Abstract: Peripheral nerve injury can induce neuroplastic changes in the central nervous system and result in neuropathic pain. This study investigated functional involvement in dorsal paratrigeminal nucleus (dPa5) and nucleus tractus solitarii (NTS) neurons projecting to the parabrachial nucleus (PBN) after trigeminal nerve injury. Anatomical quantification was performed based on phosphorylated extracellular signal-regulated kinase (pERK) expression underlying orofacial neuropathic pain associated with infraorbital nerve chronic constriction injury (ION-CCI) in rats. ION-CCI rats exhibited heat and mechanical hypersensitivity in the ipsilateral upper lip. After injection of retrograde tracer fluorogold (FG) into the contralateral PBN, ION-CCI rats received capsaicin or noxious mechanical stimulation to the upper lip. The total number of FG-labeled neurons in dPa5 and NTS did not change after ION-CCI, and pERK expression in dPa5 did not differ between sham and ION-CCI rats. In the NTS contralateral to ION-CCI, the number of pERK-immunoreactive neurons and percentage of pERK-immunoreactive FG-labeled PBN projection neurons were increased after capsaicin stimulation in ION-CCI rats. The present findings suggest that enhanced noxious inputs from the NTS to the PBN after trigeminal nerve injury modulates PBN neuron activity, which accompanies the affective components of orofacial neuropathic pain.

Keywords: nucleus tractus solitarii; dorsal paratrigeminal nucleus; parabrachial nucleus; neuropathic pain; projection neuron.

Introduction

Pain hypersensitivity caused by peripheral nerve injury can induce neuroplastic changes in the central nervous system (1). The primary structures that receive input from second-order neurons in the medullary dorsal horn are the contralateral ventral posteromedial thalamic nucleus (VPM) and bilateral (mainly ipsilateral) parabrachial nucleus (PBN) (2). The trigemino-thalamic pathway conveys orofacial noxious information to the cerebral cortex (3) mediating sensory-discriminative aspects of pain in the orofacial region (4). In contrast, the PBN sends projection axons to the amygdala, hypothalamus, periaqueductal gray (PAG), and ventrolateral medulla (5). The trigemino-parabrachial pathway mediates the emotional and/or autonomic responses accompanying orofacial pain (6). Axons from the dorsal paratrigeminal nucleus (dPa5) (7) and nucleus tractus solitarii (NTS)
(8) also terminate in the PBN. It has previously been reported that PBN projection neurons transmit nociceptive information, as do PBN projection neurons from the superficial trigeminal dorsal horn (9). The hypothesis of this study was that the affective aspects of orofacial pain would be enhanced after trigeminal nerve injury, partly because of neuroplastic changes in dPa5 and NTS-PBN pain pathways.

The dPa5, a constituent of the interstitial system of the spinal trigeminal tract (10), is a small, diffuse nucleus within the dorsal lateral medulla and is involved in the integration of somatosensory reflexes related to cardiovascular, respiratory, and pain mechanisms (7). The dPa5 also receives sensory inputs from the trigeminal nerve (7,11), which transmits cutaneous noxious inputs from the orofacial region (12). The dPa5 neurons send axons to the PBN and bilateral NTS, as well as to the contralateral VPM, dPa5, and lamina I of the trigeminal spinal subnucleus caudalis (Vc) (13). These findings suggest that the NTS, dPa5, and PBN neuronal circuit are involved in modulating orofacial nociceptive inputs.

The PBN has roles in pain processes (14), gustatory functions (15), and autonomic regulatory processes (16). The lateral parabrachial nucleus (LPBN) receives substantial noxious information and sends axons to the central amygdaloid nucleus (CeA) (17). These findings indicate that the LPBN-CeA pathway is involved in nociceptive transmission and regulation of pain-related affective components (18). Thus, the PBN is implicated in emotional and autonomic aspects of pain (19).

The NTS is involved in multiple functions, such as autonomic regulation, gustation, and visceral sensation (20,21). The caudal NTS is a site of visceral afferent integration that receives sensory afferents from trigeminal ganglion neurons (11). The superficial laminae of the Vc neurons also project to the NTS, which suggests that the NTS is involved in nociceptive transmission. Indeed, nociceptive stimulation to the orofacial region induced phosphorylation of extracellular signal-regulated kinase (ERK) in the dPa5 and NTS (9).

ERK is a member of the mitogen-activated protein kinase (MAPK) pathways and requires phosphorylation for activation. Under conditions of neuropathic pain, phosphorylated ERK (pERK) expression was increased in the Vc (22), indicating that pERK is a key signal transduction enzyme in chronic pain (23).

Thus, the purpose of this study was to evaluate if functional changes in dPa5 and NTS neurons projecting to the PBN underlie orofacial neuropathic pain in a model of infraorbital nerve chronic constriction injury (ION-CCI) in rats.

### Materials and Methods

#### Animals

The Animal Experimentation Committee of Nihon University approved the animal experimental protocols (AP16D044), which were performed according to the guidelines of the International Association for the Study of Pain (PHS Low 99-158, revised 2002). Adult male Sprague-Dawley rats (n = 21, Japan SLC, Hamamatsu, Japan) weighing 200 to 300 g were used. Data are presented from 16 rats in which fluorogold (FG) microinjections were confined to the targeted regions. Rats were housed with free access to food and water, and cages remained in a climate- and light-controlled environment (12 h:12 h light:dark cycle, lights on/off at 7:00/19:00, 23°C) for at least 5 days before experimentation. All efforts were made to minimize the number of animals used for experiments.

#### ION-CCI

Rats received anesthesia with intraperitoneal administration of 0.375 mg/kg medetomidine (ZENOAQ, Koriyama, Japan), 2.5 mg/kg butorphanol (Meiji Seika Pharma, Tokyo, Japan), and 2.0 mg/kg midazolam (Sandoz, Tokyo, Japan) mixed with saline solution. The left ION was exposed introrally (24), and a 5 to 6 mm incision was made on the buccal mucous membrane, from the incisive papilla toward the first molar, parallel with the dental arch, and 2 to 3 mm away from the palate. Two 4-0 chromic gut (lot JMZ582, Ethicon, New Brunswick, NJ, USA) ligatures, 1 mm apart, were loosely tied around the ION, and the wound was sutured. In sham rats, the ION was exposed by using the same procedure, without performing CCI.

#### Measurement of head-withdrawal threshold to heat and mechanical stimulation

The rats were trained for 10 min daily for 7 days to stay in a dim plastic pipe (diameter: 7.0 cm, length: 15.0 cm) while loosely restrained. A small trapezial hole (top width: 2.0 cm, bottom width: 1.0 cm, height: 3.5 cm) was cut in front of the box, to allow protrusion of the perioral region including the upper lips but not the orbital region (to avoid visual recognition of stimulation). After habituation, head-withdrawal reflex thresholds to mechanical and heat stimulation of the left upper lip ipsilateral to the operation site were measured before and on day 7 after the ION-CCI or sham operations. Rats were free to escape from the stimulation.

Heat stimulation was applied to the upper lip ipsilateral to ION-CCI or sham operations by using a contact thermal probe (3 × 3 mm², Intercross, Tokyo, Japan).
Stimulus velocity was automatically controlled from 35°C to threshold values (1°C/s). A cutoff of 50°C was established to prevent tissue damage. Mechanical stimuli were applied to the upper lip by using flat-tip forceps (2 × 2 mm², Panlab s.l., Barcelona, Spain). The stimulus velocity was manually controlled consecutively from 0 g to threshold values (cutoff: 55 g) at a speed of 10 g/s.

Mechanical and heat stimuli were applied three times at 5-min intervals, and the mean value of the head-withdrawal reflex thresholds was determined. The threshold values were measured in rats before ION-CCI or sham surgery “pre” and on day 7 after operations before FG injection. The experimenters were blinded to the behavioral experiments.

**FG injection into the right PBN**

On day 7 after ION-CCI or sham surgery, FG was injected into the right PBN of rats. The method used for FG injection has been described previously (9).

**Capsaicin injection or mechanical stimulation of the left upper lip**

Three days after FG injection, rats were anesthetized with pentobarbital sodium (80 mg/kg), and capsaicin was injected into the left upper lip, as described previously (9), in the sham-PBN and ION-CCI-PBN groups (n = 4 per group). In separate sham-PBN and ION-CCI-PBN groups (n = 4 per group), mechanical stimulation was applied to the left upper lip with a 100 g micro serrefine nontraumatic vascular clamp (1 × 6 mm², Fine Science Tools, North Vancouver, Canada) for 3 min (on: 20 s, off: 10 s × 6 cycles). Five min after capsaicin injection or the start of mechanical stimulation, the rats were perfused as described previously (9).

**FG injection site and brainstem immunohistochemistry**

The immunohistochemistry procedure has been described previously (9). The primary antibody was rabbit anti-phospho-p44/42 MAPK (ERK1/2) polyclonal antibody (1:500, #9101, Lot #26, Cell Signaling Technology, Danvers, MA, USA), and the secondary antibody was Alexa Fluor 568 anti-rabbit IgG (1:200, A11004, Invitrogen, Paisley, UK).

**Data analysis**

The immunohistochemical techniques have been described previously (9). Sections from three levels of the brainstem—0.5 mm rostral to the obex (+0.5 mm), obex level (0 mm), and 0.5 mm caudal to the obex (−0.5 mm)—were quantified. The average numbers of FG-labeled and/or pERK-immunoreactive (IR) neurons per section at each level were counted for the NTS (+0.5, 0, and −0.5 mm from the obex) and dPa5 (+0.5, 0, and −0.5 mm from the obex), comprising cells at the dorso-lateral edge of the main body of the trigeminal brainstem nuclear complex ipsilateral to ION-CCI or sham operations. The total numbers of positive cells were summed based on the average number of positive cells at the three brainstem levels.

**Statistical analysis**

Statistical analyses of head-withdrawal thresholds in the sham and ION-CCI groups were performed by one-way ANOVA followed by the Tukey test. Statistical analyses to compare the sham and ION-CCI groups were performed by using the Mann-Whitney U test. The data are expressed as mean ± standard error of the mean in the text and figures. The significance level was set at P < 0.05.

**Results**

**Heat hyperalgesia and mechanical allodynia in ION-CCI rats on day 7**

On day 7, head-withdrawal thresholds to heat stimulation to the left upper lip were significantly lower in ION-CCI rats as compared with those in sham rats (sham: pre = 45.17 ± 1.22°C, day 7 = 45.52 ± 0.49°C; ION-CCI: pre = 45.76 ± 0.66°C, day 7 = 39.47 ± 0.42°C; P = 0.0005; n = 4 per group; Fig. 1A) and with pre-measurement values
Head-withdrawal thresholds to mechanical stimulation were also significantly lower in ION-CCI rats on day 7 as compared with those in sham rats (sham: pre = 41.88 ± 2.63 g, day 7 = 41.24 ± 2.87 g; ION-CCI: pre = 43.39 ± 1.74 g, day 7 = 20.77 ± 2.22 g; *P* = 0.0002; *n* = 4 per group; Fig. 1B) and with pre-measurement values (*P* < 0.0001).

**FG injection site**

In most FG injection sites in the PBN, FG spread was observed in lateral and medial parabrachial nuclei (Fig. 2A-C; *n* = 4 per group).

**Distribution of pERK co-expressing FG-labeled neurons in the dPa5 and NTS**

FG-labeled pERK-expressing neurons could be identified by double immunofluorescence staining (Fig. 3A). In dPa5, FG-labeled PBN projection neurons were scattered bilaterally and were observed primarily on the FG injection side (contralateral to ION-CCI or stimulation). pERK-IR neurons were restricted to the side ipsilateral to ION-CCI and stimulation of the upper lip. pERK-IR neurons were frequently observed after capsaicin injection but rarely observed after noxious mechanical stimulation. Few FG-labeled pERK-expressing neurons...
were observed after capsaicin injection or noxious mechanical stimulation (Fig. 3B-D, Tables 1, 2).

In the NTS, FG-labeled PBN projection neurons were also scattered bilaterally. pERK-IR cells were observed bilaterally after capsaicin injection and noxious mechanical stimulation of the upper lip. Co-expression of pERK in FG-labeled PBN projection neurons was observed bilaterally (Fig. 3B, E-F, Tables 1, 2).

Quantification of FG-labeled PBN projection neurons, pERK-IR cells, and the percentage of pERK-IR PBN projection neurons in the dPa5

The total number of FG-labeled PBN projection neurons in dPa5 was not significantly different between sham and ION-CCI rats on the ipsilateral or contralateral side (capsaicin ipsilateral: sham = 31.83 ± 10.55, ION-CCI = 19.50 ± 2.64, \( P = 0.4857 \); capsaicin contralateral: sham = 131.50 ± 14.39, ION-CCI = 123.38 ± 8.89, \( P = 0.6857 \); Fig. 4A; mechanical ipsilateral: sham = 33.63 ± 5.56, ION-CCI = 31.46 ± 2.68, \( P = 0.9714 \); mechanical contralateral: sham = 116.63 ± 22.71, ION-CCI = 149.21 ± 10.09, \( P = 0.3429 \); Fig. 4B).

The phosphorylation of ERK was observed only in the ipsilateral dPa5 after capsaicin injection into the left upper lip; however, sham and ION-CCI rats did not significantly differ in the total number of pERK-IR cells (capsaicin ipsilateral: sham = 8.88 ± 3.13, ION-CCI = 15.50 ± 3.25, \( P = 0.2000 \); capsaicin contralateral: sham = 1.63 ± 0.88, ION-CCI = 6.25 ± 2.34, \( P = 0.4000 \); Fig. 4C; mechanical ipsilateral: sham = 0.63 ± 0.47, ION-CCI = 1.17 ± 0.39, \( P = 0.4000 \); mechanical contralateral: sham = 1.13 ± 0.24, ION-CCI = 0.13 ± 0.13, \( P = 0.0571 \); Fig. 4D).

No significant difference was observed in the percentage of pERK/FG-co-labeled neurons to PBN projection neurons between sham and ION-CCI rats (capsaicin ipsilateral: sham = 5.63 ± 3.32, ION-CCI = 14.3 ± 1.43, \( P = 0.3714 \); capsaicin contralateral: sham = 0.19 ± 0.19, ION-CCI = 0.09 ± 0.09, \( P > 0.9999 \); Fig. 4E; mechanical ipsilateral: sham = 0.00 ± 0.00, ION-CCI =

Table 1 Average cell counts at +0.5, 0, and −0.5 mm from the obex for FG-labeled, pERK-IR, and FG-pERK-IR neurons in rats receiving FG injection into the right-side PBN and capsaicin stimulation to the left-side upper lip. Upper panel: sham. Lower panel: ION-CCI.

<table>
<thead>
<tr>
<th>Region</th>
<th>FG-labeled</th>
<th>pERK-IR</th>
<th>FG-pERK-IR</th>
<th>FG-labeled</th>
<th>pERK-IR</th>
<th>FG-pERK-IR</th>
</tr>
</thead>
<tbody>
<tr>
<td>dPa5</td>
<td>10.61 ± 3.52</td>
<td>2.96 ± 1.04</td>
<td>0.50 ± 0.25</td>
<td>43.83 ± 4.80</td>
<td>0.54 ± 0.28</td>
<td>0.08 ± 0.08</td>
</tr>
<tr>
<td>NTS</td>
<td>27.79 ± 3.13</td>
<td>4.46 ± 1.60</td>
<td>0.63 ± 0.21</td>
<td>138.06 ± 23.33</td>
<td>2.42 ± 1.35</td>
<td>0.46 ± 0.27</td>
</tr>
</tbody>
</table>

Table 2 Average cell counts at +0.5, 0, and −0.5 mm from the obex for FG-labeled, pERK-IR, and FG-pERK-IR neurons in rats receiving FG injection into the right-side PBN and mechanical stimulation to the left-side upper lip. Upper panel: sham. Lower panel: ION-CCI.

<table>
<thead>
<tr>
<th>Region</th>
<th>FG-labeled</th>
<th>pERK-IR</th>
<th>FG-pERK-IR</th>
<th>FG-labeled</th>
<th>pERK-IR</th>
<th>FG-pERK-IR</th>
</tr>
</thead>
<tbody>
<tr>
<td>dPa5</td>
<td>6.50 ± 0.88</td>
<td>5.17 ± 1.08</td>
<td>0.08 ± 0.08</td>
<td>41.13 ± 2.96</td>
<td>0.13 ± 0.08</td>
<td>0.04 ± 0.04</td>
</tr>
<tr>
<td>NTS</td>
<td>28.75 ± 3.90</td>
<td>6.25 ± 2.34</td>
<td>0.42 ± 0.11</td>
<td>138.88 ± 9.29</td>
<td>6.38 ± 2.48</td>
<td>0.83 ± 0.47</td>
</tr>
</tbody>
</table>

Total cell counts per brain region, mean ± SEM. See the Methods for the number of sections/CNS region. Peripheral stimulation to the left upper lip contralateral to FG injection (right).
Ipsilateral Contralateral
NTS
in the NTS did not significantly differ between sham
The total number of FG-labeled PBN projection neurons
pERK-IR PBN projection neurons in the NTS
neurons, pERK-IR cells, and the percentage of
Quantification of FG-labeled PBN projection
4F).

\[ F = 0.4286; \text{Fig. 4G}; \text{mechanical ipsilateral: sham} = 129.29 \pm 15.73, \text{ION-CCI} = 113.75 \pm 1.83, P = 0.3429; \text{mechanical contralateral: sham} = 405.79 \pm 52.40, \text{ION-CCI} = 437.46 \pm 57.16, P = 0.6857; \text{Fig. 4H}).

The total number of pERK-IR cells in the NTS after capsaicin or noxious mechanical stimulation to the left upper lip was higher in ION-CCI than in sham rats bilaterally. Only the number of pERK-IR cells contralateral to capsaicin stimulation was significantly increased (capsaicin ipsilateral: sham = 13.38 \pm 4.81, ION-CCI = 31.38 \pm 3.27, P = 0.0571; capsaicin contralateral: sham = 7.25 \pm 4.04, ION-CCI = 36.38 \pm 2.66, P = 0.0286; Fig. 4I; mechanical ipsilateral: sham = 13.08 \pm 6.25, ION-CCI = 25.71 \pm 3.89, P = 0.2000; mechanical contralateral: sham = 14.50 \pm 5.08, ION-CCI = 24.96 \pm 4.83, P = 0.3429; Fig. 4J).

The percentage of pERK/FG-co-labeled PBN projection neurons after capsaicin injection was significantly higher in ION-CCI rats than in sham rats on the contralateral side (capsaicin ipsilateral: sham = 2.42 \pm 0.87, ION-CCI = 1.52 \pm 0.52, P = 0.4857; capsaicin contralateral: sham = 0.41 \pm 0.24, ION-CCI = 2.46 \pm 0.80, P = 0.0286; Fig. 4K; mechanical ipsilateral: sham = 0.74 \pm 0.33, ION-CCI = 1.93 \pm 0.68, P = 0.1143; mechanical contralateral: sham = 0.73 \pm 0.28, ION-CCI = 1.34 \pm 0.53, P = 0.4857; Fig. 4L).

Discussion
The present authors previously reported that many neurons in the dPa5 and NTS projecting to the PBN convey peripheral noxious information and that few were VPM projection neurons (9). However, how these projection neurons change functional properties after trigeminal nerve injury was not known. Therefore, this study evaluated functional changes in projection neurons from dPa5 and NTS to PBN underlying orofacial neuropathic pain associated with trigeminal nerve injury.

The present results comparing ION-CCI and sham indicate that heat hypersensitivity and mechanical allodynia occurred in the ipsilateral upper lip, that the number of FG-labeled projection neurons did not change in the dPa5 or NTS, and that there was no difference in pERK expression induced by capsaicin and noxious mechanical stimulation to the upper lip in dPa5. Conversely, the number of pERK-IR cells and percentage of pERK-IR FG-labeled PBN projection neurons in the NTS were higher after capsaicin injection to the upper lip. These

\[ 0.48 \pm 0.48, P > 0.9999; \text{mechanical contralateral: sham} = 0.29 \pm 0.18, \text{ION-CCI} = 0.00 \pm 0.00, P = 0.4286; \text{Fig. 4F}).

Quantification of FG-labeled PBN projection neurons, pERK-IR cells, and the percentage of pERK-IR PBN projection neurons in the NTS
The total number of FG-labeled PBN projection neurons in the NTS did not significantly differ between sham and ION-CCI rats on the ipsilateral or contralateral side (capsaicin ipsilateral: sham = 83.38 \pm 9.38, ION-CCI = 105.50 \pm 18.76, P = 0.6857; capsaicin contralateral: sham = 414.17 \pm 69.98, ION-CCI = 402.38 \pm 50.76, P = 0.8857; Fig. 4G; mechanical ipsilateral: sham = 129.29 \pm 15.73, ION-CCI = 113.75 \pm 1.83, P = 0.3429; mechanical contralateral: sham = 405.79 \pm 52.40, ION-CCI = 437.46 \pm 57.16, P = 0.6857; Fig. 4H).
results suggest that functional changes in PBN projection neurons in the NTS enhance noxious input and exacerbate affective aspects of persistent orofacial pain after trigeminal nerve injury.

**Function of PBN neurons**
The PBN is located in the dorsolateral pons and has essential functions in autonomic regulation (16), gustation (15), and pain (14), including autonomic responses accompanying orofacial pain (6). The medial PBN participates in taste functions, whereas the lateral PBN is implicated in nociceptive, cardiorespiratory, and immunological functions (17). Further, the LPBN sends axons to the CeA (17), and nerve injury-induced potentiation of postsynaptic currents is brought about in CeA neurons by electrical stimulation of the PBN, indicating that plastic changes occur in the CeA synaptic transmission under neuropathic pain conditions (25). The LPBN-CeA pathway is involved in nociceptive transmission and regulation of pain-related affective components (18). Thus, the PBN is strongly implicated in the emotional and autonomic aspects of pain (19). Spontaneous neuropathic pain and thermal hyperalgesia are regulated by TRPV1-positive neurons (26) activated by capsaicin, which are expressed mainly in small- or medium-diameter primary afferents (i.e., unmyelinated C- and myelinated Aδ-fibers) (27). The higher ratio of pERK-IR PBN projection neurons in the NTS after capsaicin stimulation may be attributable to the integration of noxious information in the NTS through C-fibers, which induce affective aspects of pain and autonomic regulation.

**Modulation of dPa5 and NTS-PBN neurons**
The dPa5 is involved in the integration of somatosensory reflexes related to cardiovascular, respiratory, and pain functions (7), and the NTS is also engaged in multiple functions, such as autonomic regulation, gustation, and visceral sensation (20,21). In the orofacial region, Vc, NTS, and Pa5 neurons are strongly activated by a variety of noxious stimuli of the orofacial region, and the whole body (28).

Noxious responses in dorsal horn neurons produced by peripheral nerve stimulation were significantly inhibited by glutamate microinjection or electrical stimulation in the NTS, suggesting that the NTS is involved in the control of nociceptive transmission (29). The trigeminal spinal nucleus and caudal NTS are heavily innervated by substance P (SP)-containing nerve terminals (30,31), which originate from small sensory ganglia cells, predominantly in unmyelinated primary C-fibers, and are involved in conveying nociceptive information from the periphery to the central nervous system (32). SP released from primary afferent terminals binds to neurokinin 1 (NK1) receptors in superficial spinal dorsal horn neurons (33). The NTS expresses a high density of NK1 receptors (34). NK1-immunoreactive neurons are also located in lamina I of the medullary dorsal horn (caudal trigeminal spinal nucleus) and project to the caudal NTS bilaterally (35). Collectively, NK1-expressing neurons in lamina I appear to receive somatic nociceptive information from SP-containing primary afferent neurons and convey noxious information to the NTS (35). A previous study of naïve rats revealed the existence of NK1-IR NTS neurons projecting to the PBN, but no pERK-IR neurons projecting to the PBN were observed after capsaicin stimulation of the upper lip (9). In the present study, pERK-IR NTS neurons projecting to the PBN were observed in ION-CCI rats after capsaicin stimulation but not after noxious mechanical stimulation, which suggests the possibility of enhanced C-fiber input to the NTS via pain-related receptors, such as NK1. In contrast, mechanical nociceptive input via Aβ-fibers may not be affected by trigeminal nerve injury.

A previous study reported that many pERK-IR cells express GABA in the NTS after capsaicin stimulation (28). Further, nociceptive afferents directly or indirectly activate NK1-IR GABAergic interneurons in the NTS, indicating that noxious inputs activate the NTS GABAergic intrinsic inhibitory system (36). Under neuropathic pain conditions, disinhibition-based central sensitization results from long-term depression of GABAergic interneurons due to TRPV1 activation in the spinal cord (37). Thus, past and present results imply that intrinsic GABAergic transmission, modulated by capsaicin-sensitive C-fibers in the NTS, is involved in orofacial neuropathic pain associated with trigeminal nerve injury.

**Bilateral modulation of NTS-PBN neurons**
Previous studies showed that dPa5 neurons project to the bilateral caudal NTS and contralateral lamina I of Vc (13). The dPa5-NTS pathway is known to be involved in the inhibition of pain caused by C- and Aδ-fiber stimulation (38). Further, the present group previously reported the presence of pERK-IR neurons in the superficial laminae of Vc in naïve and ION-CCI rats (9) and observed pERK-IR neurons in the NTS bilaterally and in the ipsilateral dPa5 after capsaicin and noxious mechanical stimulation to the upper lip. Taken together, past and present results suggest that the NTS receives noxious inputs through multiple pathways, encompassing primary afferents as well as inputs from dPa5 and Vc.
The PBN also projects to the midbrain PAG (5). Parabrachial targets converge, directly or indirectly, in the rostral ventral medulla (RVM) (39) and relay nociceptive information to the RVM (40). The functional connection from the LPBN to the PAG and RVM may allow the spino-parabrachial pathway to access descending control systems as part of a recurrent circuit (40). Further, ascending projections from the caudal NTS may also modulate this circuit.

In conclusion, the present findings indicate that functional changes in ascending pathways from the NTS to PBN and related multiple neuronal circuitries via the NTS-PBN pathways associated with trigeminal nerve injury are involved in orofacial neuropathic pain. Together with previous reports (17-19), the present results suggest that these functional changes in NTS-PBN pathways have a role in emotional and motivational aspects of persistent orofacial pain after trigeminal nerve injury.

Acknowledgments
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Conflict of interest
The authors declare no conflict of interest.

References