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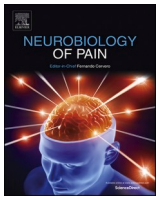

Original Research

Neurotensin-expressing lateral hypothalamic neurons alleviate neuropathic and inflammatory pain via neurotensin receptor signaling

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ABSTRACT

Persistent, severe pain negatively impacts health and wellbeing, but half of patients do not receive adequate relief from current treatments. Understanding signals that modulate central pain processing could point to new strategies to manage severe pain. Administering Neurotensin (Nts) or Nts receptor (NtsR) agonists into the brain provides analgesia comparable to pharmacologic opioids. However, the endogenous sources of Nts that modify pain processing and might be leveraged for pain relief remained unknown. We previously characterized a large population of Nts-expressing neurons in the lateral hypothalamic area (LHA^{Nts} neurons) that project to brain regions that participate in descending control of pain processing. We hypothesized that LHA^{Nts} neurons are an endogenous source of Nts and activating them would alleviate pain dependent on Nts signaling via NtsRs. To test this, we injected Nts^{Cre} mice in the LHA with AAVs to cre-dependently express either mCherry (Control) or the excitatory hM3Dq in LHA^{Nts} neurons, permitting their stimulation after treatment with the hM3Dq ligand clozapine N-oxide (CNO). Activating LHA^{Nts} neurons had no effect on thermal pain and mechanical responses in naïve mice. By contrast, both spared nerve injury- (SNI) and complete Freund's adjuvant (CFA)-induced mechanical hypersensitivity was completely reversed by CNO-stimulation of LHA^{Nts} neurons. Pretreatment with the Nts receptor antagonist SR142948 reduced CNO-mediated analgesia, indicating that LHA^{Nts} neurons alleviate chronic pain in an Nts receptor-dependent manner. Taken together these data identify LHA^{Nts} neurons as an endogenous source of Nts that modulates central pain processing and may inform future development of Nts-based targets to treat severe pain.

1. Introduction

Severe, persistent pain is prevalent, including in the United States where 20 % of adults experience chronic pain (Domenichiello and Ramsden, 2019; Yong et al., 2022). Severe chronic pain differs from acute pain in that it continues past the normal healing time, often persisting for months or years (Institute of Medicine (US) Committee on Advancing Pain Research C, and Education, 2016). As a result, it has a sustained negative impact on quality of life, causing substantial economic burden due to cost of treatment, lost productivity, and disability (Gaskin and Richard, 2012; Mansfield et al., 2016; Phillips, 2009). There are few effective interventions. Opioid analgesics have increased risk for dependence and have fueled the opioid epidemic (Stokes et al., 2019). Non-opioid pain medications have adverse effects that often preclude their use, such as weight gain (Bonomo et al., 2022; Callaghan and Feldman, 2013; Callaghan et al., 2016; Christensen et al., 2020). Better understanding of the brain cells and endogenous signals that mediate antinociception could point to strategies to leverage such systems and inform the development of effective pain treatments.

The peptide Neurotensin (Nts) is produced in the periphery and brain and has promise to modulate pain. Nts signals via the high-affinity neurotensin receptor-1 (NtsR1) and low affinity neurotensin receptor-2 (NtsR2), which are G-protein coupled receptors (Chalon et al., 1996; Vita et al., 1993; Vita et al., 1998). Nts signaling in the peripheral nervous system and spinal cord provides analgesia via yet unclear mechanisms (Chen et al., 2022; Demeule et al., 2014; Guillemette et al., 2012; Svane et al., 2022). The central Nts system also modulates pain and augmenting Nts signaling in the brain provides analgesia in a variety of

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pain modalities (Boules et al., 2013; Clineschmidt et al., 1982; Dobner, 2006). Furthermore, pharmacological stimulation of NtsR1 or −2 in the brain alleviates neuropathic pain induced by peripheral nerve injury (Demeule et al., 2014; Sarret et al., 2005; Svane et al., 2022). However, the previous difficulty in visualizing Nts expressing cells prevented determination of which brain cells release endogenous Nts to mediate the analgesic effect. Pharmacological studies were not able to resolve this question since administration of Nts or NtsR agonists into different brain regions causes varying effects, from suppression of feeding, invoking drinking, hyper or hypo-locomotion, vasodepression, and analgesia (Demeule et al., 2014). This is consistent with the role of central Nts as a neuropeptide; specific neural pathways may produce and release Nts to regionally and neurochemically distinct NtsR-expressing target cells, which can result in different physiologic effects. Defining the brain site of Nts-mediated analgesia is necessary to determine how to bias the Nts system for pain relief and avoid the adverse effects attributed to systemic/hindbrain Nts treatment (Ciriello and Zhang, 1997; Fantegrossi et al., 2005).

One promising candidate is the Nts-expressing neurons in the lateral hypothalamic area (LHA) of the brain, referred to as LHA^{Nts} neurons (Brown et al., 2018; Leinninger et al., 2011). The LHA is responsive to noxious stimuli (Dafny et al., 1996; Drago et al., 1998; Lopez et al., 1991) and electric stimulation of the LHA provides analgesia in acute and chronic pain states (Aimone et al., 1988; Aimone and Gebhart, 1987; Lopez et al., 1991). However, the LHA is heterogeneous, containing many molecularly defined neuronal populations that mediate distinct aspects of physiology (Mickelsen et al., 2019).

Some LHA cell types have been implicated in pain modulation (Bonnavion et al., 2016; Gu et al., 2023; Haghparast et al., 2020; Holden et al., 2009; Wang et al., 2022), but there is not yet a comprehensive understanding of which LHA subsets and signals released from them modulate nociception. Intriguingly, stress increases *Nts* expression in the LHA that may contribute to stress-induced analgesia (Seta et al., 2001). However, it was unknown whether LHA^{Nts} neurons mediate anti-nociception in severe pain modalities. Here we used chemogenetics to activate LHA^{Nts} neurons, revealing them as an endogenous source of Nts that contributes to pain processing.

2. Methods

2.1. Mice

All mice used in this study were bred and housed in 12 h light/ 12 h dark cycles and were cared for by Campus Animal Resources (CAR). We produced and studied male and female heterozygous $Nts^{Cre/+}$ mice (Leinninger et al., 2011) on the C57/BI6J background. All experimental protocols were conducted in accordance with the Institutional Animal Care and Use Committee (IACUC) at Michigan State University and with the Association for Assessment and Accreditation of Laboratory Animal Care and the National Institutes of Health. For experiments, 4- to 6-week-old mice were randomly assigned to control or experimental groups with littermate, age-matched, and gender-matched controls. Following stereotaxic surgeries mice were individually housed with *ad libitum* access to water and chow (Harlan Teklad # 7913) for the duration of experiment, unless specified otherwise.

2.2. Surgical procedures

Nts^{Cre} mice were anesthetized with a 2–4 % isoflurane/oxygen mixture, fitted into a stereotaxic apparatus, and given Meloxicam (5 mg/kg, i.p.) prior to using a scalpel to expose the skull. Small holes were drilled in each hemisphere of the skull to permit injection of either AAV2-hSyn-DIO-mCherry (control, Addgene, 50459-AAV2) or AAV2-hSyn-DIO-hM3Dq (Gq)-mCherry (excitatory DREADDq, Addgene, 44361-AAV2) into the LHA (300nL per side, bregma: − 1.34 mm, midline: ±0.95 mm, skull surface: −5.25 mm) per the Franklin and Paxinos brain atlas (Paxinos and Franklin, 2001). The injector was left in place for 10 min to ensure absorption of the AAV, then after retracting the hole in the skull was filled with bone wax and the incision was closed. Mice were allowed to recover from surgery for at least 2 weeks prior to testing.

2.3. Treatments

The hM3Dq DREADD-ligand clozapine N-oxide (CNO, 0.3 mg/kg) or vehicle (VEH, in this case phosphate buffered saline) were administered to mice via i.p. injection. CNO (Sigma Aldrich, Cat # C0832) was dissolved in 10 % β-cyclodextrin (Sigma Aldrich, Cat # C0926) in sterile PBS to make 20X CNO stock aliquots (1.2 mg/ml). A 20X aliquot was diluted with sterile PBS just prior to the experiment to make the 1X working solution (60 μg/mL). We then administered 5 μL of 1X CNO per gram of body weight. Unless specified otherwise, mice were treated once 30 min prior to pain testing, 1–2 hrs after onset of light cycle, via a crossover design. Thus, all mice received both VEH and CNO on different days.

For experiments examining the involvement of Nts signaling in pain regulation, 90 min before each test mice from each AAV group received i.p. pretreatment with either saline or the NtsR pan-antagonist, SR142948 (1 mg/kg) that has been reported to block signaling via NtsR1 and NtsR2 (Binder et al., 2004; Gully et al., 1997), followed 30 min later by VEH or CNO (to activate LHA^{Nts} neurons). SR142948 (TOCRIS Bioscience, 2309/1) was dissolved in 0.1 % Tween 80 in sterile PBS to make the 20x stock solution (2 mg/ml), which was diluted with sterile PBS prior to the experiment to make the 1x working solution (0.1 mg/ml). We administered 10 μ*L* of 1X SR142948 per gram of body weight.

2.4. Perfusion and Immunofluorescence

After completion of all behavioral experiments, mice received i.p. injection of VEH or CNO 90 min before perfusion. Mice were then given a lethal i.p. dose of pentobarbital sodium (Fatal Plus C II, Vortech, SKU # 035946), and transcardially perfused with 1x PBS (pH 7.4) followed by 10 % neutral buffered formalin (Epredia, Cat # 5725). Whole brains were extracted and post-fixed in 10 % formalin overnight at 4 °C then transferred to 30 % sucrose in 1x PBS for storage at 4 °C until further processing.

For immunostaining, brains were coronally sectioned (30 μm thick) using a Leica SM2010R microtome (Leica Biosystems) and collected in 1 % formalin/PBS solution. For cFOS immunohistochemistry, brain sections were blocked with 3 % normal donkey serum in 1x PBS with 0.3 % Triton X for an hour followed by incubation in goat anti-cFOS primary antibody (1:1000, Santa Cruz Biotechnology, sc-52-G). The following day the sections were washed with PBS prior to incubation with secondary antibodies conjugated to donkey anti-goat biotin (1:100, Jackson ImmunoResearch, Cat # 705-065-147), then the immunolabeled cFOS was visualized using a DAB peroxidase substrate kit per the manufacturer's protocol (Thermo Scientific, Cat # 34065). Sections were then washed in PBS prior to overnight incubation at room temperature with rabbit-dsRed/mCherry (1:1000, Takara, 632496). The next day the sections were rinsed and incubated for 2 hr in donkey anti-rabbit Alexa-568 to visualize DREADD-mCherry-expressing LHA^{Nts} neurons. Brain sections were mounted on slides for analysis using an Olympus BX53 fluorescence microscope. LHA images were obtained at 4x and 20x resolution via Cell Sens software. Images were analyzed using Photoshop (Adobe) to assess co-localization and to count cells. For DAB-labeled cFOS-expressing cells, images were taken with transmitted light and later pseudo-colored blue for ease of visualization and counting using Adobe Photoshop.

mCherry expression was used for posthoc evaluation of AAV targeting in the LHA. Mice were only included in the final data set if mCherry-expressing soma were bilaterally confined within the LHA.

Mice with mCherry soma located outside of the LHA were excluded, as were mice found to only have unilateral LHA targeting.

2.5. Thermal nociception (hot plate test)

A cylindrical plexiglass enclosure was placed on a hot plate (Ugo Basile, Hot/Cold plate). Mice were *i.p.* injected with VEH or CNO prior to thermal pain testing. After 30 mins, they were gently placed on the hot plate and their paw withdrawal latency (PWL), jump latency, and number of jumps were measured at 48 °C, 50 °C, and 52 °C. A latency of 60 secs was used as a cutoff time and mice were removed from the hot plate after cutoff time to avoid tissue damage, as described previously (Inyang et al., 2024).

2.6. Mechanical nociception (von Frey test)

Mice were acclimatized for 30 mins in clear plexiglass enclosure (10 × 10 × 13 cm^3) on a wire mesh grid floor. Von Frey filaments (Aesthesio, Cat # 514000-20C) ranging from 0.008 g to 2.0 g were used to measure mechanical allodynia, which was defined as the hind paw withdrawal response to von Frey hair stimulation, by a blinded investigator using the "up and down" method, as described previously (Chaplan et al., 1994; Laumet et al., 2015). Nociception responses were recorded when mice exhibited paw withdrawal, licking, and shaking upon filament application. Briefly, starting with the 0.4 g von Frey filament, pressure was applied perpendicular to the midplantar surface of the hind paw. Filament strength was increased until there was a paw withdrawal response or decreased until mice no longer exhibited a response.

2.7. Inflammatory pain (complete Freund's adjuvant, CFA)

To induce inflammatory pain, mice were briefly anesthetized under 2 % isoflurane and paw thickness was recorded using vernier caliper. Mice then received intra-plantar injection of 5 μl CFA (Sigma Aldrich Cat # F5881) in one hind paw and saline injection (control) in the other hind paw. Mice were returned to their home cages and inflammation was confirmed by redness and increased paw thickness due to swelling 24 h after CFA administration. Pain testing was conducted between 2-, and 6-days post-CFA injection.

2.8. Neuropathic pain (spared nerve injury, SNI)

Mice underwent SNI surgery to induce neuropathic pain (Bourquin et al., 2006; Laumet et al., 2015). The sciatic nerve in the left hind leg was exposed under isoflurane anesthesia, then the tibial and common peroneal nerves were transected, whereas the sural branch was left intact. In sham-operated mice, the nerves were exposed but remained untouched. Mice were returned to their home cages. Pain testing was conducted 7- and 28- days post-nerve injury.

2.9. RNAScope

To evaluate *Nts* expression, SNI mice were perfused 28 days after SNI injury. For *Nts* mRNA expression post CFA, a separate cohort of wildtype mice were injected with either saline in both paws or CFA in one hind paw and saline in the other hind paw. They were perfused 24 h after CFA injection to collect mRNA data. Three LHA- containing sections (30 μm thickness) were picked per mouse brain, as per (Woodworth et al., 2018). RNAScope single-plex assay (Advanced Cell Diagnostics, catalog # 322360) was performed using the manufacturer's protocol. Free floating sections were washed with 1x PBS, and incubated in Pretreatment I (RNAScope H_2O_2, Advanced Cell Diagnostics, Cat # 322335) at room temperature until bubbling stopped (45–60 min). Slices were then washed with 0.5x PBS before carefully mounted on positively charged slides (Superfrost Plus Microscope slide, Cat # 12-550-15). Once mounted, sections were washed with dH_2O and dried overnight at 60 °C in HybEZ II oven. Dried sections were incubated in 1x Pretreatment II (Target Retrieval Agent) at 99–104 °C for 5–10 min, and then washed with dH_2O, dried at room temperature followed by 100 % EtOH wash. Dried sections were then incubated in Pretreatment III (RNAScope Protease Plus, Advanced Cell Diagnostics, catalog # 322331) for 15 min at 40 °C in HybEZ II oven and then washed with dH_2O. Sections were incubated with Nts probe (Advanced Cell Diagnostics, catalog # 420441) for 2 hrs at 40 °C. For target-specific hybridization, sections were incubated in Amp1-6, followed by application of Fast-Red-A and Red-B for 10 min for visualization. Sections were washed with dH_2O then dried by briefly dipping in xylene and cover slipped using antifade mounting media.

2.10. Cell counting

The LHA was identified in 10x images relative to anatomical landmarks (e.g. located just above the fornix and lateral to the mammilothalmic tract). Cells were counted from three LHA-containing brain sections to represent the extent of the LHA (Bregma −1.22, −1.34 and −1.46). For cFOS and mCherry immunolabeling, 10x images of the left and right LHA hemisphere were collected from the chosen sections and were given a coded name to ensure blinded, unbiased analysis. Using Photoshop software, a blinded investigator counted the number of red (mCherry) and blue (DAB-labeled cFos, pseudocolored blue in Photoshop) −labeled cells in each image only in the LHA. Co-labeling was recorded only if mCherry-labelled soma encompassed a cFOS-labeled nucleus.

For quantitative analysis of *Nts* mRNA after RNAScope, 10x LHA images were analyzed by a blinded investigator using FIJI ImageJ. Corrected Total Cell Fluorescence (CTCF) was calculated by taking the integrated density (IntDen) of the region of interest (ROI) and subtracting from it the background mean (BGMean) times the area of the ROI. The formula used for this calculation is CTCF = IntDen-Area x BGMean. The Grubbs outlier test identified an outlier in the Saline-treated control group of the CFA experiment, so it was removed from the data set. Graphed data represent mean ± SEM.

For assessment of *Nts* mRNA expression in SNI mice 28 days after injury, the CTCF data was collected bilaterally from the LHA and averaged. For the CFA experiment, *Nts* mRNA data was measured 24 hr after treatment from both sides of the LHA. *Nts* expression was only increased on the side of the LHA contralateral to CFA treatment, hence only the contralateral data is graphed.

2.11. Statistics

Student's t-tests and 2-way ANOVA were calculated using Prism 7 (GraphPad). Repeated measures 2-way ANOVA with Sidak's multiple comparisons test was used when each mouse received both VEH and CNO, and when data from the same mice were collected at different time points. Ordinary 2-way ANOVA with Tukey post-test was used if mice did not receive both treatments. A p-value of < 0.05 was considered statistically significant. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

3. Results

3.1. Chemogenetic strategy successfully activates LHA^{Nts} neurons

Nts^{Cre} mice were injected bilaterally in the LHA with cre-inducible AAV-hSyn-DIO-mCherry (control) or AAV-hSyn-DIO-hM3Dq(Gq)-mCherry to express the excitatory DREADD hM3Dq in LHA^{Nts} neurons (Fig. 1A). Posthoc analysis for mCherry fluorescence confirmed LHA targeting and expression of mCherry in LHA soma and fibers of control mice (Fig. 1B) and mCherry-hM3Dq expressing soma and processes in experimental mice (Fig. 1C). To confirm CNO-mediated stimulation of hM3Dq-expressing LHA^{Nts} neurons mice were treated with either PBS as

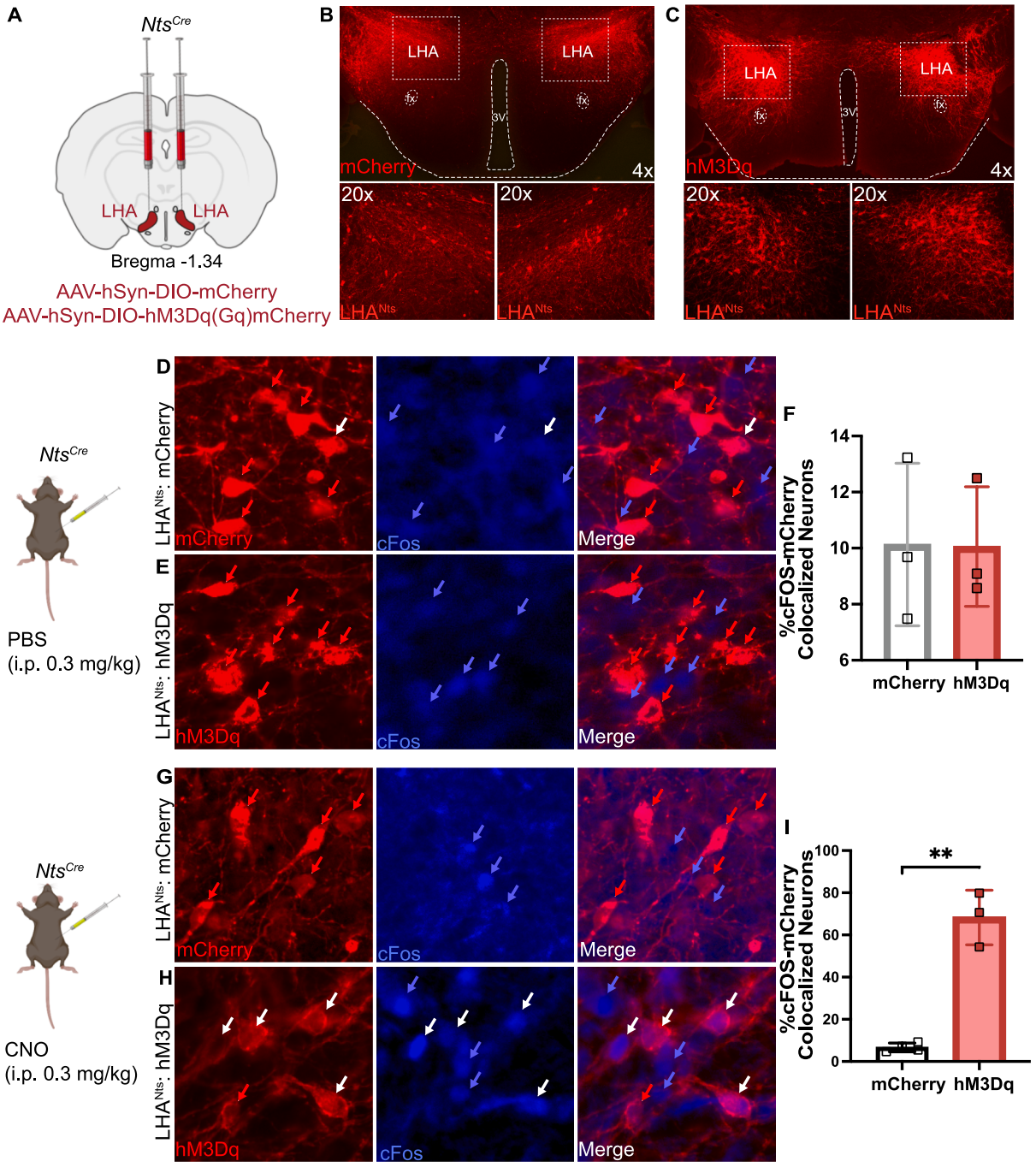

Fig. 1. Chemogenetic strategy successfully activates LHA^{Nts} neurons. A) 12 week-old male and female *Nts*^{Cre} mice were bilaterally injected in the LHA with either pAAV-hSyn-DIO- mCherry to express mCherry (LHA^{Nts}:mCherry Control mice) or pAAV-hSyn-DIO-hM3Dq(Gq)-mCherry to express excitatory hM3Dq-mCherry in LHA^{Nts} neurons (LHA^{Nts}:hM3Dq mice). B, C) mCherry fluorescence (red) in the LHA of B) LHA^{Nts}:mCherry control mice and C) LHA^{Nts}:hM3Dq mice. Top row: 4x magnification images to show AAV injection sites. Bottom row: 20x magnification images showing mCherry-expressing soma. Images representative of 6 mice per group. D-F) Immunoreactivity for mCherry (red) and cFOS (a marker of neuronal depolarization, blue) in the LHA of D) LHA^{Nts}:mCherry control mice (n = 3) and E) LHA^{Nts}:hM3Dq mice (n = 3) treated with PBS or G,H) CNO 90 min before brain collection. Red arrows indicate Nts neurons that only express mCherry, blue arrows indicate neurons that only express cFOS (middle) and white arrows indicate neurons that co-express mCherry and cFOS. F,I) Average percentage of LHA mCherry neurons co-expressing cFOS, error bars indicate SEM. **$p < 0.05$ via unpaired *t*-test with Welch's correction. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

control or CNO 90 min prior to perfusion, then brains were analyzed for mCherry/mCherry-hM3Dq (red) and cFOS (a marker of neuronal stimulation, blue). Few neurons with co-localized cFos and mCherry were observed in control mCherry-expressing and mCherry-hM3Dq-expressing neurons after PBS injections (Fig. 1D-F) but CNO significantly increased cFOS expression in hM3Dq-mCherry expressing LHA^{Nts} neurons without any change in mCherry-expressing neurons (Fig. 1G-I). These data suggest that this effect is specific to CNO-mediated treatment in DREADDq-expressing neurons and PBS treatment has no impact on hM3Dq-mCherry or mCherry-expressing *Nts*^{Cre} mice. Together, these data confirm that CNO treatment selectively activates hM3Dq-mCherry-expressing LHA^{Nts} neurons, and henceforth we used this chemogenetic method to determine whether activating them impacts nociception.

3.2. Chemogenetic stimulation of LHA^{Nts} neurons does not regulate thermal pain in Naïve mice

Early studies reported that intracisternal administration of Nts reduces acute thermal nociception (Clineschmidt and McGuffin, 1977; Clineschmidt et al., 1979), but did not verify the endogenous source of Nts mediating thermal antinociception. Here we asked whether stimulating LHA^{Nts} neurons can alter responses to thermal noxious stimuli. Nts^{Cre} mice were bilaterally injected in the LHA with Cre-recombinase dependent AAVs to express the fluorophore mCherry (LHA^{Nts}:mCherry) or hM3Dq-mCherry in LHA^{Nts} neurons (LHA^{Nts}:hM3Dq) (as in Fig. 1A). After recovery mice were treated with either VEH or CNO prior to hot plate testing at 48 °C, 50 °C and 52 °C. Thermal responses in female and male mice were similar in the LHA^{Nts}:mCherry and LHA^{Nts}:hM3Dq groups, indicating no underlying sex differences (data not shown) so data from each sex were pooled for analysis. LHA^{Nts}:mCherry mice exhibited similar thermal responses after treatment with VEH and CNO, indicating that CNO does not cause any off-target regulation in mice lacking the hM3Dq receptor (Fig. 2A, C & E). In LHA^{Nts}:hM3Dq mice, CNO-mediated stimulation of LHA^{Nts} neurons did not alter paw withdrawal latency (Fig. 2B), jump latency (Fig. 2D), or the number of jumps (Fig. 2F) in response to any of the tested temperatures. These data suggest that stimulation of LHA^{Nts} neurons does not regulate acute thermal nociception in naïve mice.

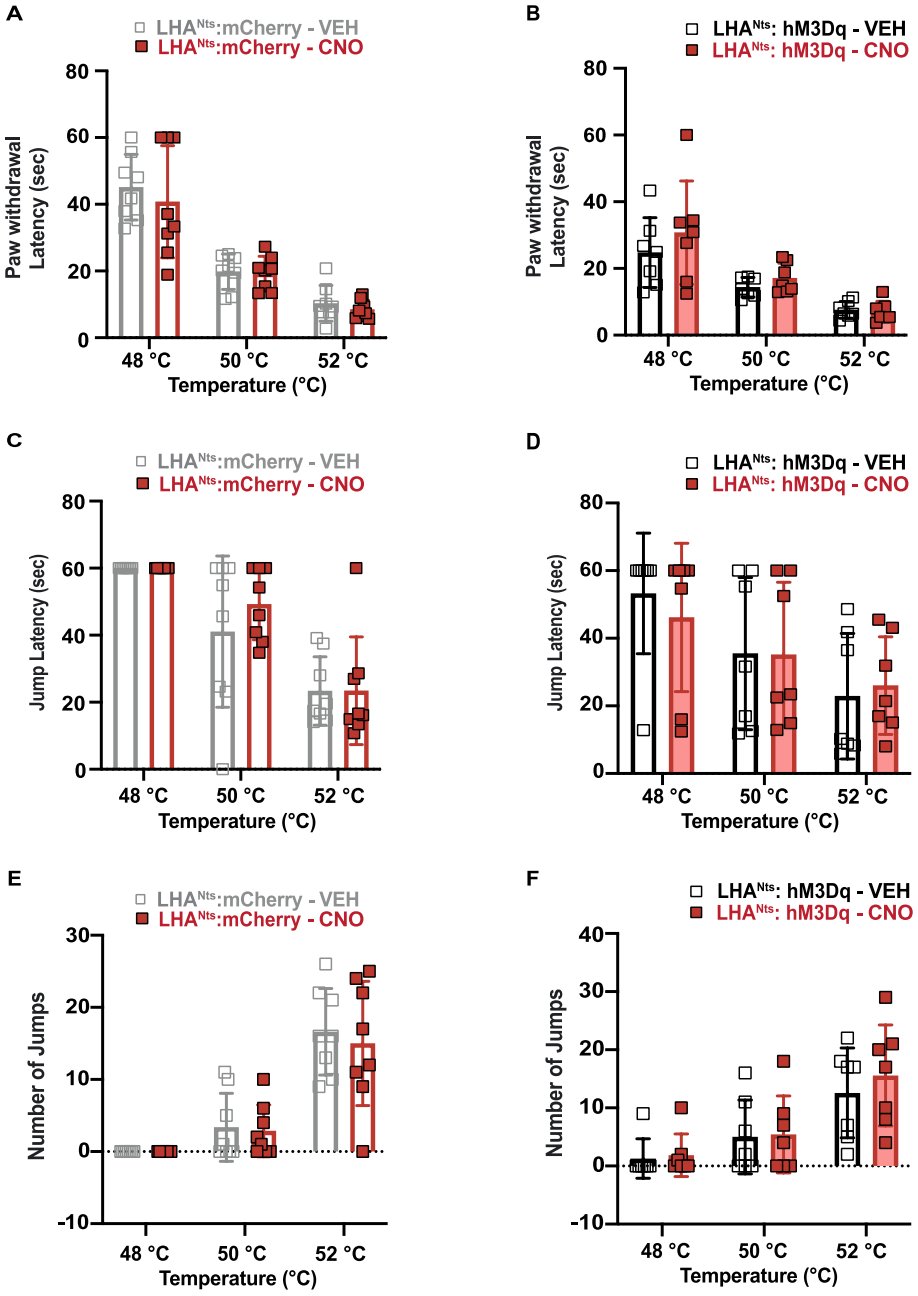

Fig. 2. Chemogenetic Stimulation of LHA^{Nts} Neurons Does not Regulate Thermal Pain in Naïve Mice. Adult *Nts^{Cre}* mice were injected in the LHA with pAAV-hSyn-DIO- mCherry to express mCherry (LHA^{Nts}:mCherry Control mice, n = 8) or pAAV-hSyn-DIO-hM3Dq(Gq)-mCherry to express excitatory hM3Dq-mCherry in LHA^{Nts} neurons (LHA^{Nts}:hM3Dq mice, n = 7) and assessed for thermal pain responses via hot plate test. A-F) Mice were placed on hot plate 30 min after i.p. treatment with VEH (control) or CNO (to activate LHA^{Nts} neurons). A, C & E) Treatment with VEH or B, D & F) CNO has no effect on paw withdrawal latency (A, B), jump latency (C, D) and number of jumps (E, F) in LHA^{Nts}:mCherry and LHA^{Nts}:hM3Dq group at 48 ℃, 50 ℃, and 52 ℃ temperature. No statistically significant differences were found between treatments as analyzed via two-way ANOVA with Sidak post-tests.

3.3. Chemogenetic stimulation of LHA^{Nts} neurons does not alter mechanical sensitivity in Naïve mice

We next tested whether activating LHA^{Nts} neurons altered mechanical sensitivity thresholds in naïve healthy mice. Mice expressing mCherry (LHA^{Nts}: mCherry) or hM3Dq-mCherry in LHA^{Nts} neurons (LHA^{Nts}:hM3Dq) were treated with VEH and CNO prior to measuring paw withdrawal threshold via the von Frey test (Fig. 3A). CNO– treatment had no significant effect on paw withdrawal in LHA^{Nts}:hM3Dq mice compared to VEH over 5 h, nor compared to LHA^{Nts}:mCherry control mice treated with VEH or CNO (Fig. 3B). These data suggest that stimulation of LHA^{Nts} neurons does not alter mechanical sensitivity in healthy mice.

3.4. Chemogenetic stimulation of LHA^{Nts} neurons attenuates persistent neuropathic pain-induced mechanical hypersensitivity

Previous studies have shown that chemical (Holden and Naleway, 2001; Holden et al., 2009) or electrical (Dafny et al., 1996; Lopez et al., 1991) stimulation of the LHA alleviates neuropathic pain in animal models. Given that LHA^{Nts} neurons are a subset of LHA neurons we asked whether specifically activating them after nerve injury would be sufficient to reverse mechanical hypersensitivity associated with neuropathic pain. To investigate this, Nts^{Cre} mice expressing either mCherry or hM3Dq in LHA^{Nts} neurons underwent a spared nerve injury (SNI) procedure to invoke neuropathic pain-associated behavior (Fig. 4A-C). LHA^{Nts}:mCherry and LHA^{Nts}:hM3Dq mice had similar paw withdrawal thresholds prior to nerve injury (Fig. 4D, 4E, Pre Injury, respectively). Consistent with prior reports (Laumet et al., 2015; Laumet et al., 2017), SNI induces acute and persistent pain hypersensitivity indicated by reduced paw withdrawal threshold. PBS treatment has no effect on nerve injured controls (LHA^{Nts}: mCherry, Fig. 4D, black data points and line) and hM3Dq-expressing mice (LHA^{Nts}: hM3Dq, Fig. 4D, red data points and line). Excitingly, CNO-mediated stimulation of LHA^{Nts}-hM3Dq expressing neurons completely reversed injury-induced reduction in paw withdrawal threshold at 7- and 28-days post-injury (Fig. 4E, red data points and line). This effect was specific to CNO-mediated effects at LHA^{Nts}-hM3Dq expressing neurons, as treatment with CNO elicited no change in pain hypersensitivity in control mice (LHA^{Nts}-mCherry) (Fig. 3E, black data points). At the molecular level, sham control mice have ample Nts expression in the LHA (Fig. 4F) but, intriguingly, Nts expression is elevated after SNI (Fig. 4G, 4H). No sex differences were observed in Nts expression between females and males nor in pain-like behaviors after activating LHA^{Nts} neurons in SNI mice (data not shown). Overall, these data support that nerve injury upregulates Nts expression in both sexes but elevated Nts expression, by itself, is insufficient to attenuate nerve injury pain. Activating LHA^{Nts} neurons in this context did alleviate acute and persistent nerve injury-induced hypersensitivity, suggesting that increasing LHA^{Nts} neuronal mediated transmitter release is required to modulate neuropathic pain-induced hypersensitivity.

3.5. Chemogenetic stimulation of LHA^{Nts} neurons attenuates inflammation-induced mechanical hypersensitivity via Nts signaling

To determine whether activating LHA^{Nts} neurons modulates inflammation-induced pain hypersensitivity, mice with mCherry or hM3Dq in LHA^{Nts} neurons (as in Fig. 1A) were injected with saline in one hind paw (Fig. 5B) and Complete Freund's Adjuvant (CFA) in the other hind paw (Fig. 5D). As expected, saline injection had no impact on paw thickness in LHA^{Nts}:mCherry or LHA^{Nts}:hM3Dq mice (Fig. 5C). By contrast, CFA induced an equivalent inflammatory response in both groups of mice, confirmed by increased paw thickness after CFA injection (Fig. 5E). Mechanical hypersensitivity was measured 24 h after saline/CFA injection using von Frey filament test. As expected, the saline-treated group showed no change in paw withdrawal threshold before (Day 0) and after saline injection. Similarly, VEH or CNO treatment also did not alter the paw withdrawal response of the saline-injected paw (Fig. 5F). In contrast, CFA treatment induced pain hypersensitivity in the LHA^{Nts}:mCherry and LHA^{Nts}:hM3Dq mice, indicated by their reduction in paw withdrawal threshold from pre-treatment baseline (Fig. 5G, Day 0 vs. CFA). Excitingly, the reduction in paw withdrawal threshold was completely reversed by CNO-mediated stimulation of the hM3Dq-expressing LHA^{Nts} neurons for at least 2 hr, as compared to VEH-treated mice (Fig. 5G). By contrast, CNO treatment had no analgesic effect on the LHA^{Nts}:mCherry control mice, which looked identical to VEH-treated mice of both groups.

LHA^{Nts} neurons express and, when activated, release multiple transmitters including the fast neurotransmitter GABA and peptides such as galanin, corticotropin-releasing hormone (CRH), and Nts itself. Any of these released signals might mediate the antinociception observed in mice with inflammatory pain after stimulation of LHA^{Nts} neurons (Fig. 5G). To evaluate whether Nts signaling via its receptors is specifically required for the antinociceptive effect, we pretreated

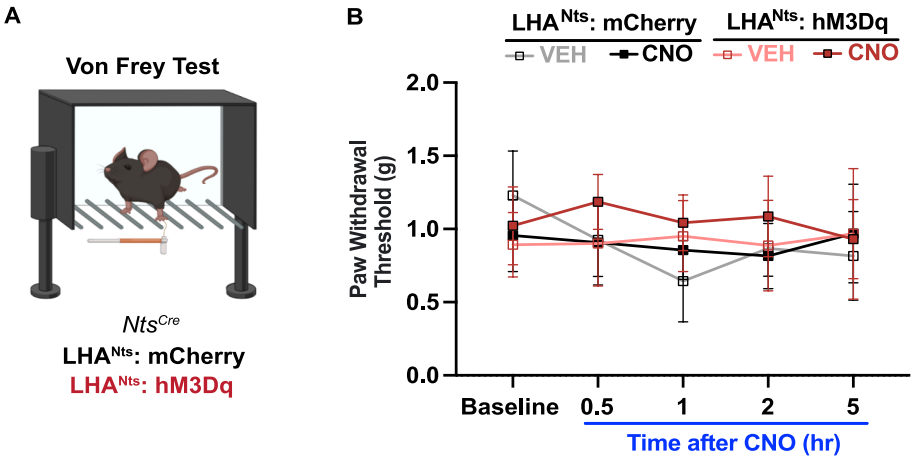

Fig. 3. Chemogenetic Stimulation of LHA^{Nts} Neurons Does Not Alter Mechanical Sensitivity in Naïve Mice. Adult *Nts^{Cre}* naive mice were injected in the LHA with either pAAV-hSyn-DIO-mCherry to express mCherry (LHA^{Nts}:mCherry) or pAAV-hSyn-DIO-hM3Dq(Gq)-mCherry to express excitatory hM3Dq-mCherry in LHA^{Nts} neurons (LHA^{Nts}:hM3Dq mice). A) Mice were assessed for mechanical sensitivity via von Frey filament test. Mice received i.p. VEH/CNO injection prior to test. B) Paw withdrawal threshold in LHA^{Nts}:mCherry mice (black, n = 8) and LHA^{Nts}:hM3Dq mice (red, n = 10) before (baseline) and after treatment with VEH and CNO. Error bars indicate SEM. No significant differences between groups as analyzed by Two-Way ANOVA with Tukey post-test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

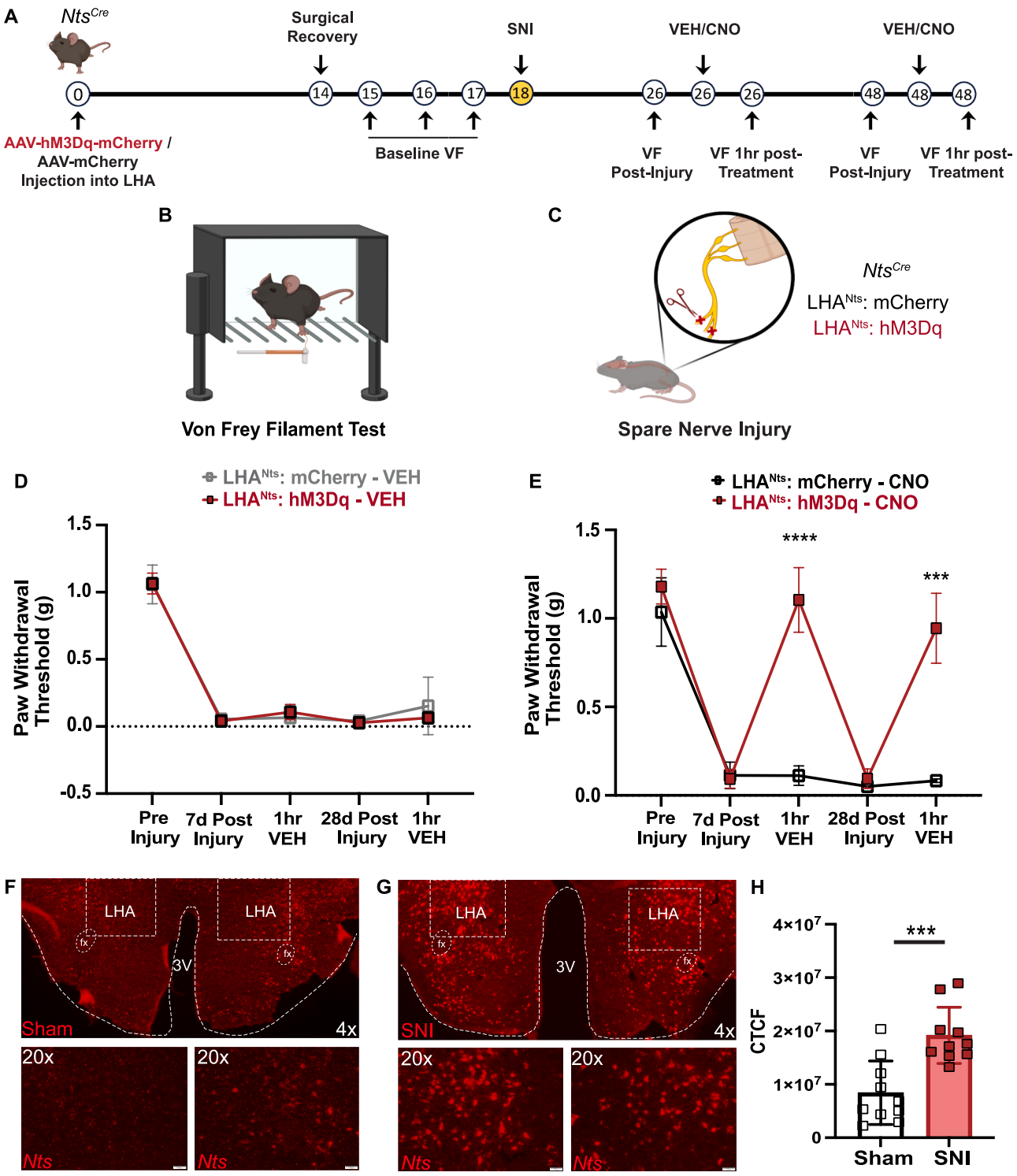

Fig. 4. Chemogenetic Stimulation of LHA^{Nts} Neurons Attenuates Persistent Neuropathic Pain-Induced Mechanical Hypersensitivity. A. Adult *Nts*^{Cre} naïve mice were injected in the LHA with either pAAV-hSyn-DIO-mCherry to express mCherry (LHA^{Nts}:mCherry) or pAAV-hSyn-DIO-hM3Dq(Gq)-mCherry to express excitatory hM3Dq-mCherry in LHA^{Nts} neurons (LHA^{Nts}:hM3Dq mice). A) Timeline of experiment, numbers indicate days. B) Mechanical pain sensitivity was measured via von Frey filament test in naïve mice. C) Mice underwent spared nerve injury (SNI) surgery to invoke neuropathic pain in one hind limb. D, E) Paw withdrawal threshold in LHA^{Nts}:mCherry (black, n = 6) or LHA^{Nts}:hM3Dq mice (red, n = 7) pre- and post-injury. Mice were assessed after 1 h of D) VEH and E) CNO treatment on Day 7, and 28 post nerve injury via Von Frey filament test. ***p < 0.001, ****p < 0.0001 via Two-Way ANOVA with Sidak post-tests. F-H) *Nts* mRNA expression in the LHA of F) sham nerve-injured mice (n = 10) and G) SNI mice (n = 10) was measured 28 days after the injury. Top images: 4x magnification. Bottom: digital magnification of boxed regions from top row, white arrows indicate LHA neurons expressing *Nts*. fx = fornix. H) To quantify *Nts* expression, Corrected Total Cell Fluorescence (CTCF) was measured bilaterally in the LHA of sham and SNI mice. *Nts* mRNA expression is significantly unregulated in SNI mice as compared to sham mice. Graph represents mean ± SEM. ***p < 0.001 via unpaired Student's *t*-test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

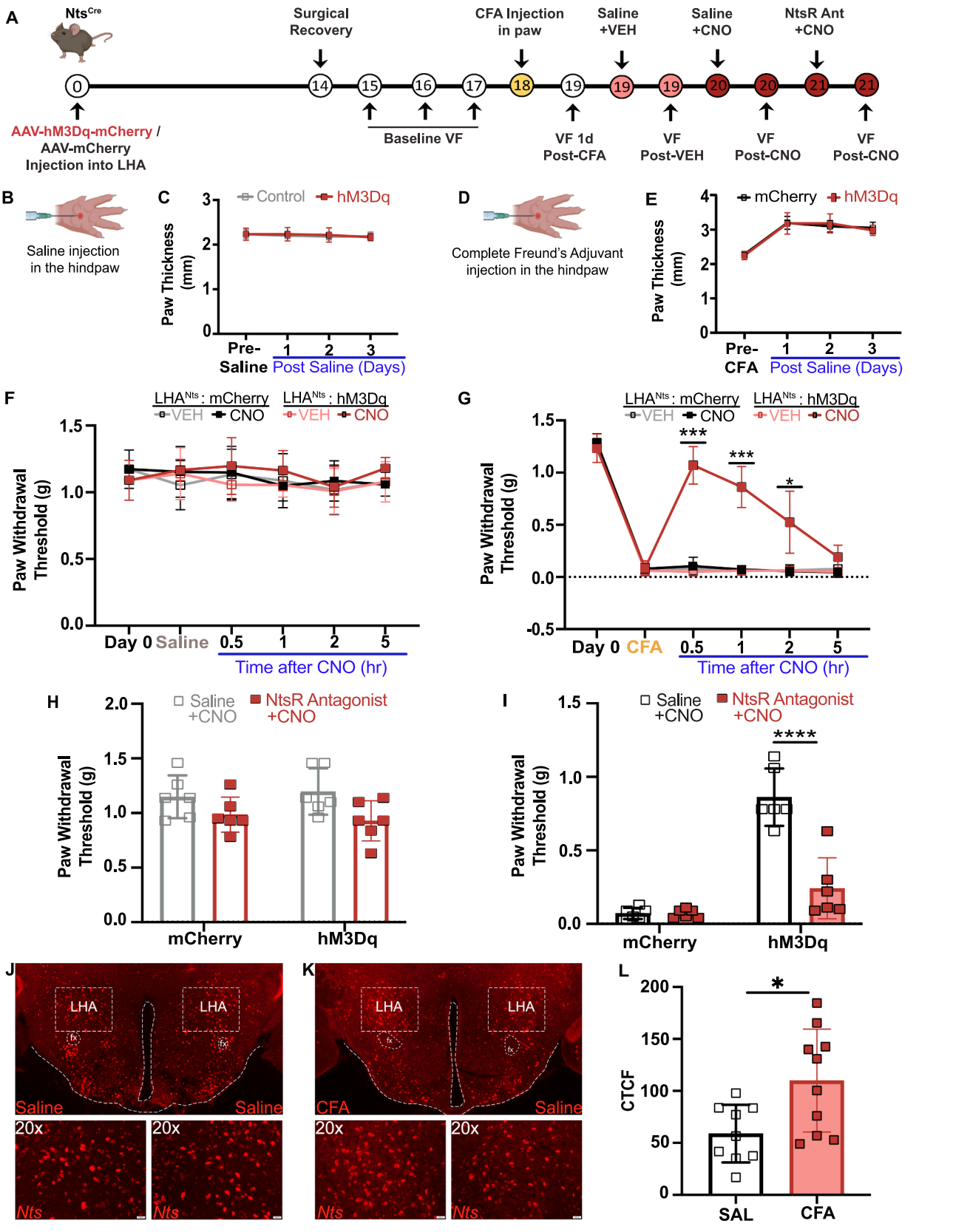

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Fig. 5. Chemogenetic Stimulation of LHA^{Nts} Neurons Attenuates Inflammation-Induced Mechanical Hypersensitivity Via Nts Signaling. Adult *Nts*^{Cre} naïve mice were injected in the LHA with either pAAV-hSyn-DIO-mCherry to express mCherry (LHA^{Nts}:mCherry) or pAAV-hSyn-DIO-hM3Dq(Gq)-mCherry to express excitatory hM3Dq-mCherry in LHA^{Nts} neurons (LHA^{Nts}:hM3Dq mice). A) Timeline of experiment in days. Mice were injected with B-C) Saline in one hind paw as control and D-E) Complete Freund's Adjuvant (CFA) in the other hind paw to induce inflammation. Paw thickness was measured before and after C) Saline and E) CFA injection. F, G) von Frey filament test to measure paw withdrawal threshold before (Day 0) and after 24 h of F) Saline (in grey) and G) CFA injection (in yellow), and 0.5, 1, 2, or 5 hr after treatment with VEH or CNO to stimulate LHA^{Nts} neurons in LHA^{Nts}:mCherry (gray/black) and LHA^{Nts}:hM3Dq mice (pink/red) mice. F) Injection with saline did not alter mechanical sensitivity in hM3Dq-mCherry mice as well as Controls. G) CFA injection induced mechanical hypersensitivity in both groups and CNO has an analgesic effect in hM3Dq-mCherry mice but not Controls. Graphs represent mean ± SEM. *$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$, Two-Way ANOVA with Tukey's multiple comparisons test. H, I) Pretreatment with Saline or NtsR pan-antagonist SR142948 (red) 1 hr prior to CNO treatment in Saline and CFA groups. ****$p < 0.0001$, Two-Way ANOVA with Sidak's multiple comparisons test. J, K) Representative images from separate cohort of wild type mice injected with either J) Saline in both hind paws or K) CFA in one hind paw and saline in the other hind paw (5 males, 5 females per treatment) and assessed for *Nts* mRNA expression (red) via RNA-Scope 24 h after treatment. Top images: 4x magnification. Bottom: digital magnification of boxed regions from top row. fx = fornix. L) To quantify *Nts* expression, Corrected Total Cell Fluorescence (CTCF) was measured in the LHA of saline-treated and CFA-treated mice. Increased *Nts* mRNA was observed specifically on the side of the LHA contralateral to the CFA-treated paw. The Grubbs outlier test identified an outlier in the Saline-treated group, which was removed. Graphed data represent mean ± SEM, Saline n = 9, CFA n = 10, *$p < 0.01$, unpaired Student's *t*-test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

mCherry and hM3Dq mice with either saline (control) or the brain permeable NtsR1 & NtsR2 pan-antagonist SR142948 followed by CNO to activate h3MDq-expressing LHA^{Nts} neurons (see the timeline of treatments in Fig. 5A). Paw withdrawal threshold of saline-treated mCherry and hM3Dq mice remained unchanged after treatment with both Saline + CNO and NtsR antagonist + CNO (Fig. 5H). On the other hand, CFA-treated LHA^{Nts}:mCherry mice had low paw withdrawal threshold that was not modified by saline + CNO or SR142948 + CNO treatment (Fig. 5I). In contrast, LHA^{Nts}:hM3Dq mice pretreated with saline and then CNO to activate LHA^{Nts} neurons increased their paw withdrawal threshold (Fig. 5I), consistent with the observations in Fig. 5G. Excitingly, pretreatment with the SR142948 pan-antagonist for NtsR1 and NtsR2 significantly blocked the antinociceptive effect of CNO-stimulated LHA^{Nts} neurons (Fig. 5I). The lack of CNO-mediated antinociception or NtsR antagonist effects in LHA^{Nts}:mCherry control mice confirms the absence of off-target drug effects and supports that chemogenetic-stimulation of LHA^{Nts} neurons mediates analgesia that depends on Nts signaling via NtsR1 and/or NtsR2. These data support that activating LHA^{Nts} neurons mediates antinociception in response to inflammatory pain that depends on Nts signaling.

Lastly, we assessed whether CFA-induced inflammatory pain modulates *Nts* expression in the LHA of wildtype mice treated with either saline in both hind paws or CFA in one hind paw and saline in the other. The mice were perfused 24 h after treatment and *Nts* expression was measured in the LHA via RNAScope. Saline-treated control and CFA-treated mice express *Nts* in the LHA (Fig. 5J-5L) with no discernable sex difference (data not shown). However, *Nts* expression is significantly elevated in the LHA of CFA-treated mice (Fig. 5K) compared to Saline-treated controls (Fig. 5J). Taken together, these data indicate that CFA-mediated inflammatory-induced pain upregulates *Nts* expression but activating the Nts-expressing LHA^{Nts} neurons is important to alleviate inflammatory pain in mice. Moreover, the analgesic effect mediated via activating LHA^{Nts} neurons is due, at least in part, to Nts signaling via NtsR1 and/or NtsR2.

4. Discussion

Despite the large body of work describing the analgesic effects of exogenous Nts treatment (Clineschmidt and McGuffin, 1977; Clineschmidt et al., 1979) little is known about the endogenous sources of Nts capable of modifying pain processing. Here we explored whether the population of neurons in the LHA that produce and release Nts, the LHA^{Nts} neurons (Patterson et al., 2015); modulate acute and persistent pain behavior. We found that chemogenetic stimulation of LHA^{Nts} neurons had no impact on acute thermal nociception and mechanical sensitivity in naïve mice over a timescale of hours. However, in models of persistent pain such as CFA- and SNI-induced mechanical hypersensitivity chemogenetic stimulation of LHA^{Nts} neurons significantly attenuated pain hypersensitivity and restored paw withdrawal threshold back to baseline levels. Furthermore, pretreatment with the NtsR1 and NtsR2 pan-antagonist (SR-142948) prior to chemogenetic stimulation of LHA^{Nts} neurons blocked the antinociceptive effect in both inflammatory and neuropathic models. Upregulation of *Nts* expression in the LHA alone was not sufficient to attenuate pain behaviors, suggesting that the LHA neurons containing Nts must be stimulated to release Nts to target regions to modify pain processing. Taken together our findings suggest that LHA^{Nts} neurons are an endogenous source of Nts and that activating them to release Nts can alleviate mechanical hypersensitivity in the context of persistent pain. In the future, augmenting endogenous LHA^{Nts} neuron-mediated Nts signaling could hold promise for treating pain.

Here we focused on the LHA because it contains a large population of Nts neurons and is anatomically positioned to coordinate many behaviors important for survival and homeostasis, including nociception. Indeed, stimulation of the entire LHA regulates descending pain processing in both acute (Esmaeili et al., 2017; Holden and Naleway, 2001; Holden et al., 2009), and persistent pain (Ezzatpanah et al., 2015; Holden et al., 2009; Holden et al., 2014; Wardach et al., 2016; Yazdi et al., 2016). Yet, these bulk modulations may have obscured the role that molecularly defined subsets of LHA neurons play in modulating pain (Bonnavion et al., 2016). Among the many types of LHA neurons, some LHA glutamatergic and hypocretin-orexin expressing populations contribute to pain processing (Esmaeili et al., 2017; Holden and Naleway, 2001; Holden et al., 2009; Wardach et al., 2016). In contrast, LHA^{Nts} neurons are exclusively GABAergic (Gu et al., 2023; Mickelsen et al., 2019; Naganuma et al., 2019) and presumably mediate distinct physiology (Brown et al., 2019; Carter et al., 2013). However, LHA^{Nts} neurons are connected with brain areas implicated in modulating pain behavior, including the ventral tegmental area (VTA), substantial nigra compacta (SNc), lateral preoptic area (LPO), and the periaqueductal grey area (PAG) (Kurt et al., 2022; Naganuma et al., 2019), an important brain region that processes noxious thermal and mechanical stimuli via descending pain pathways (Basbaum and Fields, 1978; Baskin et al., 1986; Chen and Heinricher, 2019; Liebeskind et al., 1973; Mayer and Liebeskind, 1974; Millan, 2002). Given that LHA^{Nts} neurons interface with brain regions that modulate pain processing and that Nts injected into these areas produces analgesia (Buhler et al., 2005; Buhler et al., 2008; Jennes et al., 1982) there is strong premise that LHA^{Nts} neurons are important endogenous players in pain behavior.

Intriguingly, chemogenetic stimulation of LHA^{Nts} neurons lessened nociceptive responses in the context of severe, persistent pain (inflammatory and nerve injury pain) but had no impact on thermal or mechanical nociception in naïve mice. These data suggest that the LHA^{Nts} neurons are biological modulators of persistent pain, not of acute nociception. Why this distinction? One possibility is that LHA^{Nts}-mediated influence on pain requires long-term changes to occur, such as the increased expression of *Nts* observed days after SNI or one day after CFA treatment. *Nts* expression in the LHA scaled with severity and duration of pain in our studies. Mice 28-days post SNI had *Nts* on both sides of the

LHA, whereas mice just 24-hr post CFA treatment only exhibited increased *Nts* in the LHA contralateral to the CFA-treated paw. It is conceivable that longer duration of CFA-inflammatory pain and increased severity would produce bilateral *Nts* upregulation, and this merits future investigation. Prolonged stress-induced analgesia has also been associated with increased *Nts* expression in the LHA, suggesting a potential role for LHA^{Nts} neurons in pain processing (Gui et al., 2004). Collectively, these findings support that persistent pain may biologically increase *Nts* expression in the LHA. Interestingly, upregulated *Nts* expression alone is not sufficient to provide analgesia. For example, the SNI and CFA models exhibit significantly elevated *Nts* in the LHA (Fig. 4G, Fig. 5K), yet these mice experience hypersensitivity (Fig. 4D-E and Fig. 5G, respectively). Activation of LHA^{Nts} neurons was important to reverse the hypersensitivity. This suggests that some activation-mediated release of a signal from the LHA^{Nts} neurons is required for modifying the pain processing. Nts is a strong candidate for the anti-nociceptive effect, given that it is released from LHA^{Nts} neurons and has been linked with pain relief (Behbehani et al., 1987). Indeed, pretreatment with the pan NtsR1-NtsR2 antagonist prior to stimulation of LHA^{Nts} neurons in mice with CFA-induced inflammatory pain blocked analgesia, implicating released Nts that signals via NtsR1 and/or NtsR2 as the critical mediator of pain behavior. Taken together, these data indicate that endogenous Nts released from LHA^{Nts} neurons is sufficient to reduce pain in persistent pain contexts. Whether increased expression of *Nts* within the neurons modulates the degree of pain or is required for analgesia is a question for further exploration. It is important to acknowledge that we found that severe pain increases *Nts* expression at the mRNA level, but methodological limitations prevented measurement of Nts protein. While mRNA expression often reflects protein expression this is not always the case, so future work should verify how pain impacts Nts peptide expression in the LHA. However, previous studies have shown that pharmacologically augmenting Nts peptide signaling in the brain provides antinociception after peripheral nerve injury (Guillemette et al., 2012; Tetreault et al., 2013). Given that LHA^{Nts} neurons elicited similar antinociception in our studies, augmenting LHA^{Nts} endogenous signaling may also increase Nts peptide mediated signaling and could hold potential for treating persistent pain states.

We acknowledge that there are caveats with this study and remaining questions that should be addressed with future work. One vital question is where the Nts released from LHA^{Nts} neurons acts in the brain to alleviate pain and via which receptor/s. As discussed above, LHA^{Nts} neurons project to many regions implicated in pain processing, any of which could conceivably contribute to the analgesic effects. Circuit-based analysis will be important to define the critical pathways by which the LHA^{Nts} neurons engage and modulate pain, and to identify the downstream cells of this endogenous signaling that might be pharmacologic targets for controlling pain. Moreover, further work is needed to clarify how LHA^{Nts} neuron-released Nts mediates its effects, be that signaling via NtsR1, NtsR2, or both receptor isoforms. Likewise, although our data supports an important role for Nts released from LHA^{Nts} neurons in mitigating nociception, the chemogenetic method used here causes release of all LHA^{Nts} neuronal signals and so we cannot exclude that signals other than Nts also contribute to pain processing. Given that LHA^{Nts} neurons express and release Nts as well as other neuropeptides such as galanin and CRH, along with the classical neurotransmitter GABA, these could play important roles as well (Mickelsen et al., 2019). Our data support that LHA^{Nts} induced signaling via NtsR1/NtsR2 contributes to pain processing to some degree (Fig. 5**i**) but the other released signals also merit investigation. While there is yet much to learn about the precise mechanisms by which LHA^{Nts} neurons contribute to biological pain processing, our data reveal them as an important node in the endogenous Nts system and control of pain processing. Going forward, understanding how the LHA^{Nts} neurons engage the descending pain system and modulate it may point to design of novel analgesic approaches to provide relief for severe, persistent pain.

CRediT authorship contribution statement

Rabail Khan: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Beenhwa Lee:** Writing – review & editing, Visualization, Validation, Methodology, Formal analysis. **Kufreobong Inyang:** Writing – review & editing, Visualization, Methodology, Formal analysis. **Hope Bemis:** Writing – review & editing, Visualization, Investigation, Formal analysis. **Raluca Bugescu:** Writing – review & editing, Visualization, Validation, Resources, Formal analysis. **Geoffroy Laumet:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Conceptualization. **Gina Leinninger:** Writing – review & editing, Writing – original draft, Resources, Project administration, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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