminal activation and observed increased tail-flick thresholds, fewer licks, and higher lick thresholds (**Figure 3-figure supplement 2J-L**). Thus, our acute optogenetic and chronic chemogenetic axon terminal activation experiments demonstrate that dLS neurons could bring about analgesia through their projections to the LHA.

Next, we chemogenetically stimulated the dLS neurons whose axon terminals arborize and synapse onto the LHA neurons ($dLS_{pre\text{-}LHA}$). To that end, we injected retrogradely transporting AAV (AAVRetro-Cre) in the LHA and DIO-hM3Dq-mCherry in the dLS. This intersectional genetic strategy facilitated excitatory DREADD expression exclusively in $dLS_{pre\text{-}LHA}$ neurons ($dLS_{pre\text{-}LHA\text{-}hM3Dq\text{-}mCherry}$) (**Figure 3G**). i.p administration of DCZ in the $dLS_{\ pre\text{-}LHA\text{-}hM3Dq\text{-}mCherry}$ mice had similar analgesic effects as seen with dLSGad1activation (**Figures 3H**, **3I**), indicating that activation of a specific subset of dLS neurons ($dLS_{pre\text{-}LHA}$) is sufficient for altering thermal nociception in mice. In the following experiments, we asked what the effect of chemogenetic activation of the LHA neurons downstream of dLS ($LHA_{post\text{-}dLS}$) on thermal nociceptive thresholds would be. To address this, we chemogenetically activated $LHA_{post\text{-}dLS}$ neurons by injecting AAVTrans-Cre in the dLS and AAV-DIO-hM3Dq-mCherry in the LHA (**Figure 3J**). I.p. administration of DCZ in mice expressing hM3Dq in $LHA_{post\text{-}dLS}$ neurons reduced the latency to react on the tail-flick assay, increased the number of licks, and decreased the latency to lick on the hot-plate test (**Figures 3K**, **3L**). Intriguingly, our data suggest that when activated, dLSGad1 neurons inhibit LHA neurons to cause analgesia, and so when $LHA_{post\text{-}dLS}$ neurons are artificially activated, it has the opposite effect on nocifensive behaviors and leads to hyperalgesia. In addition, given that dLS neurons project to the Hb (**Figure 3C**) and Hb neurons have been shown to have anti-nociceptive effects (Cohen and Melzack, 1985), we tested if activating Hb neurons post-

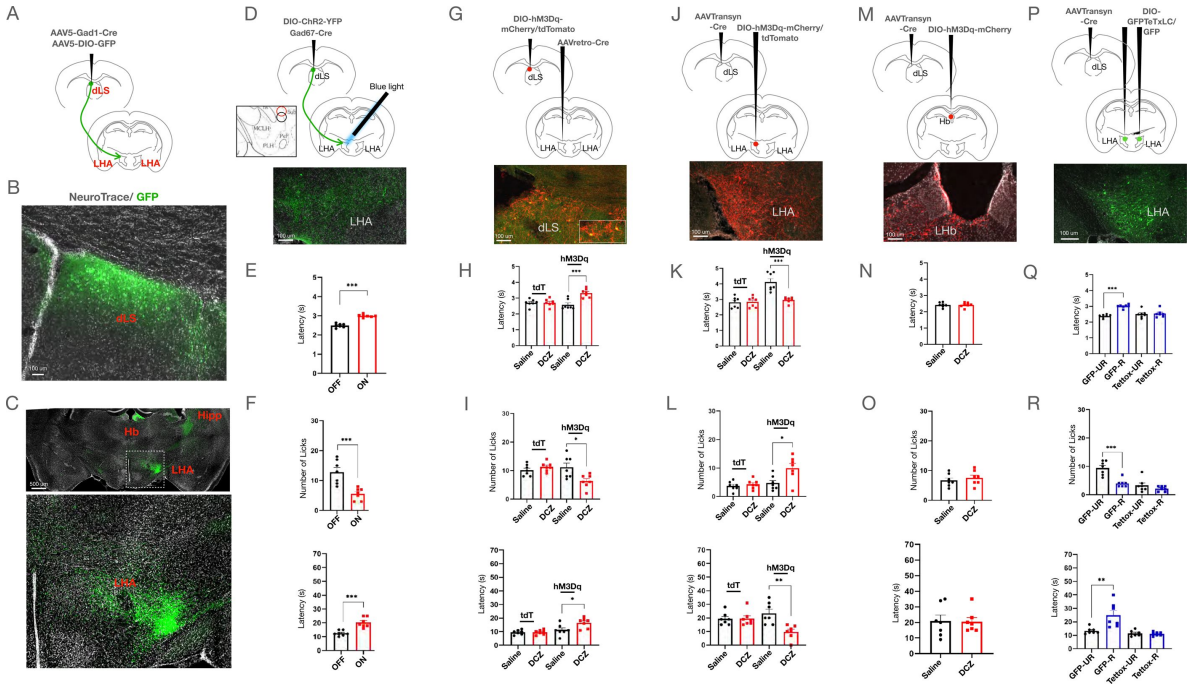


Figure 3.

dLS neurons synapse onto a small neuronal population of LHA neurons.

(A) Gad1-Cre and DIO-GFP injected in the dLS of wild-type mice. (B) GFP-positive cells seen in the dLS. (C) GFP-positive terminals from the dLS were seen in the Hippocampus (Hipp), Habenula (Hb) and Lateral Hypothalamus (LHA), marked region on LHA zoomed-in on the bottom. (D) Gad1-Cre and DIO-ChR2-YFP injected in the dLS of wild-type mice to express the excitatory opsin ChR2 in the dLS^{Gad1} neurons. The fiber was implanted in the LHA to facilitate terminal activation. ChR2-YFP-positive terminals were observed in the LHA. (E) Tail-flick latency (seconds) (2.49±0.04 compared to 2.30±0.03, respectively; t-test, ***P=0.0001, n=7) with (ON) and without (OFF) blue light illumination in $dLS^{Gad1\text{-}ChR2}$ mice (F) Licks (12.86±1.50 compared to 5.57±0.75, respectively; t-test, ***P=0.001, n=7), and latency to lick (seconds) (12.43±0.72 compared to 22.29±1.34, respectively; t-test, ***P=0.0002, n=7) on the hot plate with (ON) and without (OFF) blue light illumination in dLSGad1-ChR2 mice. (G) AAVRetro-Cre was injected in the LHA and DIO-hM3Dq-mCherry/DIO-tdTomato in the dLS of wild-type mice. Presence of mCherry-positive cells (red) co-localised with cFos-positive cells (green) in the dLS (overlap between red and green cells shown in zoom-in box). (H) Tail-flick latency (seconds) (2.58±0.12 compared to 3.32±0.10, respectively; t-test, ***P=0.0005, n=7) post saline or DCZ administration in $dLS_{pre\text{-}LHA\text{-}Gad1\text{-}hM3Dq}$ mice, with no significant difference seen in $dLS_{pre\text{-}LHA\text{-}Gad1\text{-}tdTomato}$ mice. (I) Number of licks (11.14±1.45 compared to 6.29±0.89, respectively; t-test, *P=0.0147, n=7), and latency to lick (seconds) (11.29±1.51 compared to 16.43±1.39, respectively; t-test, *P=0.0277, n=7) post saline or DCZ administration in $dLS_{pre\text{-}LHA\text{-}Gad1\text{-}hM3Dq}$ mice.. (J) AAVTransyn-Cre injected in the dLS and DIO-hM3Dq-mCherry/DIO-tdTomato in the LHA of wild-type mice. Presence of mCherry-positive cells (red) co-localised with cFos-positive cells (green) in the LHA. (K) Tail-flick latency (seconds) (2.97±0.07 compared to 4.12±0.22, respectively; t-test, ***P=0.0003, n=7) post saline or DCZ administration in $LHA_{post\text{-}dLS\text{-}hM3Dq}$ mice, with no significant difference seen in $dLS_{post\text{-}dLS\text{-}Gad1\text{-}tdTomato}$ mice. (L) Number of licks (4.71±0.94 compared to 10.00±1.60, respectively; t-test, *P=0.0149, n=7), and latency to lick (seconds) (23.00±3.11 compared to 9.43±1.93, respectively; t-test, **P=0.003, n=7) post saline or DCZ administration $LHA_{post\text{-}dLS\text{-}tdTomato}$ mice, with no significant difference seen in $dLS_{post\text{-}dLS\text{-}Gad1\text{-}tdTomato}$ mice. (M) AAVTransyn-Cre injected in the dLS and DIO-hM3Dq-mCherry in the Habenula (Hb) of wild-type mice to express the excitatory DREADD. Presence of mCherry-positive cells (red) in the Hb. (N) Tail-flick latency post saline or DCZ administration in $Hb_{post\text{-}dLS\text{-}hM3Dq}$ mice. (O) Number and latency of licks (in seconds) post saline or DCZ administration in $Hb_{post\text{-}dLS\text{-}hM3Dq}$ mice. (P) AAVTransyn-Cre injected in the dLS and DIO-Tettox-GFP/DIO-GFP bilaterally in the LH of wild-type mice. Presence of GFP-positive cells (green) in the LHA. (Q) Tail-flick latency (seconds) (2.38±0.04 compared to 3.00±0.04, respectively; t-test, ***P=0.0001, n=7) with and without restraint in $LHA_{post\text{-}dLS\text{-}GFP}$ mice, with no significant difference seen in $LHA_{post\text{-}dLS\text{-}TetTox}$ mice. (R) Number of licks (9.43±0.92 compared to 4.00±0.53, respectively; t-test, ***P=0.0003, n=7), and latency to lick (seconds) (13.29±0.89 compared to 24.86±3.47, respectively; t-test, **P=0.0072, n=7) with and without restraint in $LHA_{post\text{-}dLS\text{-}GFP}$ mice, with no significant difference seen in $LHA_{post\text{-}dLS\text{-}TetTox}$ mice.

synaptic to dLS ($Hb_{post\text{-}dLS}$) can alter thermal pain thresholds (**Figure 3M**). DCZ administration had no effect on thermal pain thresholds in $Hb_{post\text{-}dLS\text{-}hM3Dq\ mice}$ (**Figures 3N**, **3O**). Further, we tested the effects of silencing the $LHA_{post\text{-}dLS}$ neurons on RS-induced SIA (**Figure 3P**). TetTox mediated $LHA_{post\text{-}dLS}$ silencing abolished SIA (**Figures 3Q**, **3R**). In summary, the $LHA_{post\text{-}dLS}$ neurons are functionally downstream of dLS^{Gad1} neurons, and simultaneous transient activation has identical effects on nociceptive thresholds.

dLS neurons synapse onto the Vesicular glutamate transporter 2 (Vglut2)-expressing LHA neurons

We then tested if the LHA neurons receiving inputs from dLS are excitatory or inhibitory, given that LHA is composed of both *VGlut2* or *Slc17A6* and Vesicular Gamma-aminobutyric acid (GABA) Transporter (*VGat*) or *Slc32A1* expressing neurons (**Figure 4-figure supplement 1A, B**), with a relatively smaller population of VGlut2-positive neurons (**Figure 4-figure supplement 1A, B**), which have recently been implicated in pain modulation (Singewald et al., 2011). To test if VGlut2+ LHA neurons receive direct inputs from dLS^{Gad1} neurons, we took three complementary approaches. First, we injected AAV1-FlpO with anterograde transsynaptic transmission properties (AAV1-hSyn-FlpO or Transyn-FlpO) (Alexander et al., 2009; Campos et al., 2018; Nagai et al., 2020) in the dLS of VGlut2-Cre transgenic mice (**Figure 4A**). Simultaneously, we injected DIO-GFP and fDIO-tdTomato in the LHA of the same mice (**Figure 4A**). We found that 34.2 ± 9.6 % (n = 8 sections, 3 mice) tdTomato expressing cells ($LHA_{post\text{-}dLS}$) were GFP +ve, indicative of their excitatory status (**Figure 4B**). Second, we delivered anterograde transsynaptic Cre (AAV1-hSyn-Cre or Transyn-Cre) in the dLS of Rosa26-LSL-tdTomato transgenic mice to label the $LHA_{post\text{-}dLS}$ neurons with tdTomato and performed multiplex fluorescent in-situ hybridization for VGlut2 and tdTomato mRNA in the LHA (**Figure 4C**). We found that 28.5 ± 11.2 % (n = 12 sections, 2 mice) tdTomato neurons colocalized with VGlut2 (**Figure 4D**). Third, we labeled the synaptic terminals of dLS^{Gad1} neurons and post-synaptic densities of LHA-VGlut2 neurons by expressing synaptophysin-fused GFP (SynGFP) in the dLS^{Gad1} neurons and Cre-dependent inhibitory postsynaptic protein, Gephyrin fused with red fluorescent protein tagRFP (DIO-GephryrintagRFP) (Xu et al., 2012; Chen et al., 2013; Xu et al., 2012; Lerner et al., 2015) in the LHA of VGlut2-Cre mice, respectively (**Figure 4E**). Here, we noticed close apposition of green synaptophysin and red gephyrin puncta in the LHA (**Figure 4F**), suggesting that dLS^{Gad1} neurons make synaptic connections onto VGlut2 neurons in the LHA. Overall, these results suggest that inhibitory dLS axons target the excitatory populations of the LHA neurons. Since parvalbumin (PV) labels a subset of excitatory neurons in the LHA and PV-expressing neurons in the LHA have been shown to be antinociceptive (Singewald et al., 2011), we tested if $LHA_{post\text{-}dLS}$ neurons colocalize with PV-expressing cells and found little to no overlap between the two populations (**Figure 4-figure supplement 1C**). Together, our anatomical data indicate that the dLS^{Gad1} neurons are synaptically connected with the VGlut2+ve neurons in the LHA.

$LHA_{post\text{-}dLS}$ neurons are inhibited upon acute restraint

Next, we reasoned that if $LHA_{post\text{-}dLS}$ neurons are inhibited by dLS^{Gad1} neurons, then these neurons must be disengaged when mice undergo acute stress. To test this, we labeled $LHA_{post\text{-}dLS}$ neurons with GCaMP6s ($LHA_{post\text{-}dLS\text{-}GCaMP6s}$) (**Figures 5A, B**) and recorded calcium transients from these neurons as mice underwent RS (**Figure 5C**) as well as when they were exposed to noxious thermal stimulus. Surprisingly, fiber photometry recordings showed that $LHA_{post\text{-}dLSGCaMP6s}$ neurons respond to acute stress caused by physical restraint and tail hanging (**Figure 5-figure supplement 1A-D**). In addition, like dLS^{Gad1} neurons (**Figures 2H-K**), $LHA_{post\text{-}dLS\text{-}GCaMP6s}$ neurons were not engaged by noxious thermal stimuli (**Figure 5-figure supplement 1E-H**). Notably, $LHA_{post\text{-}dLS\text{-}GCaMP6s}$ neurons differed in their activity from the $dLS^{Gad1\text{-}GCaMP6s}$ neurons in one aspect — while mice struggled in the RS assay, $dLS^{Gad1\text{-}GCaMP6s}$ neurons were active for the entire duration of the struggle bouts (up to 10 seconds) (**Figure 5D**), $LHA_{post\text{-}dLS\text{-}GCaMP6s}$ neurons were active only during the initial phase of the struggle (1-3 seconds) (**Figure**

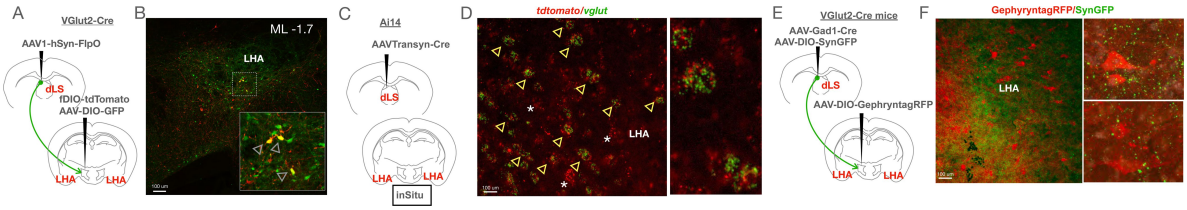


Figure 4

$LHA_{post\text{-}dLS}$ neurons are glutamatergic in nature.

(A) AAV encoding Transsyn-FlpO injected in the dLS; fDIO-tdTomato, and DIO-GFP injected in the LHA of VGlut2-Cre transgenic mice. (B) Overlapping red and green cells (yellow, seen in the zoom-in box marked by arrowheads) were seen in the LHA. (C) AAVTransyn-Cre injected in the dLS of Ai14 transgenic mice. (D) The LHA was labeled with probes against *vglut2* using in situ hybridization. Co-localisation of tdTomato-positive (red) and GFP-positive (green) cells in the LHA (overlapping areas marked by arrowheads), zoom-in of overlap on right. (E) AAVs encoding Gad1-Cre and DIO-Synaptophysin-GFP injected in the dLS and DIO Gephyrin-tagRFP in the LHA of VGlut2-Cre transgenic mice. (F) Closely apposed green synaptophysin and red gephyrin puncta seen in the LHA (zoom-in of overlap on right).

5D). This indicates that activity of pre-synaptic dLS and post-synaptic $LHA_{post\text{-}dLS}$ neurons increases in a coordinated manner at the onset of struggle in mice undergoing RS-assay. However, after the initial activity, $LHA_{post\text{-}dLS}$ neurons can potentially be suppressed by inhibitory dLS inputs causing a reduction in the firing of these post-synaptic neurons. Taken together, these data suggest that acute stress inhibits a sub-population of excitatory neurons in the LHA that are postsynaptic to inhibitory dLS neurons.

$LHA_{post\text{-}dLS}$ neurons facilitate SIA through projections to RVM

Next, in order to investigate how $LHA_{post\text{-}dLS}$ neurons facilitate RS-induced analgesia, we mapped the projections of these neurons. Expression of GFP in the $LHA_{post\text{-}dLS}$ neurons, labeled axon-terminals in the LPBN, and the RVM (**Figure 6 A-C**). In previous studies, activation or silencing of LPBN neurons did not alter the reflexive withdrawal thresholds or coping responses, such as licks in response to noxious thermal stimuli (Barik et al., 2018; Chiang et al., 2020; Han et al., 2015). However, RVM neurons are known to modulate pain bi-directionally (Fields, 2004; François et al., 2017). Importantly, SIA is opioid-dependent (Finn, 2017; Lewis et al., 1981; Vaccarino et al., 1992) (**Figure 6-figure supplement 1A**), and RVM is a major substrate for endogenous opioids (Fields, 2004). We confirmed the opioid-dependent nature of SIA induced by septal activation, by simultaneous administration of mu-opioid receptor antagonist naltrexone and DCZ in $dLS^{Gad1\text{-}hM3Dq}$ mice, which lead to the blocking of dLS activation-induced analgesia (**Figure 6-figure supplement 1B, C**). Thus, we reasoned that the projections of the $LHA_{post\text{-}dLS}$ neurons synapse onto RVM neurons and facilitate RS-induced analgesia. To that end, first, we sought to establish the anatomical location and projections of the RVM neurons ($RVM_{post\text{-}LHA}$) — that are postsynaptic to the $LHA_{post\text{-}dLS}$ neurons. We labeled the $RVM_{post\text{-}LHA}$ neurons with GFP (**Figure 6D**) and SynRuby (**Figure 6-figure supplement 1D**) separately, using the anterograde intersectional viral genetic strategy used before (AAVTrans-Cre in LHA; DIO-GFP or DIO-SynRuby in RVM). We observed that the cell bodies of the $RVM_{post\text{-}LHA}$ neurons were distributed in the midline area (**Figures 6E**, **Figure 6-figure supplement 1E**) of the medulla. We noticed abundant axon terminals of $RVM_{post\text{-}LHA}$ neurons in the LHA (**Figure 6-figure supplement 1F**), and projections in the deeper layers (VI/ VII) of the lumbar spinal cord (**Figures 6F**, **Figure 6-figure supplement 1G**). This implies that the $RVM_{post\text{-}LHA}$ neurons may modulate nociceptive thresholds through their local synaptic connections within the RVM (Marchand and Hagino, 1983), recurrent connections with the PAG, or direct interactions with spinal cord neurons. Second, using the monosynaptic retrograde rabies tracing technique (Callaway and Luo, 2015; Wickersham et al., 2007), we determined if the $RVM_{post\text{-}LHA}$ neurons are the direct postsynaptic partners of the $LHA_{post\text{-}dLS}$ neurons. We expressed G and TVA-GFP proteins in $LHA_{post\text{-}dLS}$ neurons (AAVTransyn-Cre in dLS; DIO-G; DIO-TVA-GFP in LHA) and injected 1'G-Rabies-mCherry in the RVM (**Figure 6G**). As expected, we observed the starter cells that co-expressed TVA-GFP ($LHA_{post\text{-}dLS}$) and 1'G-Rabies-mCherry (retrogradely transported from the RVM; $LHA_{pre\text{-}RVM}$) in the LHA (**Figure 6H**). Remarkably, retrogradely transported rabies-mCherry was found in the dLS neurons (**Figure 6H**). Indicating that the dLS neurons are directly upstream of the RVM projecting LHA neurons. Third, in a complementary approach, we took advantage of the GRASP synaptic labeling strategy (Kim et al., 2011), where presynaptic neurons expressed one-half of the GFP protein, and the postsynaptic neurons expressed the other half. At the functional synapses between the two GFP-subunit expressing neurons, GFP is reconstituted and can be visualized through a fluorescent microscope. In our experiments, we injected Pre-GRASP in the dLS, fDIO-Post-GRASP in the LHA, and AAVRetro-FlpO in the RVM (**Figure 6I**) of the same mice. Simultaneous injection of the three AAVs successfully labeled the synapses between the dLS and $LHA_{pre\text{-}RVM}$ neurons with GFP in the LHA (**Figure 6J**). Thus, demonstrating that the dLS neurons make synaptic connections with $LHA_{pre\text{-}RVM}$ neurons.

We then sought to understand how the $RVM_{post\text{-}LHA}$ neurons encode RS and noxious thermal stimuli, and thus, we performed fiber photometry recordings from the GCaMP6s expressing $RVM_{post\text{-}LHA}$ neurons ($RVM_{post\text{-}LHA\text{-}GCaMP6s}$) (**Figures 7A, B**). Calcium transients in the $RVM_{post\text{-}}$

Figure 5.

$LHA_{post\text{-}dLS}$ neurons are acutely engaged during the initial struggle due to physical restraint.

(A) AAVTransyn-Cre injected in the dLS and DIO-GCaMP6s in the LHA of wild-type mice to record neural activity from the $LHA_{post\text{-}dLS}$ neurons. (B) GCaMP6s-positive cells (green) and tissue injury from the fiber implant seen in the LHA, zoom-in of the marked area on the right. (C) Sample trace of neural activity when mice were allowed to move freely in the homecage (blue), and when they were under restraint (red). Peaks corresponding to neural activity (blue dashes) were seen when mice struggled in the tube. (D) Cumulative plots for calcium transients when mice struggled under restraint (5mice, 12 trials). (E) Heat maps depicting neural activity in $LHA_{post\text{-}dLS}$ (left) and dLS^{Gad1} neurons (right) during struggle in the RS assay (5 mice, 12 trials; dotted lines indicating initiation of struggle in RS assay).

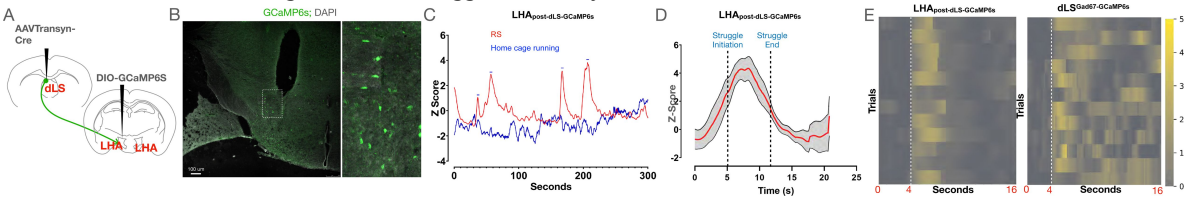
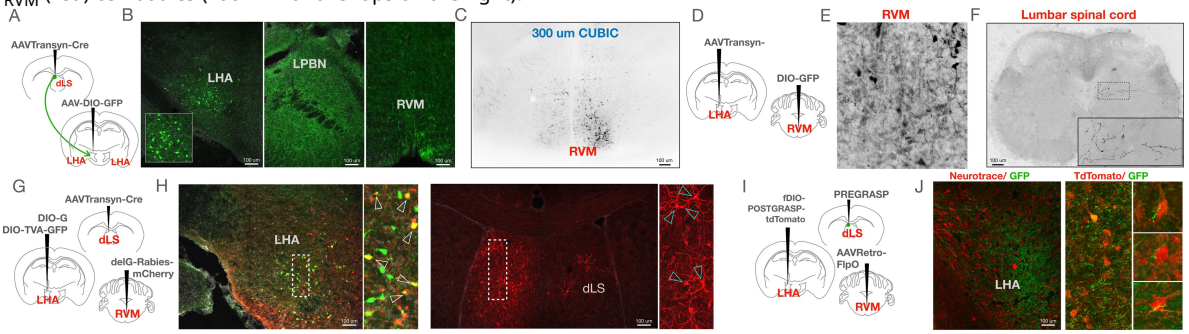


Figure 6.

RVM ON-cells are downstream of the dLS→LHA circuitry.

(A) AAVTransyn-Cre injected in the dLS and DIO-GFP in the LHA of wild-type mice to label the $LHA_{post\text{-}dLS}$ neurons. (B) GFP-positive cell bodies in the LHA (green, left; zoom-in of LHA in the box on bottom). Projections of the $LH_{post\text{-}dLS}$ neurons in the PBN, and RVM. (C) Presence of fluorescence (black) in RVM post tissue clearing of the same brains expressing GFP in the $LHA_{post\text{-}dLS}$ neurons. (D) AAVTransyn-Cre injected in the LHA and DIO-GFP in the RVM of wild-type mice to label the $RVM_{post\text{-}LHA}$ neurons with GFP. (E) GFP-positive cell bodies (black, marked by arrowheads) seen in the RVM. (F) Projections from the $RVM_{post\text{-}LHA}$ were observed in the lumbar spinal cord. (G) AAVTransyn-Cre injected in the dLS, and DIO-1'G and DIO-TVA-GFP in the LHA of wild-type mice. Three weeks later, the 1'G-Rabies-mCherry virus was injected into the RVM. (H) Starter cells (yellow) observed in the LHA. mCherry-positive cell bodies (red) seen in the dLS (marked region zoomed-in on the right with overlapping cells marked with arrowheads). (I) AAVRetro-FlpO injected in the RVM, fDIO-post-GRASP injected in the LHA, and Pre-GRASP in the dLS of wild-type mice. (J) Axon terminals from the dLS neurons (Green) were observed around the $LHA_{pre\text{-}RVM}$ (red) cell-bodies (zoom-in of overlaps on the right).



$_{LHA}$ neurons increased spontaneously when the mice were subjected to RS (**Figure 7C**). However, when the mice struggled under restraint, $RVM_{post\text{-}LHA}$ neuronal activity was suppressed (**Figures 7C, D**). The activity of the $RVM_{post\text{-}LHA\text{-}GCaMP6s}$ neurons increased when the mice shook or licked their paws on the hot-plate test (**Figure 7E**). Notably, the rise in activity of the $RVM_{post\text{-}LHA}$ neurons preceded the licks and shakes, indicating a facilitatory role of these neurons in nocifensive behaviors. To further confirm this facilitatory role, performed two additional assays. First, we recorded calcium transients from the $RVM_{post\text{-}LHA\text{-}GCaMP6s}$ neurons while the mice were on the hot plate set to a gradient of 32-52 degrees over a period of 5 minutes. We observed that the neurons started firing only once the hot plate reached noxious temperatures, with no specific activity seen at innocuous temperatures (**Figure 7F**). Second, we subjected the mice to the tail flick assay and observed a peak in neural firing preceding the tail flick instance caused by the thermal pain caused by the concentrated beam of light (**Figure 7G**). From these series of experiments, we were able to further confirm the facilitatory role of the $RVM_{post\text{-}LHA}$ neurons in nocifensive behaviors. The facilitatory pro-nociceptive population of RVM neurons is otherwise known as ON-cells (Fields et al., 1995, 1991) Thus, we hypothesized that these $RVM_{post\text{-}LHA}$ neurons are pro-nociceptive and likely ON cells. They are activated by acute stress and suppressed when the mice struggle to escape stress-causing restraint. When we calculated the area under the curve (AUC) of the Z-score of the recordings from the dLS, $LHA_{post\text{-}dLS}$, and $RVM_{post\text{-}LHA}$ neurons while the mice struggled in the RS assay, the data indicated that the dLS activity increased while at the same time $LHA_{post\text{-}dLS}$, and $RVM_{post\text{-}LHA}$ activity decreased (**Figure 8-figure supplement 1E**). Together, our data indicate that the LS inhibitory neurons engaged by acute restraint, suppresses LH activity which in turn reduces the excitability of the pronociceptive RVM neurons and result in analgesia.

To further establish that the $RVM_{post\text{-}LHA}$ neurons are ON cells, we chemogenetically activated the $RVM_{post\text{-}LHA}$ neurons (**Figures 8A, B**). Mice expressing hM3Dq in the $RVM_{post\text{-}LHA}$ responded with increased licks on the hot-plate test and a lower latency when i.p. DCZ was administered (**Figures 8C, D**). Contrary to $RVM_{post\text{-}LHA}$ activation, when we chemogenetically and silenced the $RVM_{post\text{-}LHA}$ neurons (**Figures 8E-H**), the number of licks on the hot-plate test was reduced, and the latency to lick and the tail-flick latency were increased. These findings agree with our hypothesis that the activated dLS neurons inhibit $LHA_{post\text{-}dLS}$ neurons, which in turn deactivates $RVM_{post\text{-}LHA}$ cells. Moreover, the observation that silencing bi-lateral LPBN neurons postsynaptic to LHA (**Figures 8I**, **8J**) did not affect mouse responses on hot-plate and tail-flick tests confirmed (**Figures 8K**, **8L**) that the LHA-RVM connections primarily mediate the anti-nociceptive effects of $LHA_{post\text{-}dLS}$ neurons. The results observed in the experiments with chemogenetic inhibition of $RVM_{post\text{-}LHA}$ neurons were repeated with optogenetic inhibition techniques, and similar results were obtained (**Figures 8M-P**).

Recent observations indicate that the pro-nociceptive ON cells in the RVM can be either excitatory or inhibitory (Nguyen et al., 2022). Activating the excitatory ON cells results in hypersensitivity to noxious stimuli, whereas inhibiting the same neurons results in analgesia (Nguyen et al., 2022). Since the chemogenetic activation of the pro-nociceptive $RVM_{post\text{-}LHA}$ neurons resulted in thermal hyperalgesia and inhibition leading to pain suppression, we hypothesized that the $RVM_{post\text{-}LHA}$ neurons are excitatory. Thus, to test our hypothesis, we expressed Syn-GFP in the LHA of VGlut2-Cre mice, and PSD95-tagRFP in the RVM of the same mice (**Figure 8-figure supplement 1A-D**). When we visualized the RVM with confocal and super-resolution microscopy (**Figure 8-figure supplement 1D**), we found close apposition between the GFP-expressing pre-synaptic terminals and tagRFP-expressing VGlut2 expressing RVM neurons. Thus, we concluded that the excitatory LHA neurons impinge upon the excitatory RVM-ON neurons to facilitate RS-induced analgesia. The struggle to escape restraint engages dLS inhibitory neurons. The activated inhibitory dLS neurons silence excitatory $LHA_{post\text{-}dLS}$ neurons, which consequently disengages the pro-nociceptive $RVM_{post\text{-}LHA}$ neurons to drive RS-induced analgesia (**Figure 8-figure supplement 1E-F**).

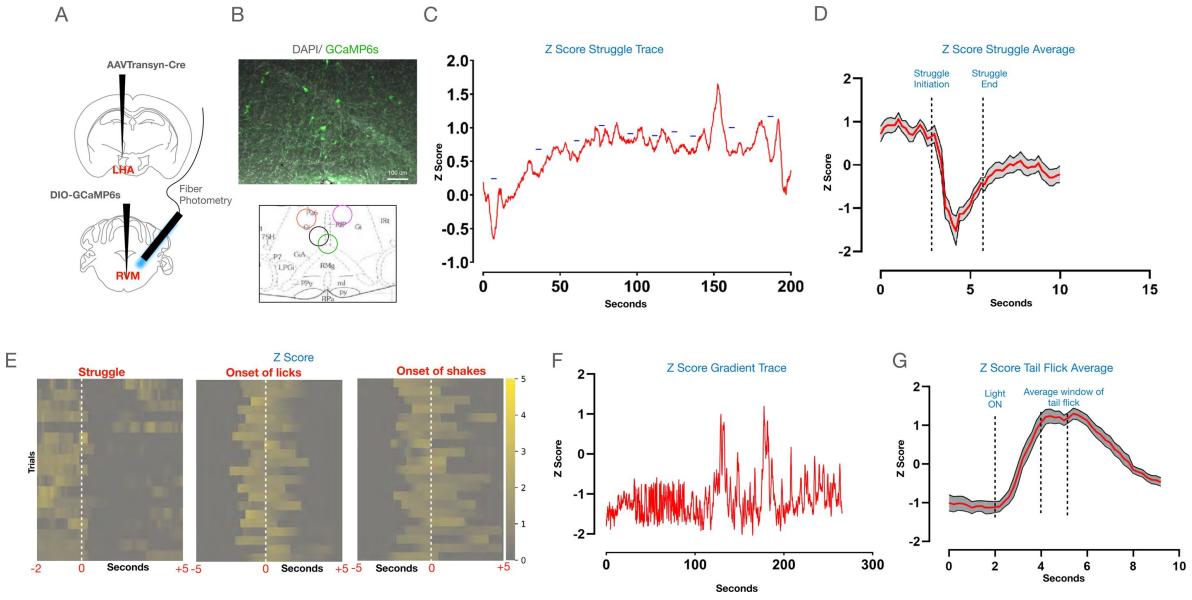


Figure 7.

$RVM_{post\text{-}LHA}$ neurons fire during nocifensive behaviors on the hotplate and respond to RS-mediated struggle.

(A) AAVTransyn-Cre was injected in the LHA with DIO-GCaMP6s in the RVM of wild-type mice (top). A fiber was implanted on the RVM over the same coordinates to perform fiber-photometry (bottom). (B) GCaMP6s-positive cells in the RVM (green) to demonstrate successful expression of GCaMP6s in the $RVM_{post\text{-}LHA}$ neurons. (C) Z-Score of calcium dynamics recorded from the $RVM_{post\text{-}LHA}$ neurons while the mice are restrained and struggling (blue dashes) in a falcon tube. (D) Average plot (individual trials) of Z-Score traces (4 mice, 16 trials) during struggle bouts of $RVM_{post\text{-}LHA}$ neurons. (E) (Left to right) Heatmaps depicting neural activity patterns during individual instances of struggle in the falcon tube, licks, and shakes on the hot plate at 52 degrees, respectively (4 mice, 16 trials). (F) Z-Score of calcium dynamics recorded from the $RVM_{post\text{-}LHA}$ neurons while the mice are on the hotplate set at a gradient from 32-52 degrees. (G) Average plot (individual trials) of Z-Score traces (4 mice, 16 trials) during tail flick assay of $RVM_{post\text{-}LHA}$ neurons.

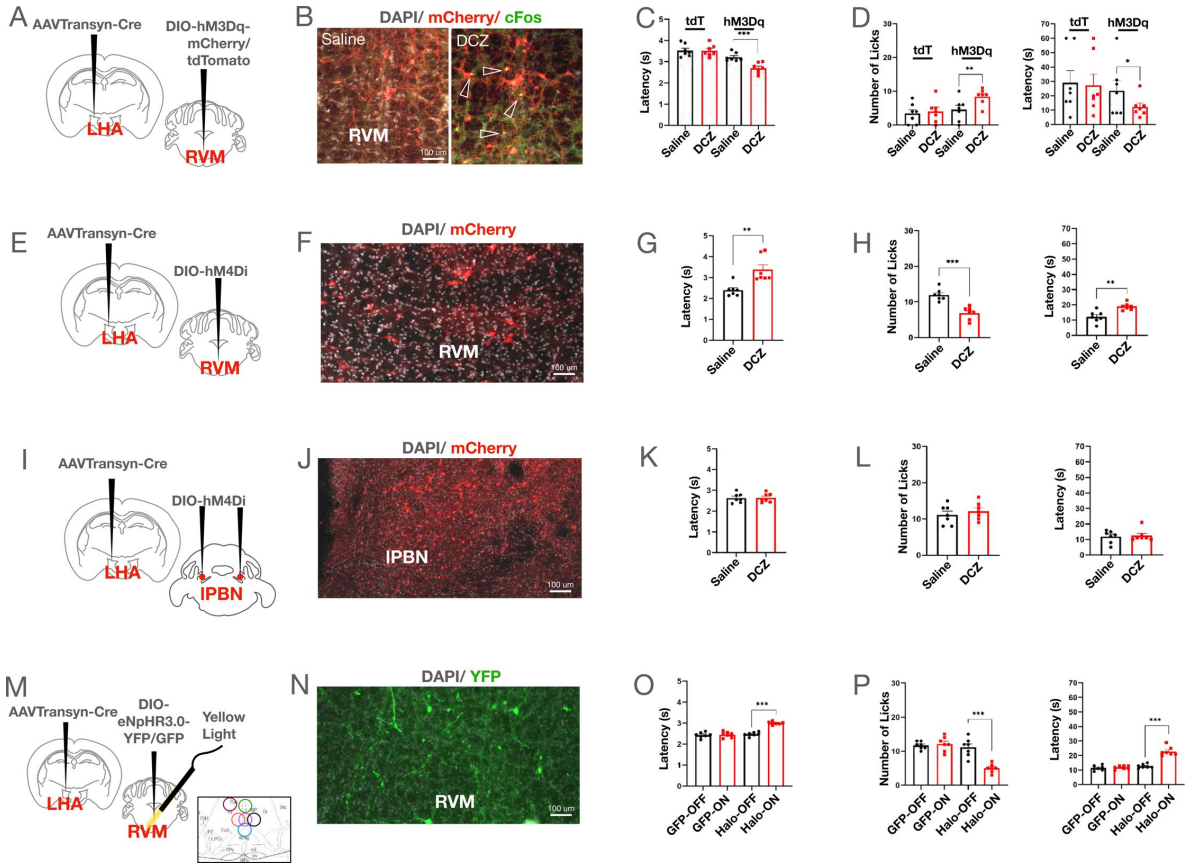


Figure 8.

Activation of $RVM_{post\text{-}LHA}$ neurons resulted in thermal hyperalgesia, while inhibition was anti-nociceptive.

(A) AAVTransyn-Cre injected in the LHA and DIO-hM3Dq-mCherry/DIO-tdTomato in the RVM of wild-type mice. (B) DCZ induced c-Fos (green) expression in the $RVM_{post\text{-}LHA\text{-}hM3Dq}$ (red) neurons (zoom-in shown on the right with overlapping cells marked using arrowheads). (C) Tail-flick latency (seconds) (3.22±0.08 compared to 2.69±0.09, respectively; t-test, ***P=0.0001, n=7) post saline or DCZ administration in the $RVM_{post\text{-}LHA\text{-}hM3Dq}$ mice, with no significant difference in $RVM_{post\text{-}LHA\text{-}tdTomato}$ mice. (D) Number of licks (4.57±1.27 compared to 8.43±0.87, respectively; t-test, **P=0.0062, n=7), and latency to lick (seconds) (23.57±7.12 compared to 12.29±2.68, respectively; t-test, *P=0.0466, n=7) post saline or DCZ administration in the $RVM_{post\text{-}LHA\text{-}hM3Dq}$ mice, with no significant difference in $RVM_{post\text{-}LHA\text{-}tdTomato}$ mice. (E) AAVTransyn-Cre was injected in the LHA and DIO-hM4Di in the RVM of wild-type mice to express the inhibitory DREADD in the $RVM_{post\text{-}LHA}$ neurons. (F) hM3Dq-mCherry expressing neurons (red) in the RVM. (G) Tail-flick latency (seconds) (2.40±0.11 compared to 3.38±0.23, respectively; t-test, **P=0.0025, n=7) post saline or DCZ administration in $RVM_{post\text{-}LHA\text{-}hM4Di}$ mice. (H) Number of licks (12.00±0.62 compared to 6.86±0.67, respectively; t-test, ***P= 0.0001, n=7), and latency to lick (seconds) (12.14±1.42 compared to 18.86±0.86, respectively; t-test, **P=0.0016, n=7) post-saline or DCZ administration in $RVM_{post\text{-}LHA\text{-}hM4Di}$ mice. (I) AAVTransyn-Cre injected in the LHA and DIO-hM4Di in the PBN (bilaterally) of wild-type mice to express the inhibitory DREADD, hM4Di in the $LPBN_{post\text{-}LHA}$ neurons. (J) hM4Di-mCherry (red) expressing neurons in the PBN. (K) Tail-flick latency (seconds) post-saline or DCZ administration in the $PBN_{post\text{-}LHA\text{-}hM4Di}$ mice. (L) Number and latency of licks (seconds) post-saline or DCZ administration in the $PBN_{post\text{-}LHA\text{-}hM4Di}$ mice. (M) AAVTransyn-Cre injected in the LHA and DIO-eNPHR3.0-YFP/DIO-GFP in the RVM of wild-type mice to label the $RVM_{post\text{-}LHA}$ neurons. Optic fiber cannulae was implanted over the RVM at same coordinates to deliver yellow light. The summary of the fiber placements are graphically represented (right corner). (N) YFP-positive cells expressing eNpHR3.0 in the $RVM_{post\text{-}LHA}$ (green) neurons in the RVM. (O) Tail-flick latency (seconds) (2.48±0.04 compared to 3.00±0.03, respectively; t-test, ***P=0.0001, n=7) with (ON) and without (OFF) yellow light illumination in $RVM_{post\text{-}LHA\text{-}eNPHR3.0\text{-}YFP}$ mice, with no significant difference seen in $RVM_{post\text{-}LHA\text{-}DIO\text{-}GFP}$ mice. (P) Number of licks (11.14±1.01 compared to 5.00±0.49, respectively; t-test, ***P=0.0001, n=7), and latency to lick (seconds) (12.71±0.52 compared to 22.86±1.18, respectively; t-test, ***P=0.0001, n=7) with (ON) and without (OFF) yellow light illumination in $RVM_{post\text{-}LHA\text{-}eNPHR3.0\text{-}YFP}$ mice, with no significant difference seen in $RVM_{post\text{-}LHA\text{-}GFP}$ mice.

Discussion

The dLS has been traditionally considered a key brain region for mediating stress responses (Anthony et al., 2014; Azevedo et al., 2020; Kubo et al., 2002; Singewald et al., 2011). However, the role of the dLS in SIA is poorly understood. In this study, we have delineated the downstream partners of dLS and elucidated the mechanisms through which dLS neurons translate stress into pain suppression. We found that the dLS neurons are specifically geared towards coping with stress and, through their connections with the spinal cord via the LHA and the RVM, inhibit responses to noxious stimuli. In conclusion, our study has comprehensively evaluated the involvement of a key neural circuit in SIA by anatomically tracing their connections, monitoring, and manipulating neural activity.

We wondered what the etiologically relevant function of SIA might be, evolutionarily what benefits SIA might provide, and how dLS plays a role in it. SIA enables animals and humans to physiologically and behaviorally evade or cope with stressors in their immediate environment. When the perceived pain is attenuated, attention is drawn toward escaping the stressor. Several experiments support this view, including studies where rats were subjected to mild electric shocks from a floor plate in either an inescapable or an escapable chamber. It was observed that analgesia occurred when the rats could not escape the chamber after experiencing the electric shocks as opposed to when they could escape (Maier et al., 1982; Terman and Liebeskind, 1986). In line with these studies, we experimentally observed SIA only when mice were restrained for enough time (~1 hour) or when dLS neurons were activated for ~30 minutes (**Figure 1-figure supplement 2C, D**). We propose that acute restraint drives animals to attempt to escape, and during these attempts dLS neurons are engaged. When the dLS neurons are activated repeatedly, it provides a short-term analgesia. SIA can be particularly pertinent in individuals with chronic pain, where pathological pain can impede the ability to react to a stressor on time. The role of dLS neurons in chronic pain is unclear. Interestingly, a recent study has shown that the dLS neurons promote both pain and anxiety (Wang et al., 2023). This is contrary to our data which shows that dLS activation has analgesic effects. The contradiction may be since dLS neurons may play opposing roles under acute and chronic stress conditions. In contrast to acute stress, mechanisms of which we have explored here, chronic stress which Wang et al. studied, is known to exacerbate pain (Wang et al., 2023). In addition, the targeted coordinates for dLS used in the study are medial compared to the ones used in our study. However, as mentioned before, lesion studies in humans or animals have consistently indicated that dLS or medial septum stimulation is anti-nociceptive, irrespective of the nature of the noxious stimulus. Notably, chronic stress can cause hyperalgesia, and the underlying circuit mechanisms are poorly understood. It will be interesting to test if the dLS-LHA circuitry is involved in the reduced pain thresholds observed in mice with chronic stress (Jennings et al. 2014).

Early studies showed that SIA could either be opioid-dependent or independent (Watkins and Mayer, 1986). Naltrexone administration blocked SIA in rats who had undergone electric shocks (Drugan et al., 1981; Maier et al., 1980). Surprisingly, it was found that acute stress can sequentially induce both opioid dependent as well as opioid-independent SIA (Grau et al., 1981). Mutant mice without functional ß-endorphin (endogenous ligand for the mu-opioid receptor, OPRM1) lacked opioid-dependent SIA (mild swim stress), however, they displayed opioid-independent SIA (cold-swim stress) (Rubinstein et al., 1996). Interestingly, opioid-dependent SIA was primarily induced when the animals underwent stress in inescapable chambers. Thus, successful induction of SIA may depend on the mode of stress delivery, and the opioid dependence may be decided by the exposure time. Our data too suggests that dLS-mediated RS-induced analgesia is opioid dependent, as we found that SIA induced by dLS activation is reversible by naltrexone administration (**Figure 6-figure supplement 1A-C**). Further, we show that RVM-ON cells play a critical role in dLS-mediated SIA. This observation is supported by a recent study that

demonstrated the necessity of kappa-opioid receptors expressing RVM neurons in SIA (Nguyen et al., 2022). Together, the restraint-induced silencing of LHA excitatory neurons by dLS may turn downstream mu-opioid receptor-expressing RVM-ON cells amenable to enkephalin and endorphin-mediated modulation and consequent analgesia. Typically, RVM-ON cells target enkephalinergic and GABAergic spinal interneurons in the superficial layers of the dorsal horn that gate pain in turn through their connections with the somatosensory primary inputs to modulate pain (François et al., 2017). However, the neurons of our interest in the RVM target the deeper layers (V/VI) in the dorsal horn and may modulate nociceptive thresholds through independent mechanisms (**Figure 6F**). In addition to the opioidergic system, SIA is known to be mediated by the endogenous endocannabinoid system (Hohmann AG). Brief and continuous electric shocks results into SIA that is not reversed by naltrexone, while can be blocked by endocannabinoid receptor antagonist. Since, the dLS mediated SIA is suppressed by naltrexone, it is unlikely is dependent on the endogenous endocannabinoid pathways.

Interestingly, LHA is known to have direct spinal projections and thus can directly modulate pain bypassing RVM (Hancock, 1976; Willis and Coggeshall, 2012). LHA neurons can modulate pain through direct orexinergic inputs to the spinal cord (van den Pol, 1999; Wang et al., 2018). The direct orexinergic LHA inputs to the spinal cord terminate in the superficial layers of the spinal cord. At the same time, the LHA neurons of our interest provide indirect inputs via RVM to the deeper layers of the spinal cord. Thus, two independent neural pathways (direct and indirect) originating from the LHA may mediate SIA. The orexinergic system housed in the LHA is recruited by acute stress and thus can supplement the LS-LHA-RVM-spinal cord circuitry in stress-induced pain modulation. We did not find direct inputs of $LHA_{post\text{-}dLS}$ neurons in the spinal cord with an AAV-mediated labeling strategy; however, these neurons can have partial overlap with the orexin population, and our method may not be sensitive enough to label the axon terminals in the spinal cord.

Notably, LHA neurons are known to respond to stress stimuli (Owens-French et al., 2022; Wang et al., 2021) and thus can cause SIA independently of dLS. The dLS independent SIA mechanisms might be driven by the direct orexinergic inputs to the spinal cord from LHA or the PV-expressing LHA neurons can mediate SIA through projections to the PAG (Siemian et al., 2021). This is reflected in our results where we observed transient activation of $LHA_{post\text{-}dLS}$ neurons when the mice struggled in the RS assay (**Figure 5D**). In addition to the excitatory projections tested here, there are inhibitory neurons in the LHA that project to the PBN (Moga et al., 1990). We primarily focused on the excitatory targets of dLS in the LHA, however, LHA is rich in GABAergic neurons and the potential roles of inhibitory targets of dLS in stress-responses remain to be tested. How both excitatory and inhibitory outputs from the LHA can modulate responses to noxious stimuli remains to be investigated. Decades of circuit tracing and functional anatomy studies have revealed synaptic targets of LHA across the brain, including the dLS (Cassidy et al., 2019). Such bidirectional connections also exist between the RVM and LHA and exploring the roles of recurrent connections between dLS-LHA/ LHA-RVM in stress-induced pain modulation will further delineate circuit mechanisms of SIA. We developed the microparticle-based CNO administration tool for projection-specific chronic DREADD ligand delivery. The same tool, by tweaking the release rates of the CNO, can be effectively used for chronic neuronal activation/silencing. Finally, the mechanistic interrogation of dLS-centric SIA circuits has revealed a hypothalamic coordinate that effectively connects dLS with RVM to influence nociceptive thresholds. We have primarily focused on thermal nociceptive thresholds, however, it will be interesting to explore how dLS-LHA circuitry influences the effects of stress on other somatosensory modalities, such as itch.

Experimental Model and Subject Details

Animals

Animal care and experimental procedures were performed following protocols approved by the IAEC at the Indian Institute of Science. The ethics approval number is CAF/ETHICS/988/2023. The animals were housed at the IISc Central Animal Facility under standard animal housing conditions: 12 h light/dark cycle from 7:00 am to 7:00 pm with ad libitum access to food and water, mice were housed in IVC cages in Specific pathogen-free (SPF) clean air rooms. Mice strains used: *Vglut2*-Cre or *Vglut2*-ires-Cre or *Slc17a6tm2*(Cre) Lowl/J(Stock number 016963); Ai14 (B6;129S6-Gt(ROSA)26Sortm9(CAG-tdTomato)Hze/J (Stock No 007905), BALB/cJ (Jackson Laboratories, USA). Experimental animals were between 2-4 months old.

Methods

Viral vectors and stereotaxic injections

Mice were anesthetized with 2% isoflurane/oxygen before and during the surgery. Craniotomy was performed at the marked point using a hand-held micro-drill (RWD, China). A Hamilton syringe (10 µL) with a glass pulled needle was used to infuse 300 nL of viral particles (1:1 in saline) at 100 nL/minute. The following coordinates were used to introduce virus/dyes: dLS-Anterior-Posterior (AP): +0.50, Medial-Lateral (ML): + 0.25; Dorsal-Ventral (DV): -2.50; LHA-AP: -1.70, ML: ±1.00; DV: -5.15; RVM-AP: -5.80, ML: +0.25; DV: -5.25; LPBN-AP: -5.34, ML: ±1.00, DV: -3.15. Vectors used and sources: ssAAV-9/2-hGAD1-chl-icre-SV40p(A) (University of Zurich, Catalog# v197-9), pAAV5-hsyn-DIO-EGFP (Addgene, Catalog# 50457-AAV 1), pAAV5-FLEX-tdTomato (Addgene, Catalog# 28306-PHP.S), pENN.AAV5.hSyn.TurboRFP.WPRE.RBG (Addgene, Catalog# 10552-AAV1), pAAV5-hsyn-DIO-hM3D(Gq)-mCherry (Addgene, Catalog# v141469), pAAV5-hsyn-DIO-hM4D(GI)-mCherry (Addgene, Catalog# 44362-AAV5), AAV9.syn.flex.GcaMP6s (Addgene, Catalog# pNM V3872TI-R(7.5)), pAAV-Ef1a-DIO-eNPHR 3.0-EYFP (Addgene, Catalog# v32533), pAAV-EF1a-double floxed-hChR2(H134R)-GFP-WPRE-HGHpA(Addgene, Catalog# v64219), AAV1-hSyn-Cre.WPRE.hGH (Addgene, Catalog# v126225), AAVretro-pmSyn1-EBFP-Cre (Donated by Ariel Levine, NIH), AAV retro-hSynapsin-Flpo (Donated by Ariel Levine, NIH), scAAV-1/2-hSyn1-FLPO-SV40p(A) (University of Zurich, Catalog# v59-1), ssAAV-1/2-shortCAG-(pre)mGRASP-WPRE-SV40p(A) (University of Zurich, Catalog# v653-1), ssAAV-1/2-fDIO-(post)mGRASP_2A_tdTomato(University of Zurich, Catalog# v651-1), pAAV-Ef1a-DIO-tdTomato (Addgene, Catalog# 28306-PHP.S), pAAV-hSyn-fDIO-hM3D(Gq)-mCherry-WPREpA (Addgene, Catalog# 154868-AAVrg), ssAAV-9/2-hSyn1-chl-dlox-EGFP_2A_FLAG_TeTxLC(rev)-dFRT-WPRE-hGHp(A) (University of Zurich, Catalog# v322-9), AAVretro-hSyn-NLS-mCherry (Donated by Ariel Levine, NIH), AAV9-DIO-GephyrinTagRFP (Donated by Mark Hoon, NIH), AAV9-DIO-PSD95-TagRFP (Donated by Mark Hoon, NIH), AAV5-hSyn-DIO-mSyp1_EGFP(University of Zurich, Catalog# v484-9). For rabies tracing experiments, rAAV5-EF1α-DIO-oRVG (BrainVTA, Catalog# PT-0023) and rAAV5-EF1α-DIO-EGFP-T2A-TVA (BrainVTA, Catalog# PT-0062) were injected first, followed by RV-EnvA-Delta G-dsRed (BrainVTA, Catalog# R01002) after 2 weeks. Tissue was harvested after 1 week of rabies injection for histochemical analysis. Post-hoc histological examination of each injected mouse was used to confirm that viral-mediated expression was restricted to target nuclei.

Optogenetic and Photometry fiber implantation

Fiber optic cannula from RWD, China; Ø1.25 mm Ceramic Ferrule, 200 µm Core, 0.22 NA, L = 5 mm were implanted at AP: 0.50, ML: +0.25; DV: -2.50 in the dLS and L= 7 mm fibers were implanted at AP: -1.70, ML: ±1.00; DV: -5.15 in the LHA, and AP: -5.80, ML: +0.25; DV: -5.25 in the RVM after AAV carrying GCaMP6s, Channelrhodopsin2 or Halorhodopsin were infused. Animals were allowed to recover for at least 3 weeks before performing behavioral tests. Successful labeling and fiber implantation were confirmed post hoc by staining for GFP/mCherry for viral expression and injury caused by the fiber for implantation. Animals with viral-mediated gene expression at the intended locations and fiber implantations, as observed in post hoc tests, were only included.

Behavioral assays

Behavioral assays for the same cohorts were handled by a single experimenter. Prior to experimentation, the experimenter was blinded to the identity of animals. The genotypes ana/or the mice with AAV1 injections were randomized within cohorts wherever possible. Mice were habituated in their home cages for at least 30 minutes in the behavior room before experiments. An equal male-to-female ratio was maintained in every experimental cohort and condition unless otherwise stated, with no significant differences seen between sexes in the responses recorded from the behavioral experiments. Wherever possible, efforts were made to keep the usage of animals to the minimum. Deschloroclozapine (DCZ) was diluted in saline (final concentration: 0.1 mg/kg) and injected intraperitoneal (i.p.) 15-20 minutes before behavioral experiments or histochemical analysis. Mice were injected with intra-plantar (i.pl.) Complete Freund's adjuvant (CFA) one day before the behavioral experiments to cause persistent inflammatory pain and thermal hypersensitivity. All the experiments were videotaped simultaneously with three wired cameras (Logitech, USA) placed horizontally and scored offline post hoc manually. The programmable hot plate with gradient function and tail flick analgesiometer (Orchid Scientific, India) were used according to the manufacturer's instructions. For optogenetic stimulations fiber-coupled laser (channelrhodopsin activation; RWD, China), fiber-coupled LEDs (for halorhodopsin stimulation; Prizmatix, Israel) were used. Prior to the behavioral testing the optic fibers were connected to the cannulae implanted in the mice brain. The animals were habituated for 30 minutes prior to the commencement of the experiments. The light-dark box tracking and estimations of the time-spent in either chamber were carried out with DeepLabCut and the data were trained and analyzed on a custom-built computer system with AMD Ryzen 9 5900x 12-core processor 24 with NVIDIA Corporation Graphics Processing Unit (GPU) (Mathis et al., 2018), (Nath et al., 2019). The RS assay was used to induce stress in the experiments reported here. In short, mice were restrained for one hour in a ventilated falcon tube, followed by testing them for stress-related and noxious behaviors using the Light Dark Box Assay, the Hot Plate Assay, and the Tail Flick Assay.

Blood corticosterone levels were measured using the Mouse corticosterone Enzyme-linked immunosorbent assay (ELISA) kit (BIOLABS, USA) by collecting blood from wild-type mice, wild-type mice subjected to RS, and dLSGad1-hM3Dq mice administered with DCZ. Plasma extracted from each of the collected blood samples were subjected to Competitive ELISA. Wells pre-coated with corticosterone antigen were incubated with the respective sample in triplicates. Biotin-conjugated primary antibody was added, followed by incubation with streptavidin-horseradish peroxidase (HRP). Finally, the samples turned from yellow to blue in color depending on the concentration of corticosterone present in the sample when Tetramethylbenzidine (TMB) substrate was added. The optical density (O.D.)of the samples was recorded using a spectrophotometer.

The mice were subjected to four primary behavioral assays for the fiber photometry experiments. In the immobilization experiments, experimenter physically restrained the mice by pressing them down by hand for approximately 10 seconds. In the tail hanging experiments, the mice were suspended upside down by their tail for 10 seconds. In the RS assay, photometry signals were recorded through the fiber coupled cannulae that passed through a modified RS-inducing falcon tube to allow unrestricted recording. On the hot plate test the mice were acclimatized to the equipment with the optic fiber connected to the cannulae a day before experimentations. During the experiments, the equipment was first allowed to reach the desired temperature and then the animals were introduced on the hot-plate test.

The collected photometry data was minimally processed with autofluorescence (405 nm) background subtraction and within trial fluorescence normalization. The median value of data points within the 10 minutes of home-cage recordings prior initiation of RS or thermal-plate

experiments was used as the normalization factor. Z-score transformation of the fluorescence data was performed with the RWD in-built software.

Immunostaining, multiplex in situ hybridization, and confocal microscopy

Mice were anesthetized with isoflurane and perfused intracardially with 1X Phosphate Buffered Saline (PBS) (Takara, Japan) and 4% Paraformaldehyde (PFA) (Ted Pella, Inc., USA), consecutively for immunostaining experiments. Fresh brains were harvested for in situ hybridization experiments. For the cFos experiments, brains were harvested 90 minutes after RS assay, and 150 minutes after i.p. clozapine N-Oxide (CNO) administration. Tissue sections were rinsed in 1X PBS and incubated in a blocking buffer (2% Bovine Serum Albumin (BSA); 0.3% Triton X-100; PBS) for 1 hour at room temperature. Sections were incubated in primary antibodies in blocking buffer at room temperature overnight. Antibodies used: cFos: Phospho-c-Fos (Ser32) (D82C12) XP® Rabbit mAb #5348, CST; RFP: 600-401-379, Rockland. Sections were rinsed 1-2 times with PBS and incubated for 2 hours in Alexa Fluor conjugated goat anti-rabbit/ chicken or donkey anti-goat/rabbit secondary antibodies (Invitrogen) at room temperature, washed in PBS, and mounted in VectaMount permanent mounting media (Vector Laboratories Inc.) onto charged glass slides (Globe Scientific Inc.). Multiplex in-situ hybridization (ISH) was done with a manual RNAscope assay (Advanced Cell Diagnostics, USA). Probes were ordered from the ACD online catalogue. We used an upright fluorescence microscope (Khush, Bengaluru) (2.5X, 4X, and 10X lenses) and ImageJ/FIJI image processing software to image, and process images for the verification of anatomical location of cannulae implants. For the anatomical studies the images were collected with 10X and 20X objective on a laser scanning confocal system (Leica SP8 Falcon, Germany) and processed using the Leica image analysis suite. For the Airy Scan Imaging, Zeiss 980 was used (NCBS Central Imaging Core facility).

CUBIC clearing and imaging

In order to visualize the fluorescent neuronal labeling in the cleared brain tissue, 300 µm thick sections were cleared by first washing with 1X PBS for 30 minutes, followed by 2 hour incubation in 50% Cubic L (TCI, Japan)solution (Susaki et al., 2020). Next, the sections were immersed and incubated in 100% Cubic L solution overnight at 37°C. The sections were preserved and imaged in the Cubic R+ (TCI, Japan) solution. For imaging the cleared sections, 10X and 20X objectives were used along with the Leica SP8 Confocal microscope.

CNO Encapsulation

CNO was encapsulated within poly-lactic-*co*-glycolic acid (PLGA, M_w 10–15 kDa, LG 50:50, PolySciTech, IN, USA) microparticles using a single emulsion method. Briefly, 100 mg PLGA and 2 mg CNO were dissolved in 1 mL dichloromethane (DCM) and mixed for 10 minutes. This mixture was homogenized (IKA® T18 digital Ultra Turrax) with 10 mL 1% polyvinyl alcohol (PVA) at 12000 rpm, resulting in an emulsion. This emulsion was added to 100 mL 1% PVA with magnetic stirring to allow DCM to evaporate. After 4 hours, the microparticles were collected by centrifugation (8000 *g*) and washed thrice with deionized water to remove PVA. The suspension was frozen, followed by lyophilization to obtain the CNO-encapsulated microparticles as powder. For experiments, the powder was weighed and resuspended in 1X PBS to get a concentration of 0.5 mg/mL.

Quantification and Statistical Analysis

All statistical analyses (t-test and one way ANOVA test) were performed using GraphPad PRISM 8.0.2 software. ns > 0.05, ∗ $P \leq 0.05$, ∗∗ $P \leq 0.01$, ∗∗∗ $P \leq 0.001$, ∗∗∗∗ $P \leq 0.0005$.

Data availability

Raw data will be made available upon request.

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Competing interests

The authors report no competing interests.

Figure legends

List of Abbreviations

- BSA: Bovine Serum Albumin
- CFA: Complete Freund's adjuvant
- ChR2: Channel rhodopsin 2
- CNO: Clozapine N-Oxide
- DCM: Dichloromethane
- DCZ: Deschloroclozapine
- dLS: dorsal Lateral Septum
- DMSO: Dimetlysulfoxide
- DREADD: Designer Receptors Exclusively Activated by Designer Drugs
- DRN: Dorsal Raphe nucleus
- ELISA: Enzyme-linked immunosorbent assay
- GABA: Gamma-aminobutyric acid
- GAD1: Glutamic acid decarboxylase 1
- GFP: Green Fluorescence protein
- GPU: Graphics Processing Unit
- Hipp: Hippocampus
- HRP: Horseradish peroxidase
- i.p.: Intraperitoneal
- ISH: In-situ hybridization

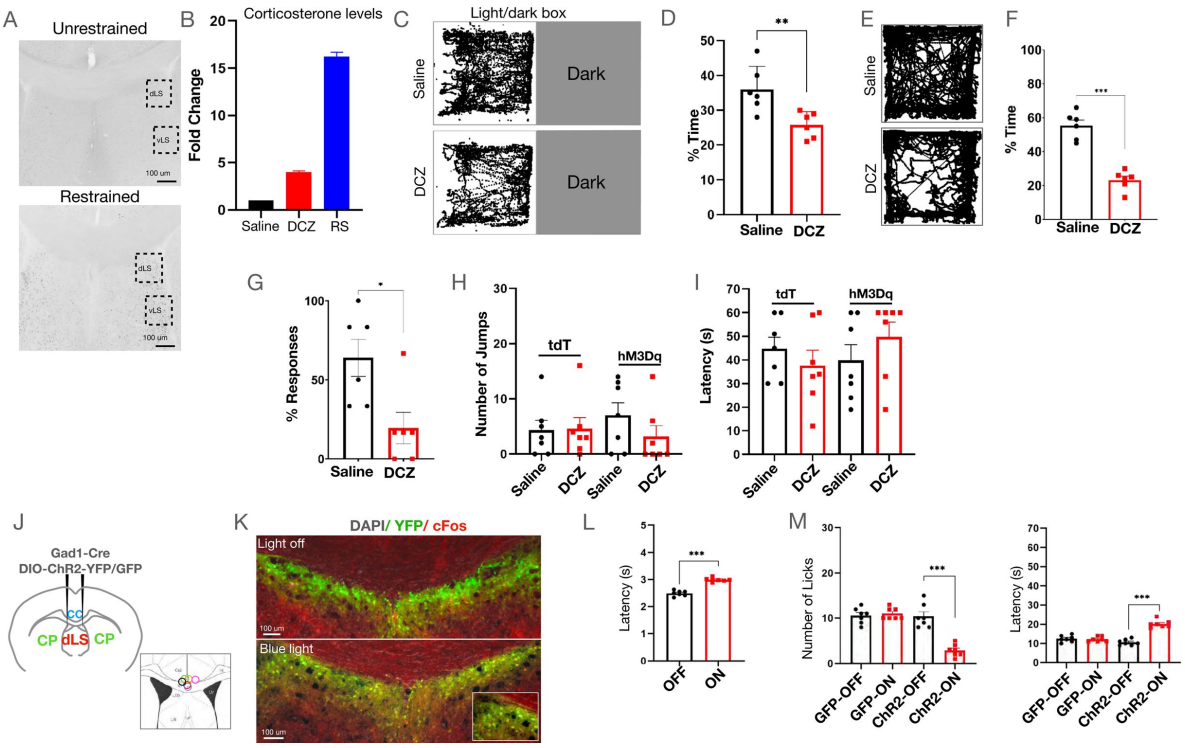


Figure 1-figure supplement 1.

(A) Acute restraint caused elevated cFos expression in the dLS and vLS of mice. (B) Blood corticosterone levels in mice administered with saline, DCZ, or subjected to RS. (C) Sample traces demonstrating time spent by dLS$^{Gad1\text{-}hM3Dq}$ mice in the lit chamber of the classic light-dark box assay to test stress-induced anxiety. (D) Time spent in the light box post-saline or DCZ administration (35.83±2.59 compared to 26.17±1.35, respectively; t-test, $^{**}P$=0.00578, n=6). (E) Sample traces demonstrating activity of dLS$^{Gad1\text{-}hM3Dq}$ mice in an open field test. (F) Percentage of time spent by dLS$^{Gad1\text{-}}$hM3Dq mice in the centre of the open field (55.33±3.21 compared to 23.17±2.36, respectively; t-test, $^{***}P$=0.0001, n=6). (G) Percentage response of mice to the von Frey Filament Test (63.89±11.72 compared to 19.44±10.01, respectively; t-test, $^{*}P$=0.0365, n=5). (H and I) Number and latency (seconds) of jumps post saline or DCZ administration on the hot plate in dLS$^{Gad67\text{-}tdTomato}$ and dLS$^{Gad67\text{-}hM3Dq}$ mice. (J) Gad1-Cre and DIO-ChR2-YFP/DIO-GFP were injected in the dLS of wild-type mice to label the dLS neurons with the opsin. Optic fiber cannulae was implanted over the dLS at the same coordinates to deliver blue light. (K) YFP-positive cells (green) observed in the dLS inhibitory neurons (zoom-in shown in the box). (L) Tail-flick latency (seconds) (2.27±0.05 compared to 3.03±0.03, respectively; t-test, $^{***}P$=0.0001, n=5) with (ON) and without (OFF) blue light illumination in dLS$^{Gad1\text{-}ChR2}$ neurons, with no significant difference in dLS$^{Gad1\text{-}GFP}$ mice. (M) Number (10.43±1.00 compared to 2.86±0.51, respectively; t-test, $^{***}P$=0.0001, n=5) and latency of licks (seconds) (10.57±0.57 compared to 20.14±0.74, respectively; t-test, $^{***}P$=0.0001, n=5) with (ON) and without (OFF) yellow light illumination in dLS$^{Gad1\text{-}ChR2}$ neurons, with no significant difference in dLS$^{Gad1\text{-}GFP}$ mice.

Figure 1-figure supplement 2.

(A) Tail-flick latencies (seconds) over an hour of $^{dLSGad1\text{-}ChR2}$ mice post blue light illumination for 30 minutes (2.33±0.05 compared to 2.74±0.01, respectively; Immediate t-test, *P=0.0321, n=4) (2.34±0.06 compared to 2.85±0.02, respectively; Post-10 minutes t-test, **P=0.00877, n=4) (2.37±0.06 compared to 2.98±0.06, respectively; Post 30 minutes t-test, **P=0.00832, n=4). (B) Tail-flick latencies (seconds) of mice with and without restraint at different time points post the RS assay (2.27±0.04 compared to 2.73±0.03, respectively; Immediate t-test, *P=0.0437, n=4) (2.30±0.05 compared to 2.87±0.06, respectively; Post-10 minutes t-test, **P=0.0057, n=4) (2.29±0.05 compared to 2.97±0.03, respectively; Post 30 minutes t-test, **P=0.0061, n=4). (C) AAVs encoding Gad1-Cre, DIO-hM3Dq-mCherry, and DIO-TetTox-GFP or DIO-GFP were injected in the dLS of wild-type mice to simultaneously label the dLS^{Gad1} neurons with the excitatory DREADD, and TetTox. Overlapping yellow cells representing hM3Dq (red), and TetTox (green) co-expressing neurons in the dLS. (D) Tail-flick latency (in seconds) in $dLS^{Gad1\text{-}TetToxGFP\text{-}hM3Dq}$ mice (2.45±0.07 compared to 2.99±0.05, respectively; t-test, **P=0.00943, n=4), and in $dLS^{Gad1\text{-}TetTox\text{-}hM3Dq}$ mice post saline or DCZ administration. (E) Gad1-Cre and DIO-Tettox-GFP/DIO-GFP injected in the dLS of wild-type mice. (F and G) Number and latency (in seconds) of jumps with and without restraint.

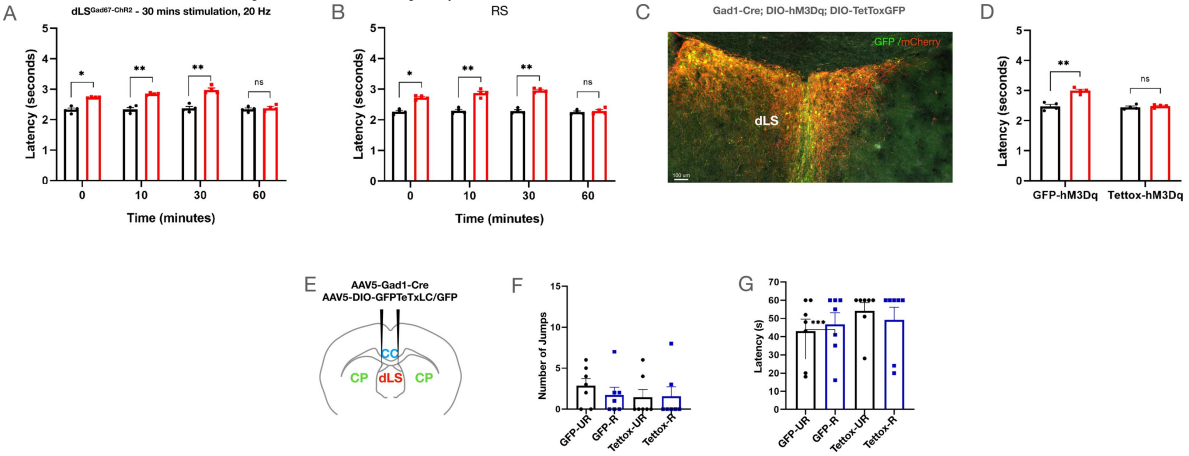
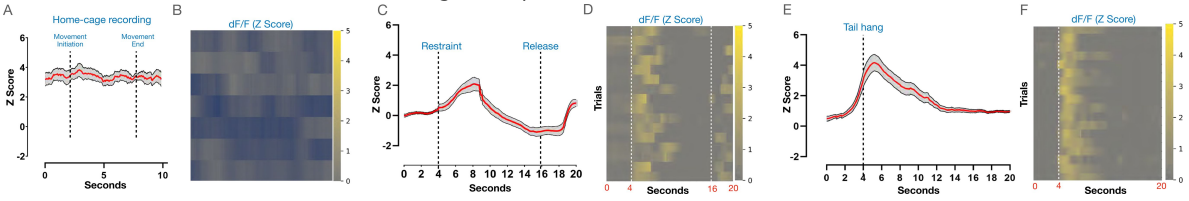


Figure 2-figure supplement 1.

(A and B) Average plot and heat map of $dLS^{GCaMP6s}$ transients for home cage activity. (C and D) Average plot and heatmap (individual trials) of Z-Score traces (5 mice, 16 trails) during immobilization. (E and F) Average plot and heatmap (individual trials) of Z-Score traces (5 mice, 18 trials) during tail suspension.



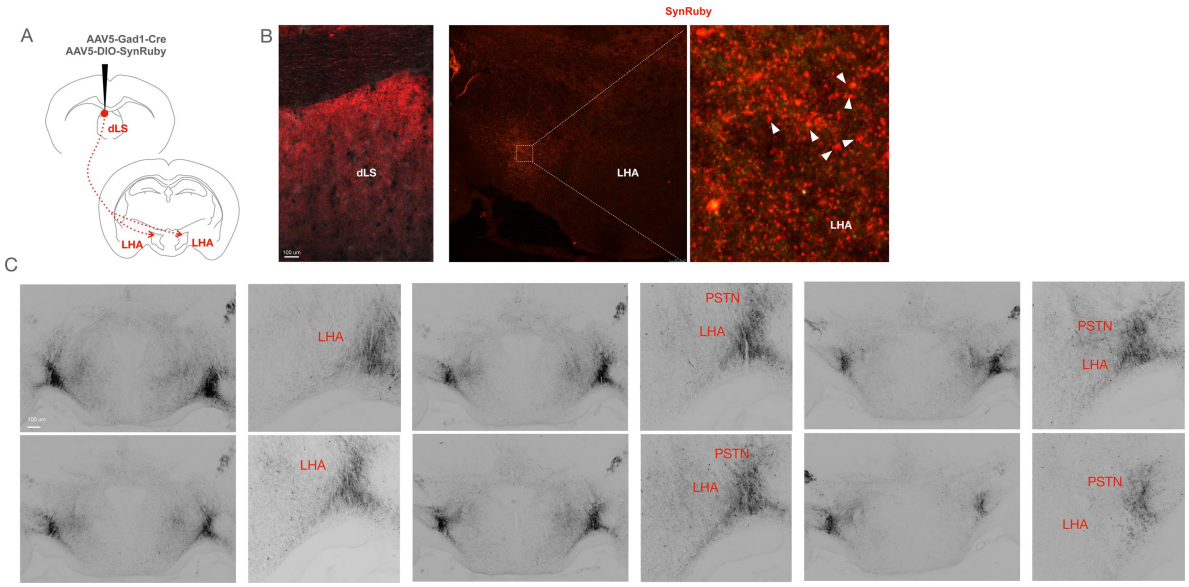


Figure 3-figure supplement 1.

(A) AAVs encoding Gad1-Cre and DIO-SynRuby injected in the dLS of wild-type mice. (B) Ruby expression was seen at the injection site and post-synaptic region (LHA), marked region on LHA shown in zoom-in on the right with synapses marked with arrowheads. (C) Rostral to caudal serial images of the LHA taken on a fluorescence microscope at 4X and 10X magnifications showing projections from the dLS, post Gad1-Cre and DIO-GFP injections in the dLS of wild-type mice.

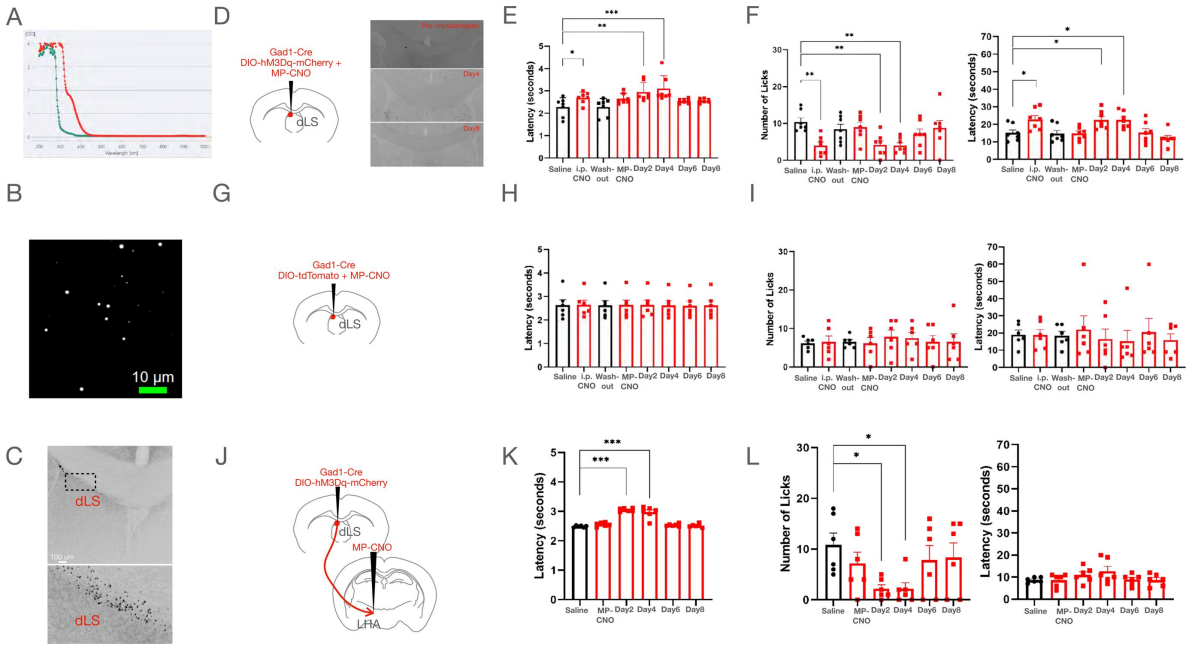


Figure 3-figure supplement 2.

(A) Absorption spectra for the vehicle Dimethysulfoxide (DMSO) (green) and CNO dissolved in DMSO (red). (B) Cy3 fluorescent beads as seen under the microscope at 100X with oil immersion. (C) Cy3 fluorescent beads injected in the dLS and images under the fluorescence microscope at 4X and 10X magnifications (marked region on dLS shown in zoom-in on the bottom). (D) Gad1-Cre and DIO-hM3Dq-mCherry stereotaxically injected in the dLS of wild-type mice to express the excitatory DREADD. 3 weeks later, CNO beads were injected stereotaxically in the dLS over the same coordinates. cFos expression was muted before MP-CNO delivery, increased after 4 days, while reduced on the 8th day of delivery (4.40±1.69 compared to 61.65±3.99, respectively; Control vs Day 4 One-way ANOVA test, ***P=0.0001, n=5), 61.65±3.99 compared to 8.69±5.31, respectively; Day 4 vs Day 8 One-way ANOVA test, **P=0.0048, n=5). (E) Tail-flick latency (seconds) (2.28±0.16 compared to 2.95±0.16, respectively; Day2 One-way ANOVA test, **P= 0.00765, n=7) (2.28±0.16 compared to 3.10±0.22, respectively; Day4 One-way ANOVA test, ***P=0.0001, n=7) over a course of 8 days post CNO beads injection. (F) Number of licks (10.43±1.09 compared to 4.14±1.14, respectively; Day2 One-way ANOVA test, **P=0.00821, n=7) (10.43±1.09 compared to 4.57±1.04, respectively; Day4 One-way ANOVA test, **P=0.0056, n=7), and latency to lick (seconds) (15.14±1.77 compared to 22.57±1.97, respectively; Day2 One-way ANOVA test, *P=0.0328, n=7) (15.14±1.77 compared to 22.57±1.76, respectively; Day4 One-way ANOVA test, *P=0.0412, n=7) over a course of 8 days post CNO beads injection. (G) Gad1-Cre and DIO-tdTomato injected in the dLS of wild-type mice. 3 weeks later, CNO beads were injected stereotaxically in the dLS over the same coordinates. (H) Tail-flick latency (seconds) over the course of 8 days post-CNO beads injection in dLS$^{Gad1\text{-}hM3Dq}$ mice. (I) Number and latency of licks (seconds) post-CNO beads injection in dLSGad1-hM3Dq mice. (J) Gad1-Cre and DIO-hM3Dq were injected in the dLS of wild-type mice. 3 weeks later, CNO beads were injected in the LHA to enable terminal activation of LHA$_{post\text{-}dLS}$ neurons. (K) Tail-flick latency (seconds) (2.49±0.01 compared to 3.05±0.02, respectively; Day2 One-way ANOVA test, ***P=0.0001, n=7) (2.49±0.01 compared to 2.97±0.08, respectively; Day4 One-way ANOVA test, ***P=0.0001, n=7) over a course of 8 days post CNO beads injection. (L) Number of licks (10.83±2.33 compared to 2.17±0.79, respectively; Day2 One-way ANOVA test, *P=0.0435, n=7) (10.83±2.33 compared to 2.17±1.22, respectively; Day4 One-way ANOVA test, *P =0.0387, n=7), and latency to lick (seconds) (10.50±1.69 compared to 31.33±4.98, respectively; Day2 One-way ANOVA test, *P=0.0245, n=7) (10.50±1.69 compared to 40.50±7.36, respectively; Day4 One-way ANOVA test, **P=0.00456, n=7) over a course of 8 days post CNO beads injection.

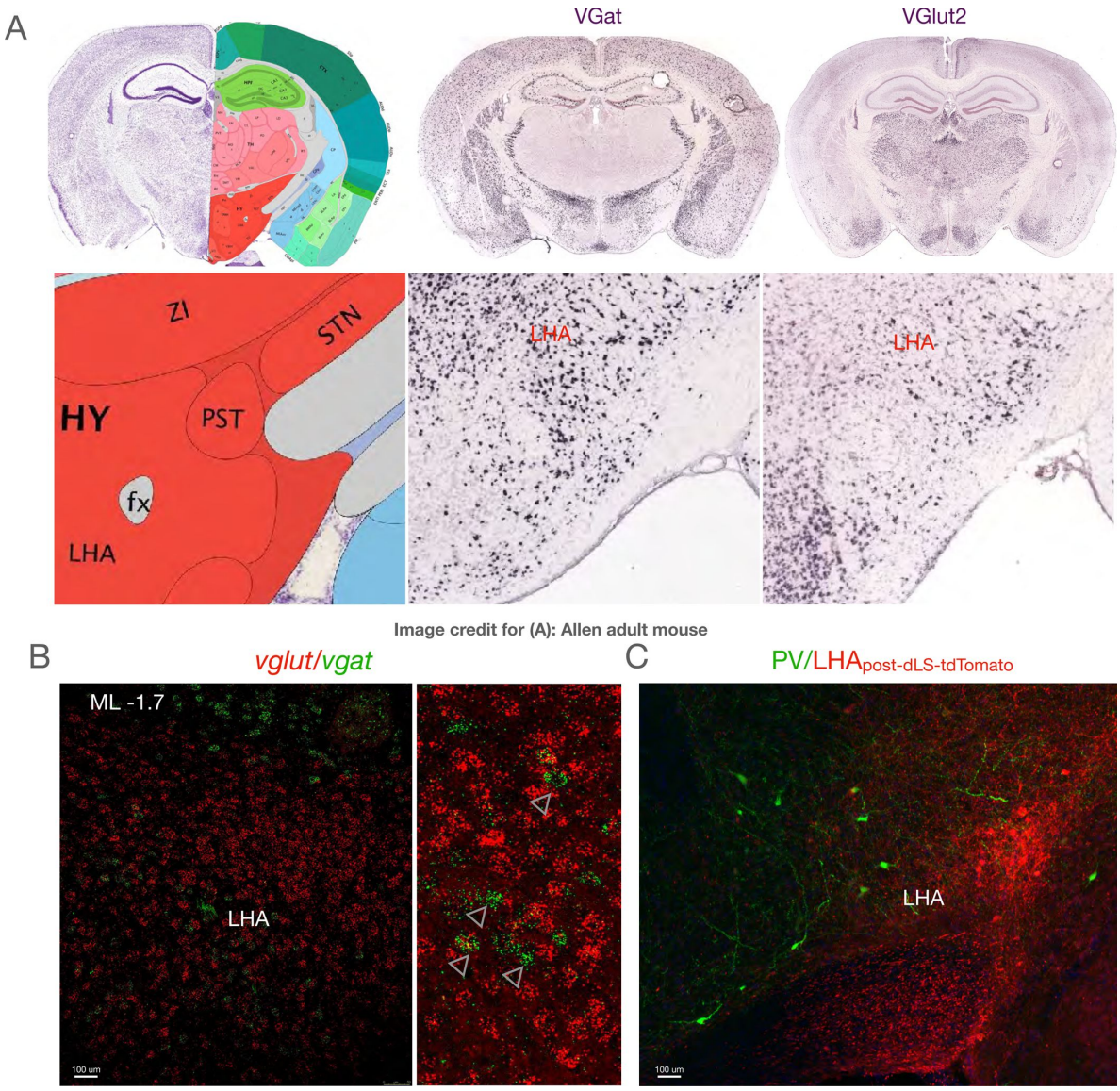


Figure 4-figure supplement 1.

(A) Allen Brain Atlas images showing the anatomical location of the LHA next to the parasubthalamic nucleus (PSTN) and *vglut/vgat* expressing cells in the LHA. (B) In situ hybridization using probes against VGlut2 and VGat performed on LHA sections of wild-type mice (red: *vglut2*, green: *vgat*) (zoom-in of overlapping cells shown on right with overlap marked using arrowheads). (C) Parvalbumin staining in the LHA sections.

Figure 5-figure supplement 1.

(A and B) Average plot and heatmap (individual trials) of Z-Score traces (5 mice, 12 trials) during immobilization of $LHA_{post\text{-}dLS}$ neurons. (C and D) Average plot and heatmap (individual trials) of Z-Score traces (5 mice, 12 trials) during tail suspension of $LHA_{post\text{-}dLS}$ neurons. (E and F) Average plots and heatmaps for dLS responses on the hot-plate at 32 deg of $LHA_{post\text{-}dLS}$ neurons (5 mice, 12 trials; dotted line indicating time point when mice were placed on the hotplate). (G and H) Average plots and heatmaps for dLS responses on the hot-plate at 52 deg of $LHA_{post\text{-}dLS}$ neurons (5 mice, 12 trials; dotted line indicating time point when mice were placed on the hotplate).

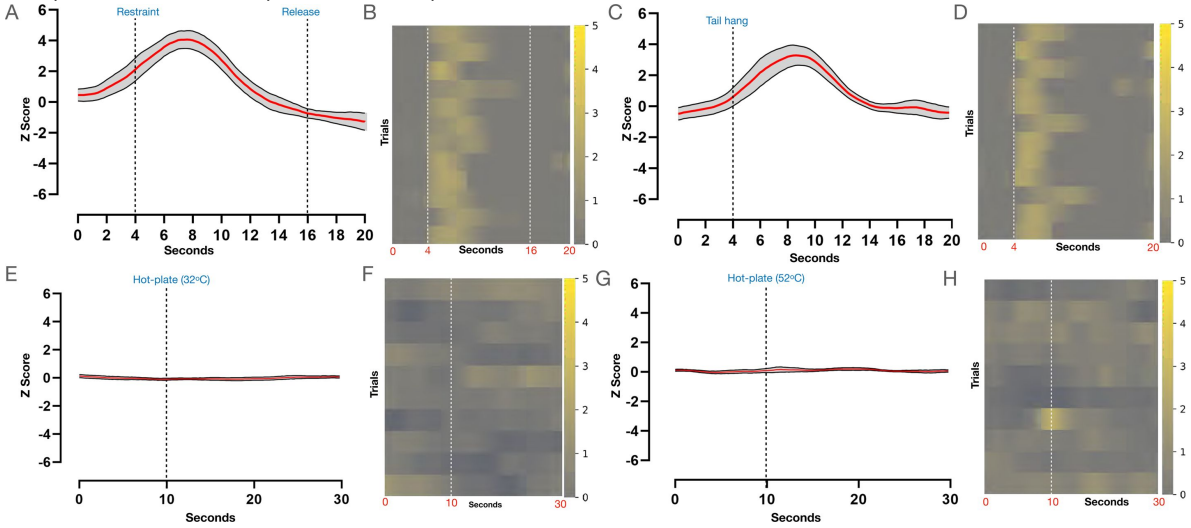
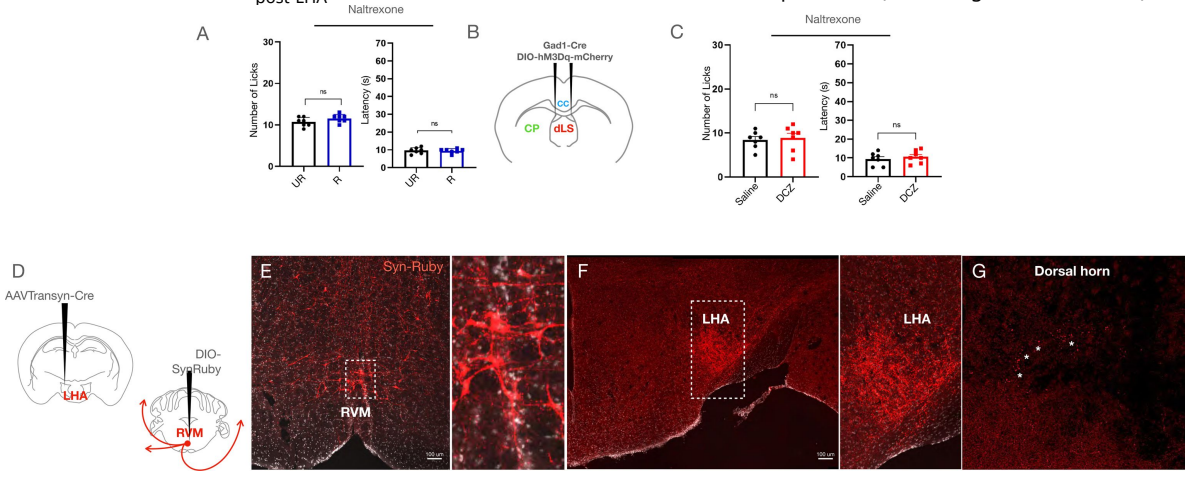


Figure 6-figure supplement 1.

(A) Number and latency to lick before and after RS in wild-type mice i.p. injected with the mu-opioid receptor antagonist naltrexone. (B) Gad1-Cre and DIO-hM3Dq were injected in the dLS of wild-type mice to express the excitatory DREADD, hM3Dq in the dLS^{Gad1} neurons. (C) Number and latency (in seconds) of licks post-saline or DCZ administration in $dLS^{Gad1\text{-}hM3Dq}$ mice post i.p. Naltrexone administration. (D) AAVTransyn-Cre injected in the LHA, and DIO-SynRuby injected in the RVM of wild-type mice. (E) Red cell bodies expressing SynRuby were observed in the RVM (zoom-in of maeked region shown on the right). (F) Red puncta of $RVM_{post\text{-}LHA}$ axon terminals observed in the LHA (zoom-in of marked region shown on the right). (G) Axon terminals of the $RVM_{post\text{-}LHA}$ neurons seen in the dorsal horn of the lumbar spinal cord (* marking distinct neurons).



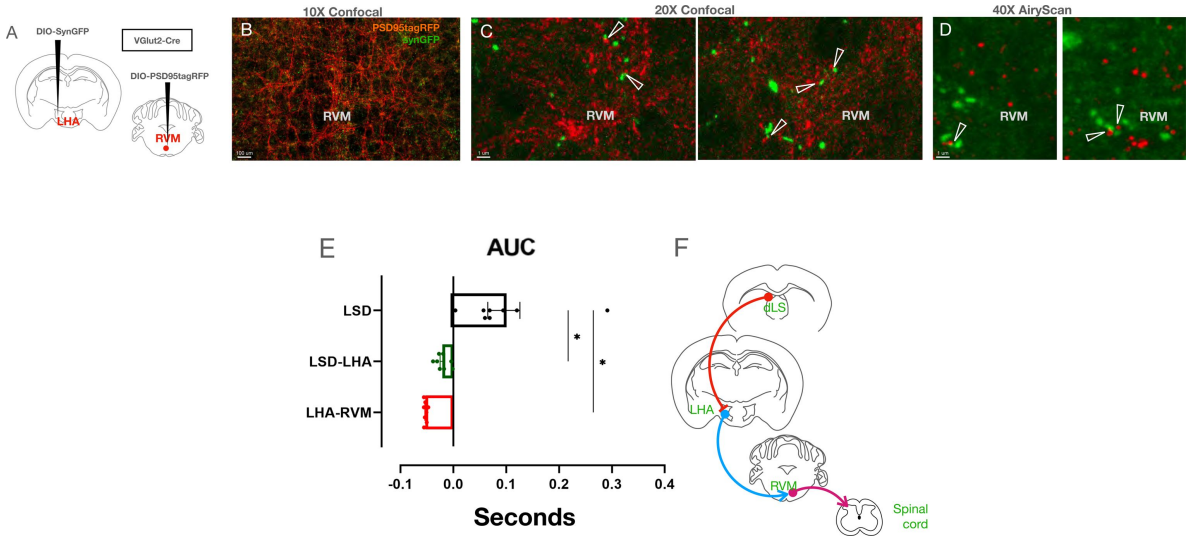


Figure 8-figure supplement 1.

(A) DIO-SynGFP injected in the LHA, and DIO-PSD95-tagRFP in the RVM of VGlut2-Cre transgenic mice. (B) Confocal images of the LHA^{VGlut2} terminals (green), and RVM^{VGlut2} dendrites (red) observed under 10X magnification. (C) Confocal images under 20X magnification of the same regions (closely apposed green and red cells marked with arrowheads, zoom-in on the right). (D) 40X Airyscan super-resolution image demonstrating close apposition between the red, and green puncta (closely apposed green and red cells marked with arrowheads, zoom-in on the right). (E) Bar graph depicting the AUC of the photometry recordings during the struggle bouts from dLS^{Gad1}, $LHA_{post\text{-}dLS}$, and $RVM_{post\text{-}LHA}$ neurons. (F) Diagrammatic representation of the dLS-centric RS-induced SIA pathway that originates from dLS and terminates in the spinal cord via LHA and RVM.

- LHA: Lateral Hypothalamic Area
- LHb: Lateral Habenula
- LPBN: Lateral parabrachial nucleus
- MP: Microparticle
- O.D.: Optical Density
- PAG: Periaqueductal gray
- PBS: Phosphate Buffered Saline
- PFA: Paraformaldehyde
- PLGA: Poly-DL-lactic-co-glycolic
- PV: Parvalbumin
- PVA: polyvinyl alcohol
- RS: Restraint Stress
- RVM: Rostral Ventromedial Medulla
- SIA: Stress Induced Analgesia
- SPF: Specific pathogen-free
- TetTox: Tetanus Toxin
- TMB: Tetramethylbenzidine
- VGat: Vesicular GABA Transporter
- Vglut2: Vesicular glutamate transporter
- YFP: Yellow Fluorescence protein

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Reviewer #1 (Public review):

The manuscript entitled "A septo-hypothalamic-medullary circuit directs stress-induced analgesia" by Shah et al., showed that the dLS-to-LHA circuit is sufficient and necessary for stress-induced analgesia (SIA), which is mediated by the rostral ventromedial medulla (RVM) in a opioid-dependent manner. This study is interesting and important and the conclusions are largely supported by the data. I have a few concerns as follows:

(1) The present data show that activation of dLS neurons produces SIA, however, this manipulation is non-specific. It may be better to see the effect of specific manipulation of stress-activated c-Fos positive neurons in the dLS using combination of the Tet-Off system and chemogenetic/optogenetic tools.
(2) Depending on its duration, and intensity, stress can exert potent and bidirectional modulatory effects on pain, either reducing pain (SIA) or exacerbating it (stress-induced hyperalgesia,SIH). Whether this circuit in the manuscript is involved in SIH.
(3) It are well-accepted that opioid and cannabinoid receptors participate in the SIA, especially, a critical role of the RVM endocannabinoid system in the SIA, why author focus their study on opioid system?
(4) Whether silencing of the dLS neurons affects stress-induced anxiety-like behaviors? Or，what is the relationship between of SIA and level of stress-induced anxiety?
(5) Please provide the direct electrophysiological evidence for confirming the efficacy of the MP-CNO.
(6) Whether LHA is a specific downstream target for SIA, whether LHA is involved in stress-induced anxiety-like behaviors?
(7) Whether LHA neurons have direct projections to the RVM? If yes, what is its role in the SIA?

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Reviewer #2 (Public review):

Shah et al. investigate the role of an understudied neural circuitry, specifically the dLS -> LHA -> RVM pathway, in mediating stress-induced analgesia. The authors use a combination of advanced techniques to provide convincing evidence for the involvement of this circuit in modulating pain under stress.

The study begins by mapping the neural circuitry through a series of intersectional tracings. Following this, the authors use behavioral tests along with optogenetic and chemogenetic

manipulations to confirm the pathway's role in promoting analgesia. Additionally, fiber photometry is employed to monitor the activity of each brain region in response to stress and pain.

While the study is comprehensive and the findings are convincing, a key concern arises regarding the overarching hypothesis that restraint-induced stress promotes analgesia. A more straightforward interpretation could be that intense struggling, rather than stress itself, might drive the observed analgesic responses.

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Author response:

> ***Reviewer #1 (Public Review):***
>
> *The manuscript entitled "A septo-hypothalamic-medullary circuit directs stress-induced analgesia" by Shah et al., showed that the dLS-to-LHA circuit is sufficient and necessary for stress-induced analgesia (SIA), which is mediated by the rostral ventromedial medulla (RVM) in a opioid-dependent manner. This study is interesting and important and the conclusions are largely supported by the data. I have a few concerns as follows:*

We thank the reviewer for finding our study “interesting”, “important”, and “conclusions are largely supported by data”.

> *(1) The present data show that activation of dLS neurons produces SIA, however, this manipulation is non-specific. It may be better to see the effect of specific manipulation of stress-activated c-Fos positive neurons in the dLS using a combination of the Tet-Off system and chemogenetic/optogenetic tools.*

We agree with the reviewer that activating the stress-“trapped” neurons will be more specific way to induce SIA through septal activation, compared to the activation of entire dLS strategy pursued by us. In most likelihood, we expect to see a robust SIA if specifically stress responsive dLS neurons are observed. We are in the process of acquiring the genetic tools required for “Trapping” stress neurons and expect to be able to perform the experiments suggested by the reviewers in the coming months.

> *(2) Depending on its duration, and intensity, stress can exert potent and bidirectional modulatory effects on pain, either reducing pain (SIA) or exacerbating it (stress-induced hyperalgesia, SIH). Is the circuit in the manuscript involved in SIH?*

As mentioned by the reviewer, it would be reasonable to suspect that the dLS neurons are involved in SIH. However, we believe that the experiments to test this hypothesis is outside the scope of this paper, since here we have focused on the circuit mechanisms for SIA. However, in the revised discussion section, we have included the possibility of dLS neurons driving SIH.

> *(3) It is well-accepted that opioid and cannabinoid receptors participate in the SIA, and the evidence is especially strong for the RVM endocannabinoid system. Given this, why did the authors focus their study on the opioid system?*

We agree with the reviewer that dLS-mediated SIA may work through neural circuits centered on RVM expressing receptors for either or both opioids and endocannabinoids. We primarily focused on the opioidergic system in the RVM as decades of mechanistic work has revealed how the ON, OFF, and neutral neurons modulate pain through the endogenous

opioids and even mediate SIA. In the revised discussion, we have included the possibility of involvement of both pain modulatory systems.

> *(4) Does silencing of the dLS neurons affect stress-induced anxiety-like behaviors? Alternatively, what is the relationship between SIA and the level of stress-induced anxiety?*

We did not test if the silencing of dLS would affect stress-induced anxiety, as our focus was on the pain modulatory effects of dLS activation. The relationships between levels of SIA and stress-induced anxiety will be interesting to explore in future. We believe we would need better behavioral assays compared to the existing ones to quantitatively measure levels of stress-induced anxiety and SIA levels.

> *(5) Direct electrophysiological evidence should be provided to confirm the efficacy of the MP-CNO.*

We agree with the reviewer that ex-vivo electrophysiology experiments will substantiate the effectiveness of the MP-CNO. However, we do not have the expertise, or the instrumentation required to perform these experiments in our laboratory.

> *(6) Is the LHA a specific downstream target for SIA, and is the LHA involved in stressinduced anxiety-like behaviors?*

Several lines of evidence points to the fact that LHA neurons are involved in stressinduced anxiety. We have also shown that the dLS downstream neurons in the LHA are activated by acute restraint by fiber photometry recordings. Thus, we expect activation of the LHA neurons will cause stress-induced anxiety. However, we wanted to focus on the pain modulation aspect of the dLS-LHA-RVM circuitry.

> *(7) Do LHA neurons have direct projections to the RVM? If yes, what is its role in the SIA?*

Our anatomical studies using transsynaptic anterograde and retrograde viral strategies in the Figure 6 shows that the LHA neurons have direct projections to the RVM, and these neurons are sufficient in driving hyperalgesia, as well as necessary for SIA.

> ***Reviewer #2 (Public Review):***
>
> *Summary:*
>
> *In this manuscript, Shah et al. explore the function of an understudied neural circuitry from the dLS -> LHA -> RVM in mediating stress-induced analgesia. They initially establish this neural circuitry through a series of intersectional tracings. Subsequently, they conduct behavioral tests, coupled with optogenetic or chemogenetic manipulations, to confirm the involvement of this pathway in promoting analgesia. Additionally, fiber photometry experiments are employed to investigate the activity of each brain region in response to stress and pain.*
>
> *Strengths:*
>
> *Overall, the study is comprehensive, and the findings are compelling.*

We appreciate the reviewer for finding our manuscript “comprehensive” and “compelling”.

> *Weaknesses:*
>
> *One noteworthy concern arises regarding the overarching hypothesis that restrainedinduced stress promotes analgesia. A more direct interpretation suggests that*

> *intense struggling, rather than stress per se, activates the dLS -> LHA -> RVM pathway that may drive analgesic responses.*

We agree with the reviewer that our data can be interpreted as “intense struggling”, rather than the “acute stress” might have altered the pain thresholds in mice. However, we would like to point out that the restraint induced stress model that we have used has been long regarded as a standard for inducing stress. Moreover, we have demonstrated that dLS activation results into acute stress by measuring the blood corticosterone levels, and showed that dLS activations caused stress-induced anxiety through lightdark box tests.

> ***Reviewer #2 (Recommendations For The Authors):***
>
> *Please find below my other comments for improvements.*
>
> *Introduction: The authors claimed that "dLS neurons receive nociceptive inputs from the thalamus and somatosensory cortices." However, citations are missing.*

We have added the citations.

> *Figure 1 B&C: Although this paper focuses on the dLS, it would be informative to also include vLS c-Fos images (maybe in a supplementary figure), given that these data appear to be already acquired. The inclusion of vLS data will provide critical information regarding potential specificity (or lack of) across LS subregions in stress responses.*

In the revised manuscript we have added the vLS c-Fos images as suggested by the reviewer.

> *Figure 1D: Quantification of Vgat vs. Vglut neurons is missing. It is unclear if the Vgat neurons are restricted to small clusters.*

We did not add the Vglut vs, Vgat quantification since from both of our experiments and publicly available data from the Allen Brain Atlas show that almost all of the neurons in the LS are gabaergic. We found very rare,0-2 Vglut2 expressing neurons per section in the the LS of the mouse brain.

> *Figure 1G: The Y-axis label is missing.*

We have added the axis in the revised manuscript.

> *Figure 2: The authors claimed that dLS neurons are preferentially tuned to stress caused by physical restraint. However, it appears that these neurons are specifically tuned to intense struggle behavior (transient) rather than stress (prolonged).*

We agree with the reviewer that the SIA observed in mice with dLS activation, can be interpreted as the effect of transient struggle behavior rather than the prolonged stress. However, we would like to point out that the acute restraint for one hour is known to produce prolonged stress, and is backed up by increased blood coticosterone levels and stress-induced anxiety (Fig1-Fig Supplementary 1).

> *Figure 4: The authors provided compelling evidence that dLS neurons synapse on LHA Vglut2 neurons. However, it is unclear if they exclusively target the Vglut2 neurons or also synapse on LHA Vgat neurons.*

We agree with the reviewer that even though the majority of the dLS downstream neurons in the LHA are glutamatergic, as now shown in the Fig. 4D, few neurons do not express Vglut and thus must be Gabaergic.

> *Figure 5D: It is unclear if the trace represents dLS or LHA calcium signal (in the main text, the authors claimed both).*

Now, we have mentioned the neurons on the LHA we have recorded from at the top of Figure 5C, D.

> *Figure 6 G&H: Presumably, ΔG-Rabies does not transmit across neurons due to the deletion of the glycoprotein (G) gene. Thus, it is unclear why dLS and LHA neurons express mCherry after injecting rabies into RVM.*

The aim of the rabies experiment was to test that the cells in the LHA that receive inputs from the dLS are the same ones that send projections downstream to the RVM. To this end, we used a monosynaptic rabies virus that has retrograde properties. Hence, when injected into the RVM, it was taken up by the terminals of the LHA neurons in the RVM and traveled to the cell bodies in the LHA. We injected the AAV1-Transsyn-Cre in the dLS, so only the cells downstream of the dLS in the LHA can express the Credependent glycoprotein (G) gene. Thus, the rabies-mCherry virus infected the LHA neurons downstream of dLS specifically, and jumped a synapse, to label the upstream dLS neurons.

> *The authors claim that "RVMpost-LHA neurons may modulate nociceptive thresholds through their local synaptic connections within the RVM, recurrent connections with the PAG, or direct interactions with spinal cord neurons." It is unclear what the "local synaptic connections within the RVM" means. It is also unclear whether there is evidence of recurrent connections between the RVM and PAG.*

We meant by local connections as intrinsic connections within the RVM, as in some or few of the RVM neurons, post LHA might be interneurons and mediating SIA by modulating the ON or OFF cells. There are some anatomical evidence for the ascending inputs from RVM to the PAG and the we have now included the citation in the mentioned section of the manuscript.

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