An NTS-CeA projection modulates depression-like behaviors in a mouse model of chronic pain

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A B S T R A C T

Depressive symptoms comorbid with chronic pain are a common health problem, but the underlying neural circuit mechanisms remain elusive. Here, we identify a glutamatergic projection from the nucleus of the solitary tract (NTS) to the central nucleus of the amygdala (CeA) that mediates depression-like behaviors in a chemotheraphy-induced neuropathic pain model. Inhibition or ablation of the glutamatergic NTS neurons alleviates depressive but not hypersensitive behaviors in these mice. The projected neurons form excitatory synapses with somatostatin-expressing neurons in the CeA. Silencing the NTS-CeA projection alleviates depressive but not hypersensitive behaviors, whereas activating the projection promotes depressive behaviors. In addition, in naïve mice, activation of the NTS-CeA projection induces obvious depressive behaviors that can be blocked by silencing the CeA somatostatin-expressing neurons. Together, we reveal a modulatory role of the NTS and its glutamatergic projection to the CeA circuit in modulating depression-like behaviors comorbid to chronic pain.

1. Introduction

The association between chronic pain and depression is becoming increasingly recognized. Prevalence studies show that approximately 65% of patients with clinical symptoms of depression have chronic pain, while up to 85% of chronic pain patients have depression (Arnow et al., 2006; Cabrera-Leon et al., 2018). Comorbid depressive symptoms frequently lead to prolonged duration and intensity of pain, thereby creating a vicious cycle between pain and depression (Goyal et al., 2014). However, the central cellular and circuit mechanisms underlying chronic pain-induced depression are only beginning to be understood (Zhang et al., 2021; Zhou et al., 2019).

The nucleus of the solitary tract (NTS), located in the dorsomedial medulla, is considered an integral part of the endogenous pain-modulatory system (Mercer Lindsay et al., 2021; Napadow et al., 2019; Todd, 2010). A recent magnetic resonance imaging (fMRI) study found that NTS is linked to pain processing (Napadow et al., 2019). Many studies have investigated the role of the NTS in modulating visceral pain processing (Menetrey and Basbaum, 1987; Nishida et al., 2021; Rhoton Jr. et al., 1966). NTS neurons are activated by noxious stimuli (Bai et al., 2019), and several agents alleviate pain symptoms by inhibiting the NTS, while activation of the NTS neurons facilitates pain reflexes (Bai et al., 2019). NTS neurons receiving sensory neuron inputs also project to many depression-related brain regions, including the locus coeruleus and the central nucleus of the amygdala (CeA) (McGovern et al., 2015a; McGovern et al., 2015b). However, an involvement of the NTS in the emotional experience of chronic pain has not been established.

The CeA is a well-known limbic region that participates in processing sensory, emotional, and depressive information, and it serves as a major output nucleus for amygdala functions (Janak and Tye, 2015; Sanders et al., 2019). It receives inputs and sends outputs to many forebrain and brainstem regions that are critical for multiple mood disorders (Han et al., 2015; Penzo et al., 2015). Recent studies have reported that the CeA might be involved in the coordination of respiration and mental illnesses (Liu et al., 2021). Another study found that CeA somatostatin-expressing (CeA SST) neurons are critical for the development of comorbidity depressive symptoms in chronic pain (Zhou et al., 2019). The CeA SST neurons are inhibited by serotonergic projection from the dorsal raphe nucleus (DRN), and disinhibition of these neurons by a decrease in DRN serotonergic inputs was identified in mice with chronic pain. By contrast, activation of DRN serotonergic projection could diminish inhibitory neuronal activity in the CeA, thereby promoting depressive behaviors.

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depression-like behaviors in chronic pain model mice but not in restraint- or social stress-related mouse models of depression (Zhou et al., 2019). CeA\(^{SST}\) neurons also participate in fear learning and anxiety development (Ahrens et al., 2018; Li et al., 2013; Penzo et al., 2015). However, whether CeA\(^{SST}\) neurons are involved in pain-related depression and whether CeA\(^{SST}\) neurons are modulated by other neurotransmitters or neuromodulators is unknown. The CeA also receives projection from NTS neurons (McDougall et al., 2017; McGovern et al., 2015b), but a role for this NTS-CeA circuit in mediating comorbid depressive symptoms in chronic pain remains to be determined.

In the present study, we used pharmacogenetic, behavioral, optogenetic, and electrophysiological approaches to dissect the anatomical and functional connections between NTS and the CeA underlying the modulation of comorbid depression in a model of chemotherapy (oxaliplatin)-induced neuropathic pain that develops profound mechanical and thermal hypersensitivity (Huang et al., 2021; Marcotti et al., 2022). We then revealed that an NTS-CeA ascending projection modulates depression-like behaviors under the state of chronic pain.

2. Materials and methods

2.1. Animals

Male C57BL/6 N, Vglut2-Cre, SST-Cre, and Ai9 mice were used for the experiments. C57BL/6 N mice were purchased from Guangdong Medical Laboratory Animal Center or Gene & Peace Co., Ltd. (Guangdong). Vglut2-Cre (JAX016963), SST-Cre (JAX010708), and Ai9 (JAX007909) mice were initially acquired from Jackson Laboratory. All mice were housed under a 12 h light/dark cycle (lights on at 7:00 a.m.) with ad libitum access to food and water. The animals were randomly allocated to different groups as appropriate. All behavioral tests were carried out during the light phase. All procedures were approved by the Animal Care and Use Committee of the Sun Yat-sen University, Guangzhou, China.

2.2. Oxaliplatin injection

Oxaliplatin (Aladdin Reagent Co., Shanghai, China) was dissolved in 5% glucose solution to a concentration of 1 mg/mL. A cycle of drug delivery consisted of intraperitoneal injections of oxaliplatin (4 mg/kg) once per day for 5 consecutive days. Control animals were injected with an equivalent volume of 5% glucose vehicle prepared in sterile distilled water. Pain behavioral tests (see below) were employed to evaluate the development of neuropathic symptoms.

2.3. Von Frey test

Animals were habituated to the testing room for at least two days before behavioral tests. The following behavioral tests were double-blindly performed, as reported previously (Liu et al., 2019). Mechanical sensitivity was tested by stimulating the mouse hindpaw perpendicularly with a series of Von Frey filaments with logarithmically incrementing stiffness (0.16–2.56 g, Stoelting, Wood Dale, IL). The withdrawal threshold was determined using the up-down method (Chaplan et al., 1994). In some experiments, we measured the withdrawal probability to mechanical stimuli.

2.4. Hargreaves test

Radiant thermal pain was investigated by determining the paw withdrawal latency to a noxious heat stimulus as the average of at least four measurements per paw over a 5 min test period using a Hargreaves apparatus, with a 20 s cutoff time set to avoid damage. Three to five replicates were acquired per hind paw per mouse, and the values for both paws were averaged.

2.5. Hotplate test

For the test of painful stimulation from heat sensitivity, a clear plexiglass cylinder was placed on the hotplate (50°C) and the mice were placed inside the cylinder. The onset of brisk hindpaw lifts and/or flinching/licking of the hindpaw was assessed.

2.6. Tail flick test

For this test of nociception, the mice were gently restrained in a cotton thread glove—a situation that did not stress the mice. The protruding one-third of the tail was then dipped into 52 °C water. The tail flick latency was recorded with a cutoff time of 10 s to avoid tissue damage.

2.7. Open field test

The animals were habituated to the testing room for at least 2 days before the behavioral tests. The locomotor activity of the mice was evaluated by the open field test performed during a 10 min period in 40 × 40 × 40 cm polystyrene enclosures, as described previously. The mice were initially placed in the center of the box and were videotaped individually. The traces were analyzed with LabState software (AniLab) as a method for measuring overall locomotion.

2.8. Forced swimming test

Animals were individually placed in a cylinder of water (10 cm diameter, 20 cm height, 22–24 °C) and made to swim for 6 min under normal light. The water depth was set to prevent the animals from touching the bottom of the cylinder with their tails or hind limbs. The time the mouse remained immobile during the last 4 min of the 6-min testing period was counted as a marker for depression. This time was defined as the time when animals remained floating or motionless, aside from the movements necessary to maintain balance in the water.

2.9. Tail suspension test

Animals were habituated to the testing room for at least two days before behavioral tests. Mice were individually suspended approximately 30 cm above the surface of a platform using adhesive tape placed approximately 5 cm from the tip of the tail. Each mouse was tested for 6 min. The time the mouse remained immobile was measured in the last 4 min as a marker of depression-like behavior. Mice were considered immobile when they did not initiate movements; immobility was considered to include passive swaying.

2.10. Sucrose preference test

Mice were housed individually and habituated with two bottles of 1% sucrose for 2 days, followed by 2 days of water. The mice were then water deprived for 24 h. The mice were presented with two bottles for 2 h, one contained water and the other contained 1% sucrose, and the position of the bottles was switched 1 h later. The amount of each fluid consumed was measured as a method for measuring anhedonia, a core symptom of depression, in rodents.

2.11. Surgery for viral injection into the brain

The mice were anesthetized with pentobarbital sodium (100 mg/kg; MYM Technologies Ltd.) and mounted in a stereotaxic apparatus (RWD Life Science Co., Ltd., 68,513). The skull was exposed through a midline scalp incision, and a unilateral or bilateral craniotomy was performed to introduce a glass microinjection pipette into the target brain regions. The craniotomy window (approximately 1.5 mm in diameter) was made using a hand-held drill (RWD Life Science Co., Ltd.) over the target area.
The glutamatergic neurons in the NTS were manipulated by bilaterally injecting AAV (adeno-associated virus) -hSyn-DIO-hM4Di-mCherry (AAV2/9, titer: 4.7 × 10^12 v.g./mL, Shanghai Taitool Bioscience Co. Ltd., S0337) into the NTS (AP, −7.56 mm; ML, ±0.4 mm; DV, −5.1–5.3 mm) of Vglut2-Cre mice in a volume of 150 nL for each side (Chen et al., 2022). The same volume of AAV-hSyn-DIO-mCherry (AAV2/5, titer: 5.1 × 10^12 v.g./mL, Shanghai Taitool Bioscience Co. Ltd., S0240–5) was used as a control.

For retrograde tracing of the NTS-CeA projections, AAV-hSyn-mCherry (AAV2/9, titer: 4.7 × 10^12 v.g./mL, Shanghai Taitool Bioscience Co. Ltd., S0238–9) was unilaterally injected into the NTS (AP, −7.56 mm; ML, ±0.4 mm; DV, −5.1–5.3 mm) of WT mice in a volume of 150 nL. The injected mice were perfused 5 weeks later.

For retrograde tracing of NTS-CeA projections, AAV2/2-retro-hSyn-Cre (AAV2/2-retro, titer: 1.0 × 10^12 v.g./mL, Shanghai Taitool Bioscience Co. Ltd., S0278-2R) was bilaterally injected into the CeA (AP, +1.3 mm; ML, ±2.7 mm; DV, −4.6 mm) of A9 mice in a volume of 300 nL. The injected mice were perfused 5 weeks later.

The functional projections from the NTS to CeA neurons were examined by injecting AAV2/9-hSyn-hChR2(H134R)-EYFP (AAV2/9, titer: 5.3 × 10^12 v.g./mL, Shanghai Taitool Bioscience Co. Ltd., S0318–9) unilaterally into the NTS, and AAV-hSyn-DIO-mCherry (AAV2/5, titer: 5.1 × 10^12 v.g./mL, Shanghai Taitool Bioscience Co. Ltd., S0240–5) bilaterally into the CeA of SST-Cre mice (7 weeks old). These mice were subjected to slice electrophysiology experiments 4 weeks later.

The CeA neurons were manipulated by bilaterally injecting AAV-hSyn-DIO-hM4Di-mCherry (AAV2/5, titer: 4.7 × 10^12 v.g./mL, Shanghai Taitool Bioscience Co. Ltd., S0193) into the CeA (AP, −1.3 mm; ML, ±2.7 mm; DV, −4.6 mm) of Ai9 mice in a volume of 300 nL. These mice were subjected to slice electrophysiology experiments 4 weeks later.

For in vivo pharmacogenetic activation experiments, mice were injected with CNO (Sigma, C0032; 1 mg/kg, i.p.) and tested for chemotherapy-induced painning-like behavior 30–40 min later.

Mice were anesthetized with an overdose of pentobarbital sodium (100 mg/kg; MYM Technologies Ltd.) and perfused transcardially with phosphate-buffered saline (PBS, HyClone), followed by PBS containing 4% paraformaldehyde (PFA, Sigma). The brains were dissected, post-fixed overnight at 4 °C in 4% PFA, and cryoprotected in 30% sucrose in PBS at 4 °C. Free-floating sections (40 μm) prepared with a cryostat (Leica CM 1950) were subjected to immunohistochemical staining. The tissue sections were blocked for 30 min at room temperature in PBST (0.3% Triton X-100) containing 5% normal donkey serum (NDS, Abcam) and then incubated with primary antibodies at 4 °C overnight and secondary antibodies at room temperature for 2–3 h. The primary antibodies used for immunohistochemistry (IHC) were rabbit anti-GFP (1:1000, Thermo Fisher Scientific) and rabbit anti-Dr channel (1:1000, Clontech). The secondary antibodies used were donkey Alexa 488-conjugated anti-rabbit IgG (1:1000, Jackson ImmunoResearch Laboratories) and Cy3-conjugated donkey anti-rabbit IgG (1:1000, Jackson ImmunoResearch Laboratories). Images were taken using a Nikon Eclipse Ni-E fluorescence microscope. Cells were counted manually or automatically using Fiji (NIH).

Stereotaxically placed micropipettes were connected to an infusion pump (Drummond, Nanoject III), and solutions were infused into the CeA at a rate of 50 nl/min. After a 0.5 min preinfusion period, the solutions were infused at a rate of 0.1 nl/minute until a total volume of 400 nL was delivered to the CeA. The solutions were infused in the following order: (1) control AAV2/2-retro-hSyn-Cre (AAV2/2-retro, titer: 1.0 × 10^12 v.g./mL, Shanghai Taitool Bioscience Co. Ltd., S0278-2R) alone, (2) AAV2/2-retro-hSyn-Cre (AAV2/2-retro, titer: 1.0 × 10^12 v.g./mL, Shanghai Taitool Bioscience Co. Ltd., S0278-2R) co-infused with 50 nl of AAV-hSyn-DIO-mCherry (AAV2/5, titer: 5.1 × 10^12 v.g./mL, Shanghai Taitool Bioscience Co. Ltd., S0240–5), and (3) AAV2/2-retro-hSyn-DIO-mCherry (AAV2/5, titer: 5.1 × 10^12 v.g./mL, Shanghai Taitool Bioscience Co. Ltd., S0240–5) alone.
The jitter was defined as the standard deviation (SD) of the averaged traces. The EPSC and IPSC amplitudes were calculated as postsynaptic currents (EPSCs or IPSCs) were manually measured from rising/falling curvature. The EPSCs and IPSCs in our study sometimes differed between the peak amplitude in a predefined window after light stimulation onset and the mean amplitude just before the EPSC or IPSC. EPSC or IPSC latency was measured as the time from the onset of light stimulation to the first intersection between the baseline and the peak amplitude. The PPR was calculated by paired-pulse stimulation (1 ms; 50%; 20 kHz; Digidata 1550; Molecular Devices). The data were acquired at a holding voltage of ~70 mV, and IPSCs were recorded at a voltage of 0 mV. For action potential recording experiments, NTS or CeA neurons were recorded in current-clamp mode using potassium-based internal solutions with addition of NBQX, CPP, and picrotoxin to ACSF. NBQX (Tocris, 1044; 10 μM) and CPP (Tocris, 0173; 5 μM) were used to block glutamate inputs. Picrotoxin (PTX, 1128; 50 μM) was used to block GABA_A receptors. The PPR was calculated by paired-pulse stimulation (1 ms; 50 ms interpulse intervals) as the ratio of the amplitude of the second response to that of the first (Britt et al., 2012).

2.15. Statistical analysis

Voltage clamp and current clamp recordings were carried out using a computer-controlled amplifier (MultiClamp 700B; Molecular Devices). During recordings, traces were low-pass filtered at 4 kHz and digitized at 20 kHz (Digidata 1550; Molecular Devices). The data were acquired with Axon Clampex 10.3 software. The amplitude and latency of the postsynaptic currents (EPSCs or IPSCs) were manually measured from the averaged traces. The EPSC and IPSC amplitudes were calculated as the difference between the peak amplitude in a predefined window after light stimulation onset and the mean amplitude just before the EPSC or IPSC. EPSC or IPSC latency was measured as the time from the onset of light stimulation to the first intersection between the baseline and the EPSC or IPSC, which could be easily identified as the point of maximal rising/falling curvature. The EPSCs and IPSCs in our study sometimes showed multiple peaks; in these cases, we measured the latency of the first EPSC or IPSC. The jitter was defined as the standard deviation (SD) of the EPSC or IPSC onset latency across individual sweeps per cell (at least five sweeps per cell). Statistical analysis was performed using Igor Pro (WaveMetrics) and Prism (GraphPad Software). All statistical analyses were two-tailed comparisons. The data were analyzed using the Mann–Whitney test, Wilcoxon signed-rank test, two-way ANOVA, and the paired t-test. All data are expressed as the mean ± SEM.

3. Results

3.1. Chronic pain induces depressive behaviors

Previous studies demonstrated that chronic pain induced depression-like behaviors in a surgical mononeuropathy model (Zhang et al., 2021; Zhou et al., 2019). We examined whether chemotherapy-induced polynepathy with widespread hypersensitivity might also induce co-morbid depressive symptoms by utilizing a rodent model of oxaliplatin-induced neuropathic pain (Huang et al., 2021). We found that the mechanical withdrawal threshold was gradually decreased during the 10-day period after 5 consecutive injections of oxaliplatin (Fig. 1A). To determine the long-term effect of oxaliplatin injections, we intraperitoneally injected mice with oxaliplatin (2 cycles of 4 mg/kg i.p. daily for 5 consecutive days with a 5-day interval). Three weeks after the first oxaliplatin chemotherapy, both mechanical and thermal withdrawal thresholds were largely diminished (Fig. 1B-D), suggesting that intra-peritoneal injections of oxaliplatin induce long-term neuropathic pain. As a control for the pain behavioral tests, we checked locomotor activity of the animals over 1 h after the 2-cycle chemotherapy and excluded the effect of locomotor activity on pain-related behaviors by showing that locomotor activity was not significantly affected (Fig. 1E). The depression-like behaviors TST, FST, OFT, and SPT were tested at 3 weeks after the first oxaliplatin administration: A 2-cycle chemotherapy regimen increased immobility time in the FST (Fig. 1F) and the TST (Fig. 1G) while decreasing preference in the SPT (Fig. 1H). Importantly, the total traveling distance in the OFT remained unaffected (Fig. S1C), indicating that the changes in the TST, FST, and SPT are shown in Fig. 1. Repeated intraperitoneal injections of oxaliplatin induce pain hypersensitivity and depression-like behaviors in mice. (A) Time course of vehicle or oxal injection-induced pain-related behaviors, two-way ANOVA. (B–C) Effects of 2-cycle vehicle or oxal injections on mechanical (B) or thermal (C) pain-related behaviors at 10 days after the first injection; Mann-Whitney test. (D) schematic illustration for drug delivery and behavioral tests. Left for (A), right for (B–C, E-H). (E) Effects of 2-cycle vehicle or oxal injections on locomotor activity; Mann-Whitney test. (F–H) Effects of 2-cycle vehicle or oxal injections on depression-like behaviors in the FST (F), TST (G) or SPT (H); Mann-Whitney test. All error bars represent the SEM. n = 7 mice in A-C, n = 12 mice in E-G, **p < 0.001. Oxal, oxaliplatin. A cycle of drug delivery included intraperitoneal injections of oxaliplatin (4 mg/kg) once per day for 5 consecutive days; control animals were injected with an equivalent volume of the 5% glucose vehicle.
unlikely to arise due to a motor deficit associated with the intervention. Taken together, these data suggest that long-term neuropathic pain leads to depression-like behaviors.

3.2. NTS glutamatergic neurons mediate depressive, but not hypersensitive, behaviors

Many studies have suggested that the NTS receives ascending projections from the dorsal horn of the spinal cord and is involved in pain processing (Mercer Lindsay et al., 2021; Napadow et al., 2019; Todd, 2010). We examined the potential involvement of the NTS in pain modulation by first performing current-clamp whole-cell recordings of NTS neurons from wild-type (WT) mice (Fig. 2A and B). We found a significant increase in the spike number in the mice after the second cycle of chemotherapy (Fig. 2C and D), indicating that neuronal activity of the NTS is enhanced in chronic pain.

We then investigated the functional role of NTS glutamatergic neurons in pain modulation with a pharmacogenetic approach (Urban and Roth, 2015). We injected an adeno-associated virus (AAV) expressing Cre-inducible hM4Di, a designer receptor exclusively inhibited by a designer drug (DREADD), into both sides of the NTS in Vglut2-Cre mice (Fig. 2E and F; Fig. S1A-S1C). We confirmed the ability of NTS neurons to suppress hM4Di by recording NTS neurons in brain slices and found that bath administration of clozapine-N-oxide (CNO) inhibited the activity of hM4Di+ neurons (Fig. 2G and H). We suppressed the activity of NTS glutamatergic neurons and found that inhibition of NTS glutamatergic neurons did not affect the mechanical and thermal hypersensitive behaviors in the mice (Fig. 2I-2L). Again, the locomotion abilities remained intact (Fig. 2M). Inhibition of NTS glutamatergic neurons alleviated the changes in the depression-like parameters of pain processing, including FST, TST, and SPT, following the 2-cycle chemotherapy treatment (Fig. 2N-2P).

Fig. 2. Silencing of NTS glutamatergic neurons attenuates pain-related and depression-like behaviors. (A) Representative image of a recorded NTS neuron in a WT mouse. (B) Summary of the spatial location of the recorded NTS neurons (n = 12 neurons). Scale bar, 300 μm. (C and D) Sample traces and statistical data for action potential firing recorded from NTS neurons in mice treated with vehicle or oxal injection (vehicle, n = 12 cells from 5 mice; oxal, n = 12 cells from 5 mice); two-way ANOVA. (E) A schematic diagram showing the sites of AAV-DIO-hM4Di-mCherry or AAV-DIO-mCherry virus injection into the bilateral NTS of Vglut2-Cre mice. (F) Expression of hM4Di-mCherry in the NTS. Scale bar, 300 μm. (G) Effect of bath application of CNO on spikes of an example hM4Di-mCherry+ NTS neuron. (H) A summary graph showing the firing rate before and after CNO application, n = 6 from 3 mice, Wilcoxon signed-rank test. (I-L) Effects of pharmacogenetic inhibition of NTS glutamatergic neurons on pain-related behaviors in von-Frey test (I), Hargreaves test (J), hotplate test (K) and tail flick test (L), two-way ANOVA (n = 10 or 12 mice). (M) Effects of pharmacogenetic inhibition of NTS glutamatergic neurons on locomotor activity, two-way ANOVA (n = 10 or 12 mice). (N—P) Effects of pharmacogenetic inhibition of NTS glutamatergic neurons on depression-like behaviors in the FST (N), TST (O) or SPT (P), two-way ANOVA (n = 10 or 12 mice). All error bars represent the SEM. *p < 0.05, **p < 0.01, ***p < 0.001. Oxal, oxaliplatin.
We confirmed the effects of hM4Di-mediated inhibition by ablating NTS glutamatergic neurons with a caspase-3-based ablation strategy (Fig. S2A). The number of NTS glutamatergic neurons decreased in mice injected with Cre-inducible taCasp3 (Fig. S2B–D). Consistently, Cre-inducible taCasp3 attenuated the depressive but not the hypersensitive behaviors following the 2-cycle chemotherapy regimen (Fig. S2E–I). Taken together, these results suggest that NTS glutamatergic neurons play a critical role in chronic pain-induced depression-like behaviors.

3.3. Monosynaptic connection between NTS and CeA

We then determined how NTS glutamatergic neurons mediate depression-like behaviors induced by chronic pain. Previous studies have suggested that the NTS mediates sensations and related mental information via ascending projections (Cheng et al., 2020; Han et al., 2018; Todd, 2010). The CeA is a well-known hub that modulates sensory and mental information (Janak and Tye, 2015; Sanders et al., 2019; Zhou et al., 2019). In addition, the CeA is known to receive projections from the NTS (McDougall et al., 2017; McGovern et al., 2015b). We confirmed the existence of projection from the NTS to the CeA by unilaterally injecting AAV-hSyn-mCherry into the NTS of WT mice and then determining mCherry expression in the CeA (Fig. 3A). Dense mCherry+ fibers were found in the CeA (Fig. 3B). The existence of an NTS-CeA projection was further verified using an AAV-based retrograde tracing strategy (Tervo et al., 2016). AAV2/2-retro-hSyn-Cre was unilaterally injected into the CeA of Ai9 mice, and numerous tdTomato+ neurons were observed in the NTS (Fig. 3C and D), suggesting that NTS neurons send glutamatergic projection to the CeA.

Given that CeAStt neurons are critical for chronic pain-induced depression (Zhou et al., 2019), we then identified the functional projection from the NTS to CeAStt neurons. We injected AAV-hSyn-ChR2-EYFP into the NTS and AAV-DIO-mCherry into the CeA of SST-Cre mice and obtained whole-cell patch-clamp recordings from mCherry+ neurons in CeA slices (Fig. 3E). We found that photostimulation of ChR2+ fibers emanating from the NTS evoked excitatory postsynaptic currents (EPSCs) but not inhibitory postsynaptic current (IPSC) components in the CeAStt neurons (Fig. 3F). Light-evoked EPSCs were blocked by bath application of the AMPA receptor antagonist 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(f)quinoxaline (NBQX; Fig. 3F and G). The latency of light-evoked EPSCs was approximately 1.2 ms (Fig. 3H), indicating a monosynaptic connection between the NTS and CeAStt neurons. We also summarized the latency jitter of the recorded neurons and found that most of these neurons had a jitter of <0.2 ms (Fig. 3I), which was consistent with a monosynaptic connection. Thus, NTS neurons form direct excitatory connections with CeAStt neurons.

Fig. 3. Synaptic inputs from the NTS to CeAStt neurons. (A) Schematic showing the site of AAV-hSyn-mCherry injection into the unilateral NTS of WT mice. (B) Images showing mCherry expression in the NTS (left) and the CeA (middle and right). Scale bars, 300 μm (left), 200 μm (middle and right). (C) Schematic showing the site of AAV2/2-retro-hSyn-Cre injection into the unilateral CeA of Ai9 mice. (D) Images showing tdTomato expression in different parts of the NTS. Scale bars, 300 μm. (E) Schematic showing the sites of AAV-hSyn-ChR2-EYFP injection into the NTS and AAV-DIO-mCherry injection into the CeA of SST-Cre mice and patch-clamp recording of CeAStt neurons. (F) Responses evoked by photostimulation of NTS ChR2+ fibers in a CeAStt neuron in ACSF (black) and in the presence of NBQX (10 μM; red). Blue bar, LED stimulation (475 nm, 1 ms). Eleven of 30 recorded CeAStt neurons from 6 mice showed light-evoked EPSCs. (G) Summary data of the amplitudes of EPSCs recorded in CeAStt neurons evoked by photostimulation of NTS ChR2+ fibers before and after the application of NBQX (n = 6 neurons from 6 mice), Wilcoxon signed-rank test. *p < 0.05. (H) Summary data of the latencies of light-evoked EPSCs (11/30 neurons) in CeAStt neurons. (I) Summary data of the jitters of light-evoked EPSCs (11/30 neurons) in CeAStt neurons. All error bars represent SEMs. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
3.4. Suppression of CeA\textsuperscript{SST} neurons relieves chronic pain-induced depression

We investigated the role of the glutamatergic NTS-CeA\textsuperscript{SST} projection in chronic pain by performing slice recording combined with optogenetics. We injected AAV-hSyn-ChR2-EYFP into the NTS and AAV-DIO-mCherry into the CeA of SST-Cre mice and treated these mice with oxaliplatin or vehicle. The numbers of ChR2-expressing neurons are similar between the groups (Fig. S3). We then performed whole-cell patch-clamp recordings from mCherry\textsuperscript{+} neurons in CeA slices (Fig. 4A). We found that photostimulation of ChR2 fibers emanating from the NTS evoked much larger EPSCs in the CeA\textsuperscript{SST} neurons in oxaliplatin-treated mice than in vehical mice (Fig. 4B and C). In each recorded CeA\textsuperscript{SST} neuron, we also calculated the paired-pulse ratio (PPR), which is related to the probability of vesicle release (Silver et al., 1998). The CeA\textsuperscript{SST} neurons showed paired-pulse depression (Fig. 4B and D), suggesting that the glutamatergic NTS-CeA\textsuperscript{SST} axons had a relatively high probability of transmitter release (i.e., that the glutamatergic synapse strength was stronger and the excitatory inputs of CeA\textsuperscript{SST} neurons were enhanced in the mice with chronic pain).

We further examined whether a gain of excitatory inputs by the NTS could induce hyperexcitability of CeA\textsuperscript{SST} neurons by recording CeA\textsuperscript{SST} neurons in slices from mice with chronic pain (Fig. 4E and F). We found an increase in the number of spikes in the CeA\textsuperscript{SST} neurons in mice exposed to chemotherapy compared to control mice (Fig. 4G and H), indicating that the neuronal activity of CeA\textsuperscript{SST} neurons was increased in chronic pain.

We also further investigated the necessity of CeA\textsuperscript{SST} neuronal hyperexcitability in comorbid depression by selectively suppressed CeA\textsuperscript{SST} neurons by introducing the Cre-inducible inhibitory molecule hM4Di into the CeA of SST-Cre mice and intraperitoneally (i.p.) injecting mice with CNO (Fig. 4I and J; Fig. S1D-S1F). We found that pharmacogenetic suppression of CeA\textsuperscript{SST} neurons significantly relieved the depressive behaviors in the mice (Fig. 4K and L), suggesting that silencing of CeA\textsuperscript{SST} neurons can counteract chronic pain-induced depression-like behaviors.

Fig. 4. Increased NTS glutamatergic inputs to CeA\textsuperscript{SST} neurons in mice with chemotherapy-induced neuropathic pain. (A) A schematic diagram showing the sites of AAV-hSyn-ChrR2-EYFP injection into the NTS and AAV-DIO-mCherry injection into the CeA of SST-Cre mice and patch-clamp recording of CeA\textsuperscript{SST} neurons. (B) Representative optically evoked EPSCs recorded in CeA\textsuperscript{SST} neurons of mice treated with vehicle or oxaliplatin. Blue bar, LED stimulation (475 nm, 1 ms). (C) The EPSCs of mice treated with oxaliplatin were larger than those of mice treated with vehicle (n = 10 neurons from 4 mice treated with vehicle, n = 8 neurons from 4 mice treated with oxaliplatin); Mann-Whitney test. (D) The PPRs (P2/P1) of NTS glutamatergic inputs to CeA\textsuperscript{SST} neurons in mice treated with oxaliplatin were significantly smaller than those in mice treated with vehicle (n = 10 neurons from 4 mice treated with vehicle, n = 8 neurons from 4 mice treated with oxaliplatin); Mann-Whitney test. (E) A schematic diagram showing the sites of AAV-DIO-mCherry virus injection into the CeA of SST-Cre mice. (F) A schematic diagram showing the recorded CeA\textsuperscript{SST} neurons (n = 10 neurons). (G and H) Sample traces of and statistical data for action potentials recorded from CeA\textsuperscript{SST} neurons in mice treated with vehicle or oxaliplatin. Blue bar, LED stimulation (475 nm, 1 ms). (I) The EPSCs of mice treated with oxaliplatin were larger than those of mice treated with vehicle (n = 10 neurons from 4 mice treated with vehicle, n = 8 neurons from 4 mice treated with oxaliplatin), Mann-Whitney test. (J) The PPRs (P2/P1) of NTS glutamatergic inputs to CeA\textsuperscript{SST} neurons in mice treated with oxaliplatin were significantly smaller than those in mice treated with vehicle (n = 10 neurons from 4 mice treated with vehicle, n = 8 neurons from 4 mice treated with oxaliplatin); Mann-Whitney test. (E) A schematic diagram showing the sites of AAV-DIO-mCherry virus injection into the CeA of SST-Cre mice. (F) A schematic diagram showing the recorded CeA\textsuperscript{SST} neurons (n = 10 neurons). (G and H) Sample traces of and statistical data for action potentials recorded from CeA\textsuperscript{SST} neurons in mice treated with vehicle or oxaliplatin. Blue bar, LED stimulation (475 nm, 1 ms). (I) A schematic diagram showing the sites of AAV-DIO-hM4Di-mCherry or AAV-DIO-mCherry virus injection into the bilateral CeA of SST-Cre mice. (J) Expression of hM4Di-mCherry in the CeA. Scale bar, 300 μm. (K and L) The effects of the inhibition of CeA\textsuperscript{SST} neurons on depression-like behaviors in the TST (K) and SPT (L); two-way ANOVA. (K) Expression of hM4Di-mCherry in the CeA. Scale bar, 300 μm. (K and L) The effects of the inhibition of CeA\textsuperscript{SST} neurons on depression-like behaviors in the TST (K) and SPT (L); two-way ANOVA (n = 9 or 10 mice). All error bars represent SEMs. *p < 0.05, **p < 0.01, ***p < 0.001. Oxal, oxaliplatin. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
3.5. An NTS-CeA projection modulates chronic pain-induced depression

We then examined the functions of NTS-CeA excitatory projection. We hypothesized that NTS glutamatergic neurons modulate pain experience via CeA\textsuperscript{SST} downstream neurons. We utilized a retrogradely expressing AAV combined with a dual-recombinase system (Li et al., 2018). AAV2/2-retro-FLEX-Flpo was bilaterally injected into the CeA, and AAV-fDIO-hM4Di-mCherry or AAV-fDIO-mGFP was injected into the NTS of Vglut2-Cre mice (Fig. 5A). The expression of hM4Di was dependent on both Cre and Flpo recombinase; therefore, only NTS glutamatergic neurons projecting to the CeA expressed hM4Di (Fig. 5B). Silencing of NTS glutamatergic neurons projecting to the CeA with CNO (i.p., 1 mg/kg) diminished pain-related and depression-like behaviors but not locomotion in mice with chronic pain (Fig. 5C-5F; Fig. S4A-S4D). The possibility that the expression of an excitatory DREADD in the NTS-CeA projection induced depression-like behaviors was also examined (Fig. 5G and H). As expected, delivery of CNO (i.p., 1 mg/kg) significantly increased the depressive behaviors in mice without affecting locomotion (Fig. 5I-5L). We determined the specificity of the NTS-CeA excitatory projection in depression modulation by investigating its roles in two other rodent models of depression. We exposed mice to chronic restraint stress (CRS) or repeated social defeat stress (SDS) and found that inhibition of CeA-projecting NTS glutamatergic neurons did not affect CRS- or SDS-induced depression-like behaviors (Fig. S4E-S4H). These data indicated that the NTS-CeA excitatory projection is necessary and sufficient for the development of depression-like behaviors, specifically in chronic pain, but not in restraint or social stress-related depression.

We examined the requirement for CeA\textsuperscript{SST} neurons in NTS-CeA excitatory circuit-induced depression by activating CeA-projecting NTS neurons, which are glutamatergic, while suppressing CeA\textsuperscript{SST} neurons. We introduced AAV-fDIO-hM3Dq-EGFP into the NTS, and AAV2/2-retro-hSyn-Flpo mixed with AAV-DIO-hM4Di-mCherry or AAV-DIO-mCherry into the CeA of SST-Cre mice (Fig. 6A-6C). This revealed that activation of the NTS-CeA pathway induces obvious depressive behaviors in naive mice, but these behaviors can be alleviated by silencing the CeA somatostatin-expressing neurons (Fig. 6D-6F). Together, these results suggested that CeA-projecting NTS glutamatergic neurons induce depression-like behaviors via CeA\textsuperscript{SST} neurons.

4. Discussion

The major findings of this study include the following: (1) A glutamatergic projection from the NTS innervates and modulates the activity of the somatostatin-expressing neurons in the CeA. (2) This NTS-CeA circuit is activated under chronic pain and ultimately results in an
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increased firing activity of the CeA somatostatin-expressing neurons. (3) Overactivity of the CeA somatostatin-expressing neurons underlies the depression-like behaviors developed under chronic pain. (4) Inhibiting the firing of the CeA somatostatin-expressing neurons reverses the depressive, but not the hypersensitive pain behaviors. Taken together, our results reveal the functional role of an NTS-CeA projection in mediating depressive behavior comorbid to a state of chronic pain.

The NTS receives somatosensory inputs from the dorsal horn of the spinal cord (Todd, 2010), and vagal input from nodose ganglion neurons innervating visceral organs and thereby processes various sensory signals (Napadow et al., 2019; Singh et al., 2020). We hypothesize that chronic pain induces depression symptoms via activating the NTS-CeA circuit. An increase in glutamatergic neuronal activity in both the NTS and the CeA accompanied the depression-like behaviors in the mice after the second cycle of chemotherapy. In addition, we found that inhibiting the NTS glutamatergic neurons alleviated depression-like behavior but not hypersensitive pain behavior, suggesting that this neuronal population may have a specific role in the emotional-affective dimension of chronic pain.

The CeA is well recognized as a critical structure for the emotional-affective dimension of pain and pain modulation (Liang et al., 2020; Senn et al., 2014; Zhou et al., 2019). Two recent studies (Liang et al., 2020; Zhou et al., 2019) have identified a glutamatergic projection from the thalamic paraventricular nucleus contributing to the role of CeA in mechanical hypersensitivity and a serotonergic projection from the dorsal raphe nucleus to CeA. We previously showed that NTS glutamatergic neurons innervate the CeA and CeA accompanied the depression-like behaviors in the mice after the second cycle of chemotherapy. Our recent study further extends this line of evidence by adding a new glutamatergic circuit and a widespread polyneuropathic model to the list. Our anatomic results show that NTS neurons form excitatory synapses with CeA neurons. Given that CeA neurons accept serotonergic innervation from various mental state-related regions and play an important role in mediating depression and anxiety (Liu et al., 2021; Sanders et al., 2019; Zhou et al., 2019), the NTS glutamatergic projection may directly facilitate depression-like behaviors in the mice receiving a 2-cycle administration of oxaliplatin via CeA neurons. This is supported by the following three pieces of evidence: First, excitatory synapses between NTS and CeA neurons were stronger in mice with chronic pain. Second, specific inhibition of CeA-projecting NTS glutamatergic neurons alleviated the depression-like behaviors, whereas selective activation of NTS glutamatergic neurons projecting to the CeA induced depression-like behaviors. Finally, activation of the NTS-CeA projection induced obvious depressive behaviors in naive mice, but these behaviors could be blocked by silencing the CeA somatostatin-expressing neurons. We also investigated the potential role of NTS GABAergic neurons in addition to glutamatergic neurons. Consistent with previous studies showing that NTS GABAergic neurons mainly form circuits with hindbrain areas, the bed nuclei of the stria terminalis, and the paraventricular hypothalamic nucleus (Shi et al., 2021), we found no NTS GABAergic neurons projecting to CeA in mice with chronic pain.

Interestingly, in our study, inhibiting the glutamatergic NTS-CeA projection has no effect on depression-like behavior in mice with chronic stress, suggesting that this circuit is specifically recruited and thus has a role in the emotional-affective dimension of chronic pain. In mice with strong stresses, such as acute life-threatening conditions, the possibility exists that more NTS glutamatergic neurons are recruited to produce depression. Although we emphasized the crucial role of NTS glutamatergic neurons, other NTS subpopulations may also play roles in chronic pain-induced depression-like behaviors. Notably, the AAV-based retrograde strategy applied here cannot label all the CeA-projecting NTS glutamatergic neurons; thus, we cannot exclude the possibility that the unlabeled glutamatergic neurons or other neuron subpopulations among the NTS neurons project to the CeA and play a role in the emotional-affective dimension of chronic pain. Additionally, we have missed a control group for the DREADD experiments.

5. Concluding remarks

In closing, we identified a functional role for an NTS-CeA ascending projection in mediating depression-like behaviors under a state of chemotherapy-induced neuropathic pain. Inhibition of this projection might be a direct and useful therapeutic method for the treatment of comorbid depressive symptoms in chronic pain.
Author contributions

XH, YZ, and XR designed experiments. Animal breeding, behavioral testing, data analysis, and the construction of the manuscript was performed by SH, XH, and JZ. SH, XH, JZ, and XR performed experiments. All authors contributed to editing and revisions of the manuscript.

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Availability of data and materials

The data used and/or analyzed during the current study are available from the corresponding author on reasonable request.

CRediT authorship contribution statement

Shilang He: Methodology, Data curation, Visualization, Investigation. Xuelin Huang: Conceptualization, Methodology, Jun Zheng: Conceptualization, Methodology, Validation. Yuehong Zhang: Methodology, Validation. Xiangcai Ruan: Conceptualization, Supervision.

Declaration of Competing Interest

The authors declare that they have no competing interests.

Data availability

Data will be made available on request.

Acknowledgements

Not applicable.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nd.2022.105893.

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