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Basic Study

Nucleus tractus solitarius mediates hyperalgesia induced by chronic pancreatitis in rats

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Abstract

BACKGROUND

Central sensitization plays a pivotal role in the maintenance of chronic pain induced by chronic pancreatitis (CP). We hypothesized that the nucleus tractus solitarius (NTS), a primary central site that integrates pancreatic afferents apart from the thoracic spinal dorsal horn, plays a key role in the pathogenesis of visceral hypersensitivity in a rat model of CP.

AIM

To investigate the role of the NTS in the visceral hypersensitivity induced by chronic pancreatitis.

METHODS

CP was induced by the intraductal injection of trinitrobenzene sulfonic acid (TNBS) in rats. Pancreatic hyperalgesia was assessed by referred somatic pain *via von Frey* filament assay. Neural activation of the NTS was indicated by immunohistochemical staining for Fos. Basic synaptic transmission within the NTS was assessed by electrophysiological recordings. Expression of vesicular glutamate transporters (VGluTs), N-methyl-D-aspartate receptor subtype 2B

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(NR2B), and α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor subtype 1 (GluR1) was analyzed by immunoblotting. Membrane insertion of NR2B and GluR1 was evaluated by electron microscopy. The regulatory role of the NTS in visceral hypersensitivity was detected *via* pharmacological approach and chemogenetics in CP rats.

RESULTS

TNBS treatment significantly increased the number of Fos-expressing neurons within the caudal NTS. The excitatory synaptic transmission was substantially potentiated within the caudal NTS in CP rats (frequency: 5.87 ± 1.12 Hz in CP rats *vs* 2.55 ± 0.44 Hz in sham rats, $P < 0.01$; amplitude: 19.60 ± 1.39 pA in CP rats *vs* 14.71 ± 1.07 pA in sham rats; $P < 0.01$). CP rats showed upregulated expression of VGluT2, and increased phosphorylation and postsynaptic trafficking of NR2B and GluR1 within the caudal NTS. Blocking excitatory synaptic transmission *via* the AMPAR antagonist CNQX and the NMDAR antagonist AP-5 microinjection reversed visceral hypersensitivity in CP rats (abdominal withdraw threshold: 7.00 ± 1.02 g in CNQX group, 8.00 ± 0.81 g in AP-5 group and 1.10 ± 0.27 g in saline group, $P < 0.001$). Inhibiting the excitability of NTS neurons *via* chemogenetics also significantly attenuated pancreatic hyperalgesia (abdominal withdraw threshold: 13.67 ± 2.55 g in Gi group, 2.00 ± 1.37 g in Gq group, and 2.36 ± 0.67 g in mCherry group, $P < 0.01$).

CONCLUSION

Our findings suggest that enhanced excitatory transmission within the caudal NTS contributes to pancreatic pain and emphasize the NTS as a pivotal hub for the processing of pancreatic afferents, which provide novel insights into the central sensitization of painful CP.

Key words: Rat; Chronic pancreatitis; Visceral hypersensitivity; Nucleus tractus solitarius; Excitatory synaptic transmission

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Core tip: This study investigated the role of the nucleus tractus solitarius (NTS) in the pathogenesis of visceral hypersensitivity in a rat model of chronic pancreatitis (CP). We demonstrated that CP rats exhibited potentiated excitatory synaptic transmission within the caudal NTS. This potentiation may be mediated by enhanced glutamate release and the recruitment of membrane glutamate receptors. Inhibiting both the excitatory synaptic transmission and neural activity of caudal NTS neurons alleviated visceral hypersensitivity in CP rats. Our results suggest the caudal NTS as a primary central site that processes pancreatic pain, as well as a potential therapeutic target for the treatment of chronic visceral pain in patients with CP.

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INTRODUCTION

Persistent pain is the cardinal feature of chronic pancreatitis (CP) with a profound socioeconomic impact^[1]. The management of painful CP remains a major challenge because current therapeutic approaches fail to produce satisfactory results. More effective therapies require the discovery of novel targets based on new advances in our understanding of the neurobiology of painful CP^[2,3]. Anatomical knowledge indicates that pancreatic sensory information enters the central nervous system *via* both sympathetic and vagus nerves. The former runs through the celiac plexus and reaches the lower thoracic spinal cord *via* the splanchnic nerve, while the latter runs through the nodose ganglion and connects with the nucleus tractus solitarius (NTS)

via the abdominal vagus^[4]. Previous studies concerning the central processing of painful CP usually center on the thoracic spinal dorsal horn^[5-7]. In general, prolonged stimulation from peripheral sensitization facilitates aberrant excitation of dorsal horn neurons. This process is referred to as central sensitization and results in visceral hypersensitivity in chronic pancreatic pain^[8-10]. Unfortunately, much less focus has been directed on the role of the NTS in painful CP.

The NTS is a key relay station for primary visceral afferents located within the dorsomedial medulla oblongata. It is commonly recognized that the rostral third of the NTS is implicated in gustatory and oral somatosensory regulation^[11], while the caudal two-thirds is a major hub for general visceral sensation^[12]. An increasing body of morphological evidence has demonstrated both mechanical^[13,14] and chemical^[15-17] gastrointestinal noxious stimuli induced overexpression of Fos within the NTS, suggesting the involvement of the NTS in the processing of visceral pain. However, the cellular and molecular mechanisms underlying the role of the NTS in visceral pain have not been reported.

Glutamate is the major excitatory neurotransmitter within the NTS. Excitatory synaptic transmission is generally mediated by the action of glutamate on two different ionotropic receptors, the N-methyl-D-aspartate receptor (NMDAR) and the α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA), which play a major role in the processing of visceral sensation within the NTS^[18,19]. Accumulating preclinical studies have provided evidence of plasticity changes in glutamatergic synaptic transmission within the NTS, including the upregulation, modification, and membrane insertion of NMDAR and AMPAR, under pathological circumstances such as chronic hypertension and hypoxia^[20-22]. Our previous study also showed that excitatory synaptic transmission within the NTS was enhanced during chronic myocardial infarction (CMI) induced visceral pain^[23]. Both chemical lesion and pharmacological inhibition of the NTS exerted analgesic effects in cardiac visceral pain^[23,24]. Considering these, we hypothesized that excitatory synaptic transmission within the NTS is potentiated and contributes to visceral hypersensitivity in painful CP.

In the present study, we first examined the expression of Fos within the NTS in rats with CP induced by intraductal trinitrobenzene sulfonic acid (TNBS) injection. Electrophysiological approach was subsequently performed to explore the changes in excitatory synaptic transmission within the NTS. Then, immunoblottings and electron microscopy were applied to unravel the potential molecular basis for these changes. Finally, we proved the pain-facilitating role of the NTS in the pathogenesis of painful CP *via* pharmacological method and chemogenetics.

MATERIALS AND METHODS

Animals and experimental design

Eighty-one male Sprague-Dawley rats (250-280 g) were provided by the Experimental Animal Center of the Fourth Military Medical University (Xi'an, China). All procedures were performed according to the guidelines of the International Association for the Study of Pain^[25] and approved by the Institutional Animal Care and Use Committee of the Fourth Military Medical University. Every measure was taken to minimize the discomfort of the animals. The animals were acclimatized to laboratory conditions (23 °C, 12 h/12 h light/dark, 50% humidity) for two weeks prior to experimentation and were provided with water and food *ad libitum* 12 h before and after the induction of pancreatitis, during which food and water were withdrawn. Animals for tissue collection were euthanized by an overdose of 2% pentobarbital sodium (i.p., 100 mg/kg), while those for surgery were deeply anesthetized *via* 2% pentobarbital sodium (i.p., 40 mg/kg).

Experiment 1: Evidence of increased expression of Fos-immunoreactive (-ir) neurons within the NTS in CP rats. Eight rats were equally divided into two groups (sham and TNBS groups). Pancreatitis was induced by intraductal infusion of 0.4 mL of 2% TNBS (Sigma, St. Louis, MO, USA) as previously reported^[26]. The sham rats received an equal volume of saline. On postoperative day (POD) 14, brain tissues were sampled for Fos immunostaining.

Experiment 2: Effect of painful CP on excitatory synaptic transmission within the NTS. Eighteen rats were equally divided into two groups (sham and TNBS groups). On POD 14, brainstem sections containing the NTS were obtained for electrophysiological recordings.

Experiment 3: Effect of painful CP on the expression of vesicular glutamate

transporters (VGluTs), NMDAR subunit NR2B, and AMPAR subunit GluR1 within the NTS. Twelve rats were equally divided into four groups (sham, TNBS POD 7, POD 14, and POD 28 groups). All TNBS-treated rats were sacrificed according to aforementioned time points and processed for NTS sampling for VGluT1, VGluT2, NR2B, and GluR1 immunoblotting.

Experiment 4: Evidence for painful CP induced membrane insertion of NR2B and GluR1 within the NTS. Six rats were equally divided into two groups (saline and TNBS groups). On POD 14, the brainstems were sampled for NR2B and GluR1 electron microscopy.

Experiment 5: Effects of CNQX (AMPA antagonist) and AP-5 (NMDAR antagonist) microinjection into the NTS on visceral hypersensitivity of CP rats. Nineteen rats were divided into three groups (6 rats in saline group, 6 rats in CNQX group, and 7 rats in AP-5 group), and received cannula implantation upon the NTS 7 days before TNBS treatment. On POD 14, the abdominal withdraw threshold (AWT) was monitored before and after drug microinjection into the NTS.

Experiment 6: Effects of activation or inactivation of NTS excitatory neurons on visceral hypersensitivity of CP rats. Eighteen rats were equally divided into three groups (CaMKIIa-Gq, CaMKIIa-mCherry, and CaMKIIa-Gi groups), and received virus injection into the NTS 7 d before TNBS treatment. On POD 14, the AWT was monitored before and after clozapine-N-oxide (CNO) treatment.

von Frey test

As a measure of referred abdominal mechanical hypersensitivity in CP rats, AWT was monitored by *von Frey* filament (VFF; Stoelting, Kiel, WI, United States) assay^[26]. For the VFF assay, the belly area designated for stimulation was shaved 3 d before testing. The animals were habituated in the testing apparatus until calming down. VFFs with increased forces from 0.16 g to 26 g were applied to the abdomen 5 times, each for 5-8 s with a 5 min interval. The minimal force that elicited at least 3 times of withdrawn responses was considered as the AWT.

Immunohistochemical staining

On POD 14, the rats in Experiment 1 were deeply anesthetized and then perfused with 100 mL of 0.01 mol/L phosphate buffer saline (PBS, pH 7.4), followed by 500 mL 4% paraformaldehyde (PFA) fixative solution in 0.1 mol/L of phosphate buffer (PB, pH 7.4). After perfusion, the brains were removed, placed in 30% sucrose solution for 24 h at 4 °C, and then cut into coronal sections at 35 μm thickness into six dishes as six sets of every sixth serial sections. Next, the sections in the first set were incubated in 10% normal donkey serum for 40 min at room temperature (RT) to block nonspecific immunoreactivity, and then incubated overnight at 4 °C with the following antibodies in sequence: (1) Mouse anti-Fos (1:500; Abcam, Cambridge, MA, United States) for 24 h at 4 °C; (2) Biotinylated donkey anti-mouse (1:500; Millipore, Billerica, MA, United States) for 4 h at RT; and (3) Avidin-biotin complex (1:200; ABC kit, Vector, Burlingame, CA, United States) for 2 h at RT. The sections were then reacted with 0.05 mol/L Tris-HCl buffer containing 0.02% DAB (Dojin, Kumamoto, Japan) and 0.003% H₂O₂ for visualizing Fos. Finally, the slices were mounted onto glass slides, and coverslipped for examination *via* light microscopy (AH-3, Olympus, Tokyo, Japan). The images were captured and analyzed using a Fluoview 1000 microscope (Olympus). For Fos-ir neuron counting, at least four sections per brain region from each rat were counted for the average number of Fos-ir neurons in the sham and TNBS groups (four rats per group) by an investigator who was blind to the experiment. We limited our quantifications to the medial prefrontal cortex (mPFC), anterior cingulate cortex (ACC), insular cortex (IC), paraventricular thalamic nucleus (PVT), central medial thalamic nucleus (CM), amygdala, periaqueductal gray (PAG), dorsal raphe nucleus (DR), parabrachial nuclei (PBN), and NTS, all of which are closely related to visceral pain^[17,27].

Whole-cell patch-clamp recordings

On POD 14, the rats in Experiment 2 were anesthetized and coronal brainstem sections (300 μm) containing the NTS were cut at 4 °C with a vibratome in oxygenated ACSF (in mmol/L: 124 NaCl, 2.5 KCl, 2 MgSO₄, 2 CaCl₂, 25 NaHCO₃, 1 NaH₂PO₄, 37 glucose, pH 7.4). The sections were then transferred to a recovery chamber with oxygenated ACSF at RT for 1 h. For whole-cell patch-clamp recordings, the section was placed in a recording chamber maintained with oxygenated ACSF at 28 °C. The potassium-based intracellular solution within the micropipettes (8-10 MΩ) contained the following (in mmol/L): 120 K-gluconate, 5 NaCl, 0.2 EGTA, 10 HEPES, 2 MgATP, 0.1 Na₃GTP, 1 MgCl₂, and 10 phosphocreatine (adjusted to pH 7.2 with KOH, 290

mOsm). Neurons with small diameters (<15 μm), which were assumed to receive excitatory afferents^[28,29], were visually preselected in the commissure and medial part of caudal NTS neurons. Before recording, a concentric bipolar electrode was placed on the adjacent solitary tract and stimulus pulses were applied at a minimal voltage (1-15 V, 0.1 ms pulse width). Only neurons with a clear monosynaptic evoked excitatory postsynaptic currents (EPSC) peak were then processed for spontaneous EPSCs (sEPSCs) recording. sEPSCs were recorded for at least 10 min with the membrane clamped at -60 mV, and sEPSCs from 5 to 10 min were statistically analyzed. All recordings were conducted in the presence of picrotoxin (100 μM , Sigma) to block GABA_A receptor-mediated inhibitory synaptic currents. All recordings were performed with a MultiClamp 700B amplifier (Axon Instruments, Sunnyvale, CA, United States) and digitized at 5 kHz with DigiData 1550B (Axon Instruments). Data were discarded if the access resistance (10-30 M Ω) changed by 15% during the experiment.

Western blot analysis

Brainstem tissues from the rats in Experiment 3 were harvested in cold ACSF. Total protein was prepared for VGluT1, VGluT2, NR2B, and GluR1 assays according to our previous study^[50]. Subsequently, 30 μg of protein from each sample (quantitatively measured by bicinchoninic acid protein assay; Thermo Scientific; Rockford, IL, United States) was subjected to 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) electrophoresis and electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore, Billerica, MA, United States). After blocking with 5% DifcoTM skim milk in Tris-buffered saline with Tween (TBST) for 2 h, the membranes were incubated overnight at 4 °C with the following primary antibodies: Mouse anti-VGluT1 (1:500; Millipore); mouse anti-VGluT2 (1:500; Synaptic Systems, Goettingen, Niedersachsen, Germany); rabbit anti-pNR2B-Tyr¹⁴⁷² (1:500; Millipore); rabbit anti-NR2B (1:500; Abcam, Cambridge, United Kingdom); rabbit anti-GluR1 (1:500; Millipore); and rabbit anti-pGluR1-Ser⁸⁴⁵ (1:500; Millipore). The immunoblots were then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (goat anti-rabbit/mouse, 1:5000; Amersham Pharmacia Biotech, Piscataway, NJ, United States). To verify equal loading, we also probed the membranes with rabbit anti-GAPDH (1:5000; Beijing TDY BIOTECH CO., Beijing, China). Bands were visualized by an enhanced chemiluminescence (ECL) detection method (Amersham Pharmacia Biotech) and exposed to films. The scanned images were quantified and analyzed with ImageJ software. Target protein levels were normalized against the GAPDH levels and are expressed as fold changes relative to those of the sham group.

Electron microscopy

The rats in Experiment 4 were deeply anesthetized and perfused with 100 mL of 0.01 mol/L PBS followed by 500 mL of fixative containing 4% PFA and 0.05% glutaraldehyde in 0.1 mol/L PB. The brainstem was immediately removed and postfixed in the same fixative without glutaraldehyde at 4 °C for 4 h, and the lower medulla oblongata was then transversely cut on a vibratome (Microslicer DTM-1000; Dosaka EM, Kyoto, Japan) into 50 μm sections. The sections were further divided into four sets in four dishes. The sections in the first two dishes were subsequently processed for NR2B and GluR1 staining.

Sections were cryoprotected in 0.05 mol/L PB containing 30% sucrose and 10% glycerol for 30 min. The sections were freeze-thawed and then incubated in a blocking solution containing 20% normal goat serum in 0.05 mol/L Tris-buffered saline (TBS; pH 7.4) for 30 min, followed by incubation with rabbit anti-GluR1 (1:200; Millipore) or rabbit anti-NR2B (1:200; Abcam) at 4 °C for 12 h. After washing in TBS, the sections were incubated for 8 h in anti-rabbit antibody conjugated to 1.4 nm gold particles (1:100; Nanoprobes; Stony Brook, NY, United States). The sections were then processed as follows: (1) Post-fixation with 1% glutaraldehyde in 0.1 mol/L PB for 10 min; (2) Silver enhancement with an HQ Silver Kit (Nanoprobes); (3) 1% OsO₄ in 0.1 mol/L PB for 1 h; and (4) 1% uranyl acetate in 70% ethanol for 1 h. After dehydration, the sections were mounted on glass slides and embedded in epoxy resin (Durcupan; Fluka, Buchs, Switzerland). Under an electron microscope, the immunoreactivities were determined by the presence of the immunogold-silver particles distributed in the cytoplasm of the somata and dendrites. An asymmetric synaptic junction containing more than two immunogold-silver particles was considered as an immunoreactive postsynaptic structure. The average distance between individual gold particles and the closest edge of the postsynaptic active zone in each asymmetric synapse within the commissure and medial parts of the caudal NTS was counted.

Drug microinjection and behavioral tests

The rats were deeply anesthetized and secured on a stereotaxic apparatus (RWD Life Sciences, Shenzhen, Guangzhou, China). Guide cannulas (26 gauge; RWD life sciences) were implanted into the caudal NTS (14.00 mm caudal to the bregma)^[31]. Briefly, a partial occipital craniotomy was performed and the dura and arachnoid were incised to expose the dorsal surface of the brainstem at the level of the obex. The calamus scriptorius was identified and served as the anterior/posterior and lateral stereotaxic zero coordinate. The guide cannula was implanted in the NTS at coordinates that were 0.8 mm rostral and 0 mm lateral to the calamus scriptorius and 0.3 mm ventral to the dorsal surface of the medulla^[24]. On POD 14, intra-NTS injections were delivered through an injector cannula (30 gauge), which was located 0.2 mm lower than the guide. A microsyringe (10 μ L, Hamilton, NV, United States) was connected to the injector by a thin polyethylene tube and was driven by a motorized pump (ALCBIO, Shanghai, China). AP-5 (50 mmol/L, 0.4 μ L) or CNQX (20 mmol/L, 0.4 μ L) was infused into the caudal NTS at a rate of 0.05 μ L/min, with an equivalent volume of saline as the control. All injections were followed by an additional 5 min before removal of the injection cannula. The AWT was measured before TNBS treatment, and 30 min before and after drug microinjection on POD 14. Injection sites were verified *post hoc*, and the rats with inaccurate sites were excluded.

Chemogenetics and behavioral tests

To specifically modulate the activity of caudal NTS excitatory neurons, we injected Calmodulin-dependent Protein Kinase IIa (CaMKIIa) promoter-driven virus (rAAV2/9-CaMKIIa-hM4Di/hM3Dq-mCherry, 0.25 μ L/30 min, BrainVTA, Wuhan, Hubei, China) into the caudal NTS 7 d before TNBS treatment, with an equivalent amount of rAAV2/9-CaMKIIa-mCherry injected as the sham group. The virus was injected into the NTS by pressure through a glass micropipette (tip diameter 15–25 μ m) attached to a Hamilton microsyringe (1 μ L) at coordinates that were 0.8 mm rostral and 0 mm lateral to the calamus scriptorius and 0.5 mm ventral to the dorsal surface of the medulla. AWT tests were performed before TNBS treatment, and 1 h before and after CNO administration (*i.p.*, 3 mg/kg) on POD 14. Injection sites were verified *post hoc*, and the rats with inaccurate sites were excluded.

Data analysis

The results are expressed as the mean \pm SEM. Statistical analysis between two groups was tested by Student's *t*-test (SPSS 17.0). Statistical comparison among multiple groups was performed using one-way ANOVA followed by the LSD *post hoc* test. Analyzed numbers for each experiment are indicated in the corresponding figures. $P < 0.05$ was considered statistically significant.

RESULTS

TNBS treatment increases Fos expression within caudal NTS

Fos is widely used as an indicator of neuronal activation under the condition of pain stimuli^[32]. In the present study, we selected POD 14 as the time point for Fos immunostaining, when rats exhibited robust visceral hypersensitivity^[33]. As the NTS is a long brain region along the rostral-caudal axis, we calculated the number of Fos-ir neurons from five different coronal sections that covered the whole NTS. As shown in **Figure 1**, no significant change in Fos expression was identified in the rostral NTS (Bregma -14.16: TNBS 142.25 ± 14.08 vs saline 143.25 ± 25.13 ; Bregma -14.88: TNBS 226 ± 21.41 vs saline 158.75 ± 23.56 ; $P > 0.05$; **Figure 1A and B**). However, more Fos-ir neurons were identified in the caudal NTS of the CP rats (Bregma -13.44: TNBS 238.5 ± 27.49 vs saline 155 ± 15.35 ; Bregma -14.16: TNBS 108 ± 6.99 vs saline 72.5 ± 6.34 ; Bregma -14.88: TNBS 76.75 ± 11.06 vs saline 41.75 ± 3.19 ; $P < 0.05$; $n = 4$ sections from 4 rats per group; **Figure 1C-E**). In the caudal NTS, more abundant Fos expression was observed in the medial (SolM) and commissural (SolC) regions of the NTS than in the lateral (SolL) region (**Figure 1C-E**) of the CP rats. These data provide morphological evidence for the activation of the caudal NTS under the condition of painful CP.

Apart from the NTS, many other pain-related areas exhibited higher levels of Fos expression in the CP rats (**Supplementary Figure 1**). In the cortical areas (**Supplementary Figure 1A-C**), TNBS treatment induced prominent Fos staining in the mPFC, ACC, and IC. In the subcortical areas (**Supplementary Figure 1D-E**), Fos expression was mainly found in the PVT and CM as well as the amygdala. In the midbrain (**Supplementary Figure 1F**), TNBS treatment augmented Fos expression within the PAG and DR. In the brainstem (**Supplementary Figure 1G**), abundant Fos expression was also observed in the lateral part of the PBN. These observations suggest that these classical brain regions associated with the sensory and emotional aspects of pain are

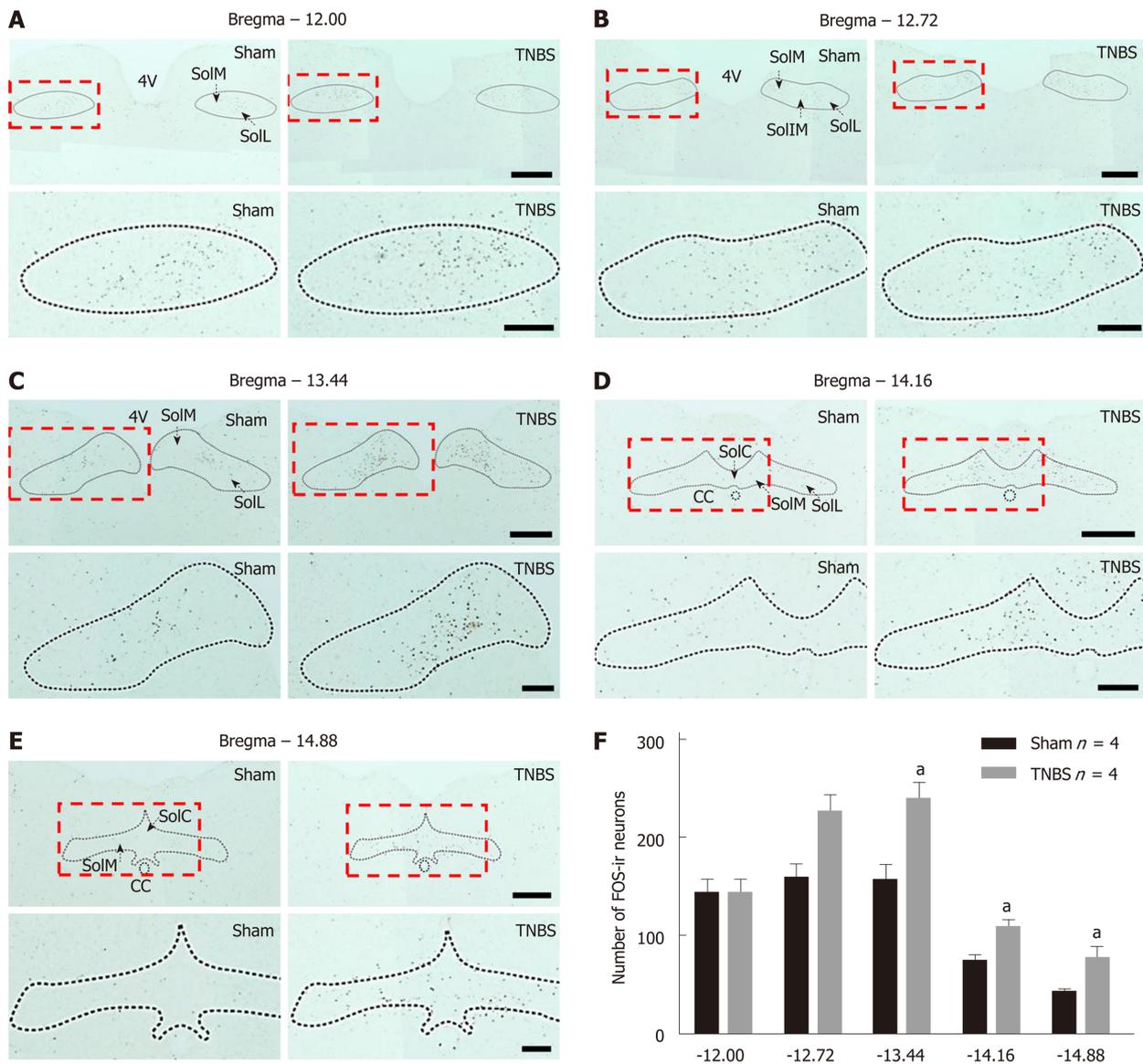


Figure 1 The number of Fos-expressing neurons is upregulated within the nucleus tractus solitarius in rats with chronic pancreatitis. A-E: Immunohistochemical staining for Fos within different coronal sections of nucleus tractus solitarius (NTS) in sham (left) and chronic pancreatitis (right) rats. The rectangles in the top panels are further magnified in the bottom panels. Bars = 500 μ m in top panels and 200 μ m in bottom panels; F: Histogram showing the qualification of Fos-expressing neurons within different parts of NTS in saline and trinitrobenzene sulfonic acid (TNBS)-treated rats. $n = 4$ rats per each group, unpaired t -test, $^*P < 0.05$, TNBS vs sham. 4V: 4th ventricle; CC: Central canal; SolIM: Nucleus of the solitary tract, intermedial part; SolM: Nucleus of the solitary tract, medial part; SolL: Nucleus of the solitary tract, lateral part; SolC: Nucleus of the solitary tract, commissural part; TNBS: Trinitrobenzene sulfonic acid.

activated during painful CP.

TNBS treatment enhances excitatory synaptic transmission within caudal NTS

We subsequently assessed whether there were electrophysiological changes in basal synaptic transmission within the caudal NTS in CP rats. The second-order neurons within the SolM and SolC, which receive sensory inputs from the solitary tract^[34], were recorded under voltage-clamp mode. Example recordings of sEPSCs from caudal NTS neurons in both groups are shown in Figure 2A. Comparing to the sham rats, the CP rats showed a rightward shift in the cumulative probability of the sEPSC amplitude (Figure 2B, left), with a leftward shift in the inter-event intervals (Figure 2B, right). The mean sEPSC amplitude (Figure 2C, left) was significantly higher (19.60 ± 1.39 pA vs 14.71 ± 1.07 pA; $P < 0.01$; $n = 13$ and 14 in the CP and sham rats, respectively) in the CP rats than that in the sham rats. The mean frequency (Figure 2C, right) was also significantly higher (5.87 ± 1.12 Hz vs 2.55 ± 0.44 Hz, $P < 0.01$) in the CP rats. These results suggest that increased presynaptic transmitter release probability and postsynaptic responsiveness both likely contribute to enhanced synaptic transmission in the caudal NTS of CP rats.

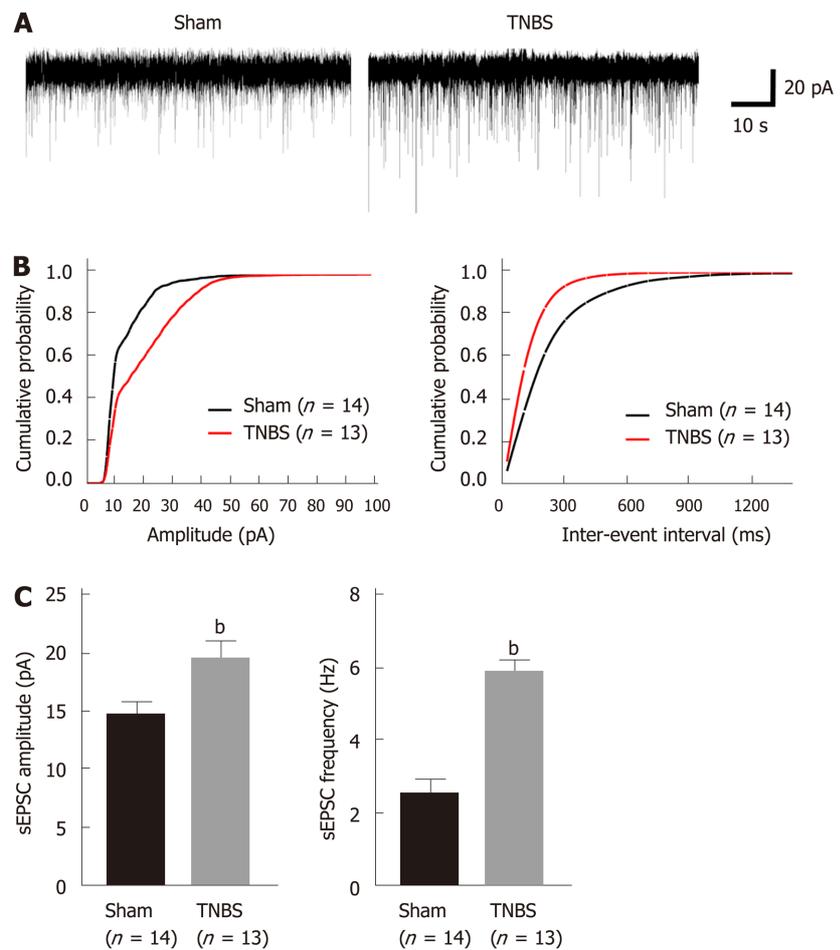


Figure 2 Rats with chronic pancreatitis have increased amplitude and frequency of spontaneous excitatory postsynaptic currents in the caudal nucleus tractus solitarius. A: Representative spontaneous excitatory postsynaptic currents (sEPSCs) recorded from a sham (left) rat and a trinitrobenzene sulfonic acid (TNBS)-treated (right) rat holding at -60 mV; B: Cumulative amplitude (left) and inter-event interval (right) histograms of sEPSCs recorded from sham and chronic pancreatitis (CP) rats; C: Summary plots showing the frequency (left) and amplitude (right) of sEPSCs from sham and CP rats. Unpaired *t*-test, ^b*P* < 0.01, TNBS vs sham. sEPSC: Spontaneous excitatory postsynaptic currents; TNBS: Trinitrobenzene sulfonic acid.

TNBS treatment increases expression of VGluT2 within caudal NTS

VGluTs are essential for glutamatergic synaptic transmission through glutamate packaging and subsequent exocytotic release upon stimulation within the central nervous system. The variation in the level of VGLUTs could have a major impact on synaptic transmission^[35,36]. Both VGluT1 and VGluT2 terminals are present while VGluT2 terminals predominate within the NTS^[37]. To examine whether there are expressional changes of VGluTs within the caudal NTS in CP rats, biochemical studies were performed to measure the levels of VGluT1 and VGluT2 at different time points. Biochemical analysis showed the expression of VGluT2 was upregulated within the caudal NTS along the course of CP (Figure 3A and B), with no significant change in the VGluT1 level between the sham and CP rats (Figure 3A and C). These data suggest that CP stimulates the secretion of glutamate, which may account for the increased presynaptic transmitter release within the caudal NTS.

TNBS treatment increases expression of GluR1 and NR2B within caudal NTS

NMDAR is a tetra-heteromeric ligand-gated ion channel composed of an obligate NR1 subunit and NR2A-D subunits^[38]. Among these, NR2B participates in various synaptic signaling events and is implicated in a series of functions, including learning memory and chronic pain^[39]. AMPAR is also known to be a heteromeric tetramer containing GluR1-4. Although GluR2 is the most abundant subunit within the NTS^[18], GluR1 exhibits expressional changes and may play a pivotal role in synaptic plasticity under pathological conditions, including chronic visceral pain^[21,22]. Considering these, we examined the expression changes of these two representative glutamate receptor subunits within the caudal NTS. Biochemical analysis showed that CP triggered a

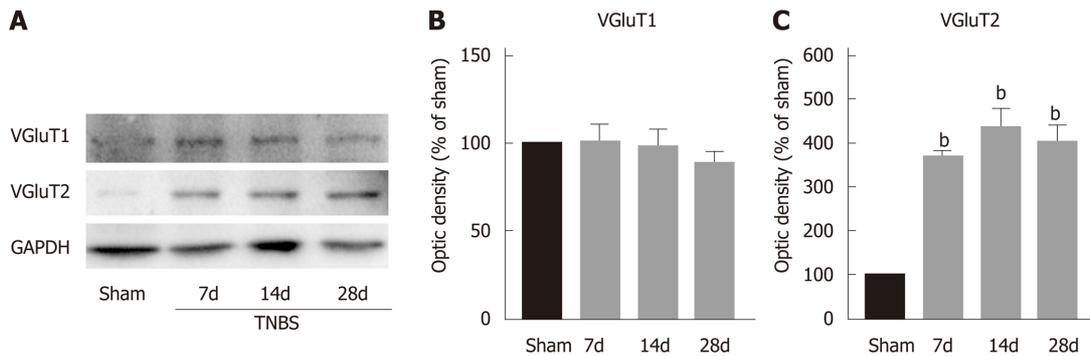


Figure 3 Trinitrobenzene sulfonic acid treatment upregulates the expression of vesicular glutamate transporter 2 within the caudal nucleus tractus solitarius. **A:** Representative Western blots for VGLUT1 and VGLUT2 within the caudal nucleus tractus solitarius (NTS) on postoperative days (POD) 7, 14, and 28; **B:** VGLUT1 amount within the caudal NTS showed no changes after trinitrobenzene sulfonic acid (TNBS) treatment; **C:** VGLUT2 amount within the caudal NTS was significantly increased on POD 7, 14, and 28 after TNBS treatment. $n = 3$ rats per each group, one-way ANOVA, ^a $P < 0.05$, ^b $P < 0.01$, TNBS vs sham. TNBS: Trinitrobenzene sulfonic acid; VGLUT1: Vesicular glutamate transporter 1; VGLUT2: Vesicular glutamate transporter 2.

long-term increase in the expression of GluR1 (Figure 4A and B) and NR2B (Figure 4A and C) within the caudal NTS.

Phosphorylation is an important posttranslational modification for membrane targeting of glutamate receptors^[40]. The phosphorylation of cortical NMDAR, particularly its NR2B subunit, leads to enhanced synaptic NMDARs *via* inhibiting NMDAR endocytosis and mediates the chronification of pain^[41]. Likewise, cortical GluR1 phosphorylation also contributes to the localization and function of AMPAR during chronic pain^[42-45]. To confirm whether similar posttranslational modifications occur during painful CP within the caudal NTS, we measured the phosphorylation status of NR2B and GluR1 using phosphorylation site-specific antibodies. Our data showed that both pNR2B (Figure 4D and E) and pGluR1 (Figure 4D and F) were robustly upregulated along the course of CP. These findings suggest that TNBS treatment leads to the upregulation and modification of glutamate receptors, which may provide postsynaptic evidence for enhanced synaptic transmission within the caudal NTS.

Enhanced membrane insertion of NR2B and GluR1 within caudal NTS in CP rats

Both NMDAR and AMPAR are dynamically shifting in and out of the membrane according to synaptic activity, which, in turn, determines synaptic strength^[46,47]. Our biochemical data showed the upregulation of pNR2B and pGluR1 in CP rats, indicating the accumulation of postsynaptic NR2B and GluR1 within the caudal NTS. By pre-embedding electron microscopy, we observed that more NR2B particles were closer to the synaptic active zone in the caudal NTS neurons from the CP rats (221.2 ± 14.33 nm, $n = 152$ synapses/3 rats) than those from the sham rats (332.3 ± 20.9 nm, $n = 156$ synapses/3 rats, $P < 0.001$) (Figure 5A-C). Similarly, more GluR1 particles were closer to the synaptic active zone in the CP rats (209.7 ± 12.87 nm, $n = 150$ synapses/3 rats) than the sham rats (266.1 ± 15.94 nm, $n = 145$ synapses/3 rats, $P < 0.001$) (Figure 5D-F). These results strongly suggest that CP induces postsynaptic accumulation of NR2B and GluR1 within caudal NTS neurons.

Inhibiting excitatory synaptic transmission within caudal NTS relieves CP-related visceral pain

We subsequently assessed whether inhibiting NTS glutamatergic transmission could alleviate the behavioral manifestations of pancreatic nociception in CP rats (Figure 6A and B). The behavioral results showed that microinjection of both CNQX and AP-5 into the caudal NTS increased the AWT in the CP rats on POD 14 (7.00 ± 1.02 g in CNQX group, 8.00 ± 0.81 g in AP-5 group and 1.10 ± 0.27 g in saline group; $n = 6$ for saline and CNQX groups and 7 for AP-5 group; $P < 0.001$; Figure 6C). These behavioral results indicate that excitatory glutamatergic transmission within the caudal NTS contributes to the visceral hypersensitivity of CP rats.

Inhibiting excitatory caudal NTS neurons relieves CP-related visceral pain

Finally, we utilized chemogenetics to reversibly, bidirectionally modulate the activity of caudal NTS neurons in CP rats. We targeted both inhibitory and excitatory designer receptors exclusively activated by designer drugs (hM4Di and hM3Dq DREADD)^[48] to excitatory caudal NTS neurons by injecting rAAV2/9-CaMKIIa-

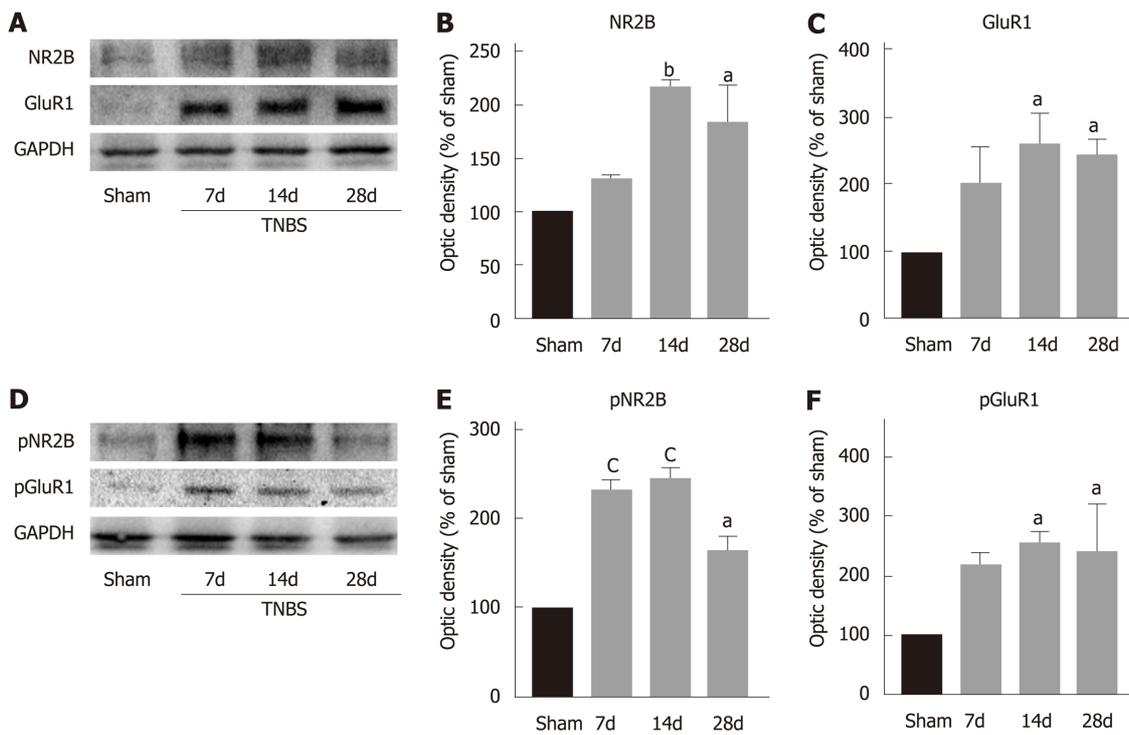


Figure 4 Trinitrobenzene sulfonic acid treatment upregulates the expression of N-methyl D-aspartate receptor subtype 2B and α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor subtype 1 within the caudal nucleus tractus solitarius. A: Representative Western blots for NR2B and GluR1 within the caudal nucleus tractus solitarius (NTS) on postoperative days (POD) 7, 14, and 28; B: The amount of NR2B within the caudal NTS was significantly increased on POD 7, 14, and 28 after trinitrobenzene sulfonic acid (TNBS) treatment; C: The amount of GluR1 within the caudal NTS was significantly increased on POD 7, 14, and 28 after TNBS treatment; D: Representative Western blots for pNR2B and pGluR within the caudal NTS after TNBS treatment; E: The amount of pNR2B within the caudal NTS was significantly increased after TNBS treatment; F: The amount of pGluR1 within the caudal NTS was significantly increased after TNBS treatment. $n = 3$ rats per each group, one-way ANOVA, ^a $P < 0.05$, ^b $P < 0.01$, ^c $P < 0.001$, TNBS vs sham. GluR1: α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor subtype 1; NR2B: N-methyl-D-aspartate receptor subtype 2B; TNBS: Trinitrobenzene sulfonic acid.

hM3Dq/hM4Di-mCherry into the caudal NTS (Figure 7A and B). Behavioral results showed that inhibiting excitatory caudal NTS neurons alleviated pancreatic hyperalgesia while activating them exerted no significant effects on the AWT of CP rats on POD 14 (13.67 ± 2.55 g in Gi group, 2.00 ± 1.37 g in Gq group and 2.36 ± 0.67 g in mCherry group; $n = 6$ in each group; $P < 0.01$, Gi vs mCherry; Figure 7C). These indicate an important role of excitatory caudal NTS neurons in the production of CP-induced visceral pain.

DISCUSSION

NTS and visceral pain

NTS is a second-order subnuclear complex processing gustatory and general viscerosensory afferents in its rostral and caudal regions, respectively. Peripheral visceral afferents terminate in topographically distinct regions of the NTS^[49-51]. For gastrointestinal inputs, vagal afferents from the upper digestive tract preferentially terminate in the central subnucleus of the intermediate NTS, while those from the subdiaphragmatic digestive tract target the SolM and SolC^[50,52-54]. A growing body of evidence using neural activation markers, such as Fos and phosphorylated extracellular signal-regulated kinases, suggests that the NTS is activated upon acute noxious gastrointestinal stimuli, as well as chronic ileitis pain^[13-16,55,56]. Our previous studies also showed the overexpression of Fos within the NTS, especially the SolM and SolC, in both irritable bowel syndrome (IBS) and CMI related visceral pain^[17,23]. In accordance with these findings, we observed an increased number of Fos-expressing neurons within the SolM and SolC in TNBS-treated rats, which provided morphological evidence for the involvement of the NTS in CP-related visceral pain^[17,23].

Apart from the NTS, we observed that a series of brain areas exhibited elevated expression of Fos during painful CP. Among these regions, the mPFC, ACC, IC, and amygdala are involved in the emotional dimension of pain^[57]; the PAG is the cardinal region for descending pain modulation^[58]; the PVT is a midline thalamic nucleus that

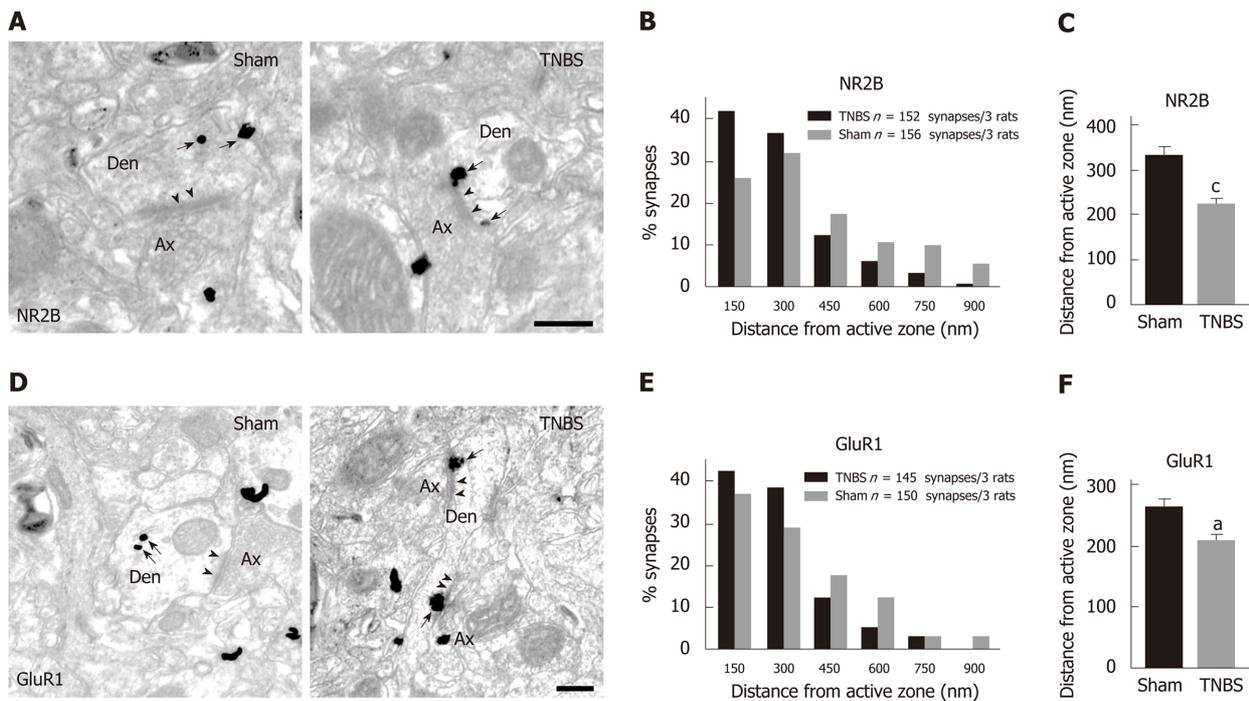


Figure 5 Trinitrobenzene sulfonic acid treatment results in accumulation of postsynaptic N-methyl D-aspartate receptor subtype 2B and α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor subtype 1 in the caudal nucleus tractus solitarius. **A:** Representative electron microscopy images showing immune-gold particles labeled N-methyl-D-aspartate receptor subtype 2B (NR2B) distributed in an asymmetric synapse within the caudal nucleus tractus solitarius (NTS) of sham (left) and chronic pancreatitis (CP) (right) rats; **B:** Percentage of NR2B particles in 150 nm bins as a function of distance from the nearest edge of synapses; **C:** Averaged distances of NR2B from the edge of synapses of sham rats and CP rats; **D:** Representative electron microscopy images showing immune-gold particles labeled GluR1 distributed in an asymmetric synapse within caudal NTS of sham (left) and CP (right) rats; **E:** Percentage of α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor subtype 1 (GluR1) particles in 150 nm bins as a function of distance from the nearest edge of synapses; **F:** Averaged distances of GluR1 from the edge of synapses of sham rats and CP rats. Bar = 250 nm in A and D. ^a $P < 0.05$, ^c $P < 0.001$, TNBS vs sham. Ax: Axon; Den: Dendrite; GluR1: α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor subtype 1; NR2B: N-methyl-D-aspartate receptor subtype 2B; TNBS: Trinitrobenzene sulfonic acid.

encodes the affective and sensory components of pancreatic nociception^[27]; and the PBN is another crucial relay center for general visceral inputs^[59]. Previous morphological evidence demonstrated that the NTS sends direct projections toward the PBN, PVT, and amygdala^[60], which may then recruit a broad cortical and subcortical pain network, as well as the descending modulatory system for the processing or modulation of visceral pain. These potential pathways merit further investigations for understanding the neurocircuitry mechanisms of visceral pain.

Enhanced excitatory synaptic transmission within NTS during painful CP

The NTS is a major integrative center receiving peripheral cardiovascular, respiratory, and gastrointestinal inputs^[61]. Glutamatergic viscerosensory afferents enter *via* the solitary tract and connect with second-order NTS neurons^[62,63]. Our previous study showed that the excitatory synaptic transmission within the NTS was enhanced in chronic cardiac pain. In the present study, we also recorded second-order caudal NTS neurons and observed that both the frequency and amplitude of the sEPSCs were significantly increased in CP rats, suggesting that both presynaptic and postsynaptic mechanisms may be involved.

VGluTs are selectively located in glutamatergic terminals, the content of which changes under pathological states^[18,64]. VGluT2-containing terminals are found throughout the NTS, with VGluT1-positive terminals are mainly distributed within the lateral NTS^[37]. For vagal afferent fibers, cardiac vagal fibers mainly express VGluT1 while other afferents predominantly express VGluT2 or both subtypes^[65]. Herein, our biochemical results showed that the content of VGluT2 (expressed in pancreatic afferents), instead of VGluT1 (irrelevant to pancreatic afferents), significantly increased in CP rats, which may support our functional evidence showing the increased frequency of sEPSCs within the NTS. One limitation here is that the increased expression of VGluT2 may not only reflect the sensitization of vagal primary afferents because VGluT2-containing terminals within the NTS also originate from upper brain structures^[18].

Among AMPAR subunits, GluR2-containing AMPARs are impermeable to Ca^{2+} ,

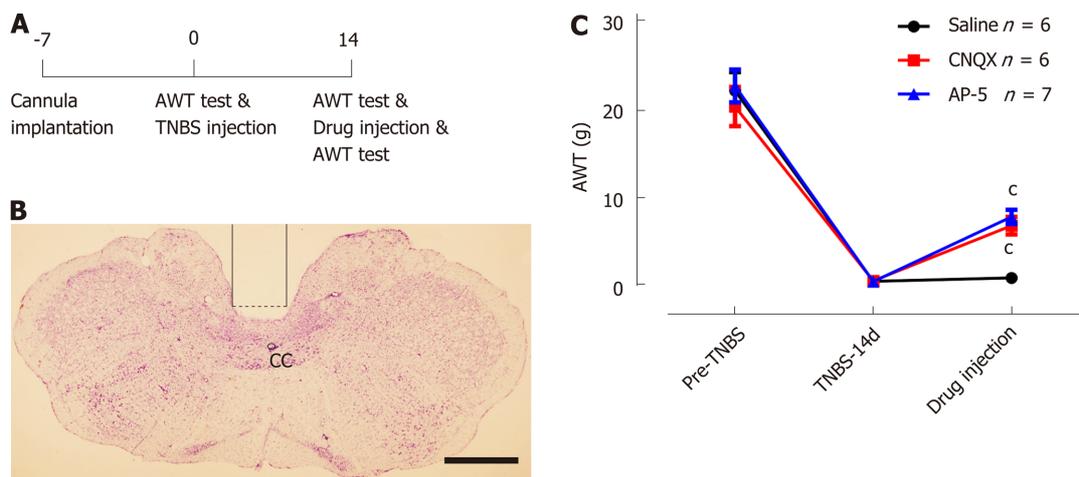


Figure 6 Administration of 6-cyano-7-nitroquinoxaline-2,3-dione and amino-5-phosphonopentaic acid into the caudal nucleus tractus solitarius alleviates chronic pancreatitis induced visceral hypersensitivity. A: Schematic diagram of behavioral test; B: A representative section showing the site of cannula implantation within the caudal nucleus tractus solitarius. Bar = 1 mm; C: Microinjection of 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and amino-5-phosphonopentaic acid (AP-5), instead of saline, increases the abdominal withdraw threshold in chronic pancreatitis rats on postoperative day 14. $n = 6$ rats in saline and CNQX-treated groups and 7 rats in AP5-treated group, one-way ANOVA, $^*P < 0.001$, CNQX or AP5 groups vs saline group. AWT: Abdominal withdraw threshold; CC: Central canal; CNQX: 6-cyano-7-nitroquinoxaline-2,3-dione; AP-5: Amino-5-phosphonopentaic acid; TNBS: Trinitrobenzene sulfonic acid.

while GluR2-lacking AMPARs possess high Ca^{2+} permeability^[66]. It is well-documented that both spinal and cortical GluR1 plays an important role in triggering synaptic plasticity induced by chronic pain^[67,68], which also holds true in the case of the NTS. In accordance with our study^[23], we also observed the upregulation and postsynaptic recruitment of NTS GluR1, which may contribute to the postsynaptic enhancement of the NTS during painful CP. In addition, emerging evidence has also indicated structural and functional changes of GluR1 within the NTS in chronic hypertension and hypoxia^[21,22]. This evidence implicates the role of NTS AMPA receptors in synaptic plasticity under pathological conditions.

The NMDAR is pivotal for triggering postsynaptic accumulation of GluR1 during the induction of long-term potentiation within the cerebral cortex. NR2B is a predominant subunit that determines the synaptic trafficking of the NMDAR and plays an important role in learning memory and chronic pain^[67,69]. Within the NTS, it is reported that NR2B maintains synaptic transmission during high frequency firing^[70]. Our data herein showed the upregulation and membrane recruitment of NR2B within the NTS in CP rats. Considering these findings, we speculated that excessive glutamate release from sensitized pancreatic afferents triggers over-activation of NR2B and subsequent membrane trafficking of Ca^{2+} -permeable GluR1; the excessive calcium influx then initiates subsequent intracellular signaling pathways to phosphorylate GluR1 and NR2B, which promotes their membrane trafficking and long-term potentiation of NTS excitatory transmission. Another limitation of our study is that we did not examine whether there are expression or post-modifical changes of other glutamate receptor subunits in painful CP. The detailed signaling mechanisms underlying the recruitment of NR2B and GluR1 also warrant further investigations.

NTS as a viable therapeutic target for painful CP

The role of the NTS in visceral pain has been demonstrated in several rodent models. For cardiac pain, both chemical lesion and pharmacological inhibition of NTS glutamate receptors ameliorated acute or chronic cardiac pain^[23,24], while microinjection of glutamate into the NTS facilitated acute inflammatory cardiac pain^[24]. Moreover, chemical NTS lesion also relieved peritonitis-related visceral pain^[71]. Our data herein showed both blocking excitatory synaptic transmission within the NTS *via* CNQX and AP-5 and chemogenetic inactivation of excitatory NTS neurons ameliorated visceral hypersensitivity in CP rats. However, chemogenetic activation of NTS excitatory neurons did not aggravate visceral hypersensitivity in CP rats. This may be because NTS excitatory neurons have reached their peak activity under the condition of CP; thus, further enhancing their activity fails to elicit more pain sensation. One limitation in our pain-evaluating method is that withdrawal responses to pressure on the abdominal wall are an indirect marker of visceral sensitization and do not necessarily implicate the pancreas itself^[72]. Thus, measuring

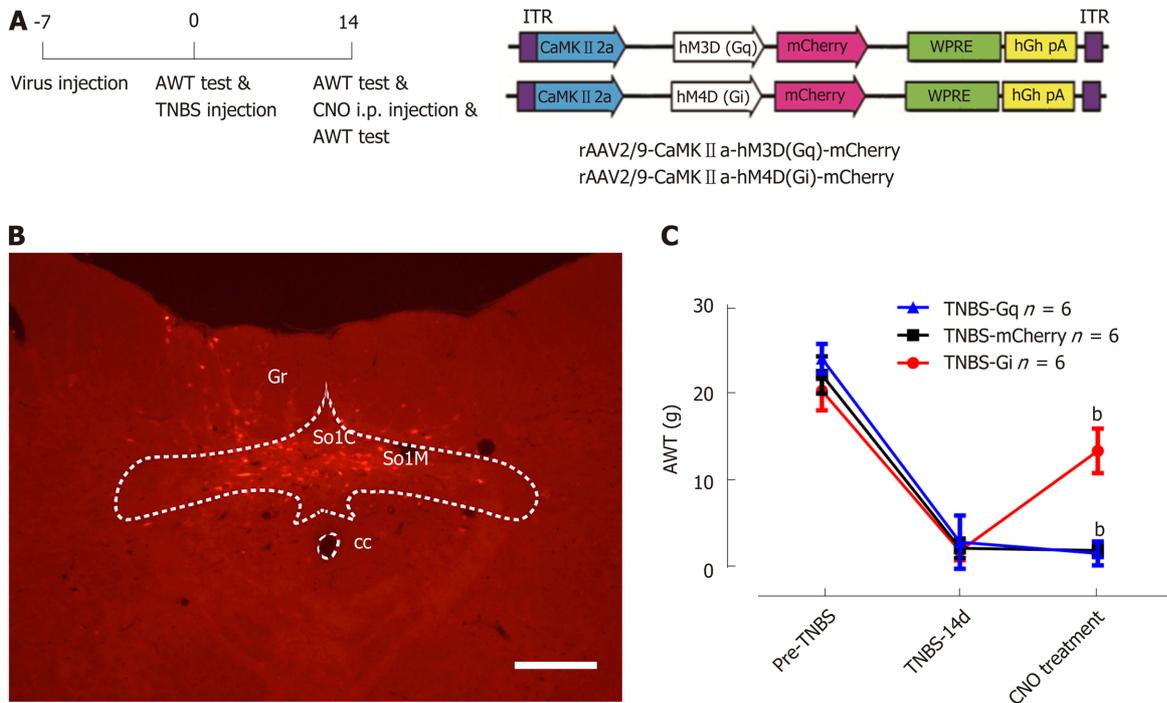


Figure 7 Inactivating excitatory neurons within the caudal nucleus tractus solitarius alleviates visceral hypersensitivity in rats with chronic pancreatitis. **A:** The schematic diagram of behavioral experiment (left) and rAAV2/9-CaMKIIa-hM4Di/hM3Dq-mCherry construct (right); **B:** A representative image showing that the expression of hm4Di-mCherry was confined within the commissure and medial nuclei of the caudal nucleus tractus solitarius (NTS). Bar = 0.4 mm. **C:** Inhibiting caudal NTS excitatory neurons *via* i.p. clozapine-N-oxide injection significantly increases the abdominal withdraw threshold (AWT) in chronic pancreatitis rats, while activating these neurons does not influence the AWT. *n* = 6 rats in mCherry, Gq, and Gi groups, one-way ANOVA. ^b*P* < 0.01. Gi vs mCherry. AWT: Abdominal withdraw threshold; CC: Central canal; CNO: Clozapine-N-oxide; So1M: Nucleus of the solitary tract, medial part; So1C: Nucleus of the solitary tract, commissural part; Gr: Gracile nucleus; TNBS: Trinitrobenzene sulfonic acid.

defensive behaviors induced by pancreas stimulation may be a better way to evaluate the pain related to pancreatic neuropathy.

Current clinical treatment of painful CP relies on combination analgesic therapy, such as opioids and gabapentin, which achieve desirable analgesic efficacy yet inevitably severe adverse effects, such as drug addiction, emotional disorder, and cognitive impairment^[73,74]. Therefore, there is an urgent need for the development of effective alternatives for painful CP. Our study supports the NTS as a major hub for processing pancreatic pain, which may pave the way for therapeutics aiming at modulating the activity of the NTS in the clinical remedy of refractory pancreatitis pain. To the best of our knowledge, acupuncture at orofacial acupoints has been applied clinically to treat visceral pain for a long time^[75], which is a result of acupuncture-induced inhibition of NTS hyperactivity^[76,77]. In addition, NTS activity can be therapeutically modulated electrically *via* noninvasive transcutaneous vagus nerve stimulation in the treatment of acute and chronic pain^[78]. We are optimistic that these neuromodulatory approaches targeting at the NTS will tremendously benefit patients suffering from painful CP.

In conclusion, we have provided morphological, biochemical, physiological, and behavioral evidence showing that enhanced excitatory synaptic transmission within the caudal NTS contributes to hyperalgesia during painful CP. Both presynaptic and postsynaptic mechanisms are involved in this process. The data provided herein deepen our understanding of central sensitization in pancreatic pain and supports the NTS, apart from the thoracic spinal cord, as another important central site for the processing of pancreatic pain, which may provide new clues for gastroenterologists and pain physicians in the treatment of CP.

ARTICLE HIGHLIGHTS

Research background

Central sensitization plays a pivotal role in the maintenance of chronic pain induced by chronic pancreatitis (CP). Previous studies concerning the central processing of painful CP usually center on the spinal dorsal horn. However, much less focus has been directed on the nucleus tractus solitarius (NTS), another primary central site for pancreatic afferents, during painful CP.

Research motivation

We aimed to investigate the plastic changes of caudal NTS as well as its role in the development of painful CP in rats.

Research methods

Chronic pancreatitis was established by intraductal injection of trinitrobenzene sulfonic acid (TNBS) in rats. Abdomen mechanical hypersensitivity was assessed by *von Frey* filament test. Then, immunohistochemical staining for Fos was performed to lay morphological evidence for NTS activation during painful CP, while patch-clamp recordings were performed to explore the changes of basic excitatory transmission of NTS in CP rats. Next, the expression of VGluT1, NMDAR subunit NR2B, and AMPAR subunit GluR1 was analyzed by immunoblottings. Membrane insertion of NR2B and GluR1 was evaluated by electron microscopy. Finally, AMPAR antagonist CNQX or NMDAR antagonist AP-5 was microinjected into the NTS to block the glutamatergic transmission on postoperative day (POD) 14 and then abdominal withdraw threshold (AWT) was tested. Chemogenetic method was also utilized to activate or inhibit the activity of excitatory NTS neurons on POD 14 and then AWT was tested.

Research results

TNBS treatment increased the number of Fos-expressing neurons within the caudal NTS. Both the frequency and amplitude of sEPSC of second-order neurons within the caudal NTS were substantially potentiated in CP rats. TNBS treatment upregulated the expression of VGluT2, and enhanced the phosphorylation and postsynaptic trafficking of NR2B and GluR1 within the caudal NTS. Blocking excitatory synaptic transmission increased the AWT of CP rats. Chemogenetic inhibition of the excitability of excitatory NTS neurons also significantly attenuated pancreatic hyperalgesia.

Research conclusions

These data suggest that both presynaptic and postsynaptic mechanisms contribute to the enhanced excitatory transmission within the caudal NTS, which mediates visceral hypersensitivity under the condition of painful CP.

Research perspectives

The results obtained deepen our understanding of central sensitization in pancreatic pain and support the caudal NTS, apart from the thoracic spinal cord, as another important central site for the processing of pancreatic pain, which may provide new clues for gastroenterologists and pain physicians in the treatment of CP.

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