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Partial Infraorbital Nerve Ligation as a Model of Trigeminal Nerve Injury in the Mouse: Behavioral, Neural, and Glial Reactions

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Abstract: Trigeminal nerve damage often leads to chronic pain syndromes including trigeminal neuralgia, a severely debilitating chronic orofacial pain syndrome. Options for treatment of neuropathic pain are limited in effectiveness and new approaches based on a better understanding of the underlying pathologies are required. Partial ligation has been shown to effectively mimic many of the qualities of human neuropathic pain syndromes. We have devised a mouse model of trigeminal neuralgia using a partial infraorbital nerve ligation (pIONL) that induces persistent pain behaviors and morphological changes in the brainstem. We found that the pIONL effectively induced mechanical allodynia lasting for more than 3 weeks. Cell proliferation (bromodeoxyuridine), activation of astrocytes and microglia in the ipsilateral caudal medulla, and persistent satellite cell reaction in the ipsilateral ganglion were observed. Neurochemical markers calcitonin gene-related peptide, substance P were decreased in medullary dorsal horn ipsilateral to the injury side, whereas substance P receptor NK1 expression was increased after 8 days. Nerve injury marker ATF3 was markedly increased in ipsilateral trigeminal ganglion neurons at 8 days after pIONL. The data indicate that partial trigeminal injury in mice produces many persistent anatomical changes in neuropathic pain, as well as mechanical allodynia.

Perspective: This study describes the development of a new mouse model of trigeminal neuropathic pain. Our goal is to devise better treatments of trigeminal pain, and this will be facilitated by characterization of the underlying cellular and molecular neuropathological mechanisms in genetically designed mice.

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Key words: Infraorbital nerve, caudal medulla, allodynia, neuron, glia.

Orofacial pain disorders encompass a wide range of conditions including trigeminal neuralgia, temporomandibular joint disorders, periodontal pain, and atypical face pain. Great advances in the understanding of the mechanisms that underlie neuropathic pain states have come from the development of animal models such as the partial sciatic nerve ligation model of Seltzer's et al.⁴² For the trigeminal nerve there is the infraorbital chronic constriction injury (CCI) with loose ligatures in rat, developed by Vos et al^{52,53} and

adapted from the sciatic CCI model of Bennett and Xie.⁵ These models reproduce important aspects of trigeminal neuralgia, including signs of abnormal spontaneous pain-related behavior, mechanical allodynia^{51,52} and heat hyperalgesia.²⁴ There are also studies of complete transection or crush injury of infraorbital, or alveolar or lingual nerves to analyze deafferentation or regeneration mechanisms in rats.^{6,22,23,25,37,39,41,47} Abnormal spontaneous discharges from trigeminal cell bodies and upregulated glial fibrillary acidic protein (GFAP) expression in satellite cells have been detected in both mandibular and maxillary divisions in rats as long as 2 months after inferior alveolar (maxillary) nerve crush¹⁰ with some persistent behavioral consequences.¹²

The generation of a trigeminal pain model for studies using knock-out and transgenic mice would offer a promising approach to the identification of novel biochemical factors that contribute to persistent trigeminal pain conditions. Behavioral approaches to the study of

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nociceptive pain have been used in mouse,⁵⁰ but, to our knowledge, reliable and readily performed mouse models of neuropathic trigeminal pain have not been published. We think that this is an important goal because of some unique features of trigeminal nerve organization, the special tissues that it innervates, and special clinical problems of orofacial pain.⁴³

We have adapted the partial sciatic nerve ligation model of Seltzer et al⁴² to the infraorbital branch of the trigeminal nerve of mice. Partial sciatic nerve injury results in a decrease in substance P (sub P), calcitonin gene-related peptide (CGRP) and an increase in neurokinin-1 (NK-1/sub P) receptor immunoreactivity in the dorsal horn of rats^{17,19,27} and mice.³³ Glial cells in the central nervous system also respond to the peripheral insults, both microglia and astrocytes are activated after inferior alveolar nerve and mental nerve transaction in medullary dorsal horn in rats⁴⁰ and sciatic nerve ligation in mice.^{55,56} Here we investigate the behavioral and anatomical changes in wild-type C57B1/6 mice (Charles River Laboratories, Wilmington, MA) with partial infraorbital nerve ligation. This model provides an approach for future studies with mice in which genetic dissection of mechanisms for trigeminal neuropathic pain would be possible.

Materials and Methods

Animals

C57B1/6 male adult mice (Charles River Laboratories) weighing 22 to 32 g (12–16 weeks old) were used in these experiments. Mice were group-housed, in self-standing plastic cages (28 cm long × 16 cm wide × 13 cm high) within the animal core facility at the University of Washington, and maintained in a specific pathogen-free housing unit. Mice were transferred 1 week before behavior testing into a colony room adjacent to the testing room to acclimate to the testing environment. Housing rooms were illuminated on a 12-hour light-dark cycle with lights on at 7 AM. Food pellets and water were available ad libitum. Procedures with mice were approved by the institutional animal care and use committee, in accordance with the 1996 NIH Guide for the Care and Use of Laboratory Animals.

Surgical Preparation: Partial Ligation of the Infraorbital Nerve

The unilateral partial infraorbital nerve ligation (pIONL) was performed under direct visual control using a Zeiss surgical microscope ($\times 10$ –25; Carl Zeiss, Inc., Jena, Germany) (Fig 1A). The animals were anesthetized with sodium pentobarbital (nembutal, 80 mg/kg ip). They were kept warm with a heat lamp and foil blanket, their eyes were treated with lubricating ophthalmic ointment (Akorn, Buffalo Grove, IL). The mouse was taped to a sterilized cork board, the skin along the top of the snout was shaved, iodine treated, and a mid-line incision was made to expose nasal and maxillary bone. All tools were gas sterilized before surgery and then washed and

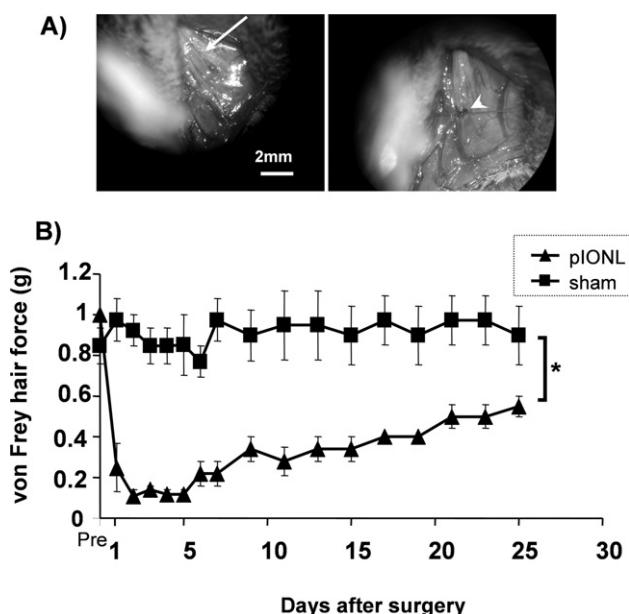


Figure 1. Images showing the surgical approach used during partial infraorbital nerve ligation (pIONL) and mechanical allodynia produced after pIONL. **A**, Exposure of right ION (arrow) and tight ligature with 7-0 thread (arrowhead) at the lateral half of nerve. Scale bar: 2 mm. **B**, Response thresholds to usually innocuous tactile (von Frey hair) stimuli in sham and pIONL mice during the 25 days after surgery. Mice with pIONL developed significant allodynia as evident from the decreased thresholds to tactile stimulation compared with mice receiving sham surgeries. The mechanical allodynia for ligated mice lasted for >3 weeks. Asterisks indicate significant decreases in response thresholds in pIONL-operated mice compared with the sham-operated mice on the days noted (* $P < .001$, ANOVA, nonparametric analysis). Data are presented as mean \pm SEM von Frey hair threshold in grams; $n = 6$ to 8 per time point.

heat-treated (glass beads at 250°C) for each animal. The infraorbital part of the right ION was initially exposed 1 to 2 mm rostral to infraorbital fissure on the maxillary bone using blunt dissection with small scissors. The ION was gently isolated using fine forceps without damaging nearby facial nerve branches. Approximately one-third to one-half the diameter of the nerve was tightly ligated with 7-0 silk suture (Surgical Specialties Corporation, Reading, PA) by passing the suture needle completely under the lateral aspect of the nerve and then up through the middle. The incision was closed using silk sutures (5-0) after confirming hemostasis. For the sham-operated mice, the ION was exposed on the right side using the same procedure, but the ION was not touched or ligated. The mice received one analgesic (buprenorphine, 0.05 mg/kg) treatment at the end of the surgery. The operated mice were able to eat and drink unaided soon after waking up, the body weight returned back to or exceeded preoperative weights after the first week.

Behavioral Testing

Stimulus-Evoked Responses: Mechanical Allodynia

The mice were tested 1 day before surgery, daily during the first postoperative week, and on alternate days

after that for a period of 25 days. All experiments were carried out in a quiet room between 8 AM and noon to avoid diurnal variations. Body weight was measured every time before testing. On the day of testing, mice were habituated to handling and testing equipment 20 to 30 minutes before experiments. A graded series of von Frey filaments (Semmes-Weinstein monofilaments; Stoelting, Wood Dale, IL) was used for mechanical stimulation of ipsilateral ION territory. The filaments produced a bending force of 0.02, 0.04, 0.07, 0.16, 0.4, 0.6, 1.0, and 1.4 g. The mice stood on a metal mesh with a porous plastic cup (diameter, 8 cm) covering them. Von Frey hairs were then inserted from below through the mesh when the animal's head was steady resting or alert status.⁸ The stimuli were applied within the ION territory, near the center of the vibrissa pad, on the hairy skin of the right (ipsilateral) side and involved brief bending of the filament. The threshold was taken with a response of a brisk withdrawal of the head followed by an uninterrupted series of at least 3 face-grooming strokes directed to the stimulated facial area. The time period between each filament stimulus was about 2 to 3 minutes. The stimulation always began with the filament producing the lowest force and stopped when threshold was found. The threshold response had to occur within 2 seconds to be considered a head withdrawal response. Unresponsive mice received a maximal score of 1.4 g.

Nonevoked Behaviors: Face-Grooming Behavior

Mice were placed individually in small transparent plastic cages (14 cm × 16 cm × 13 cm) without bedding. A video camera was placed 0.8 m at the side of the cage and positioned so that the image of the mouse head was observed. Mice were habituated in the cage for 15 minutes and then recorded 15 minutes per day for 7 days. Duration of face grooming actions was recorded when the forelimbs contact facial region and ear grasps.

Immunohistochemistry in Brainstem and Trigeminal Ganglion Sections

Mice were anesthetized with sodium pentobarbital (nembutal, 100 mg/kg ip) and intracardially perfused with 4% para-formaldehyde in phosphate buffer (PB, 0.1 M sodium phosphate; pH 7.4). The brainstem and trigeminal ganglia (TG) were dissected, postfixed 2 hours, cryoprotected with solution of 30% (wt/vol) sucrose in PB at 4°C overnight and cut into series of 40-μm sections (brainstem) or 20-μm sections (TG) with microtome. Briefly, sections were washed 3 times in phosphate-buffered saline (PBS), blocked in PBS containing 0.1% Triton X-100 and 4% normal goat serum for 1 hour, and incubated overnight with primary antibodies. Primary antibody concentrations were as follows: Rabbit anti-CGRP (1:5000; Chemicon, Temecula, CA), rabbit anti-NK1 receptor (1:1000; Chemicon), rabbit anti-Substance P (1:5000; Chemicon), rabbit anti-ATF3 (1:50; Santa Cruz Biotechnology, Santa Cruz, CA), rat anti-CD11b (1:200; Serotac, Oxford, UK), rabbit anti-GFAP (1:3000; Dakopatts, Glostrup, Denmark), and mouse anti-bromodeoxyuri-

dine (BrdU) (6 μg/mL; Chemicon, Temecula, CA) or rat anti-BrdU (1: 50; Abcam, Cambridge, MA). For BrdU staining, the animals were treated with intraperitoneal injection of BrdU 100 mg/kg once per day for 7 days before perfusion. The brainstem sections were treated with 2N HCl for 60 minutes at 37°C, followed by 2 10-minute washes in 0.1 M borate buffer before incubation overnight with primary antibody. Sections were then washed with PBS, and detection was carried out using the rhodamine or fluorescein conjugated fluorescent secondary antibodies (1:250; Jackson ImmunoResearch, West Grove, PA). Antibodies were diluted in a solution containing 0.1% Triton X-100 and 4% normal goat serum in PBS. The sections were rinsed in PBS for 30 minutes and then mounted on gelatin-coated slides with Vectashield mounting medium (Vector Laboratories, Burlingame, CA) and sealed with nail polish for microscopy. The sections were viewed with a Nikon Eclipse E600 fluorescence microscope (Tokyo, Japan) or a Leica SL confocal microscope (Leica Microsystems, Inc., Bannockburn, IL) located in the W.M. Keck Imaging Facility at the University of Washington. Relative staining intensities were analyzed using NIH Image J version 1.62 software (National Institutes of Health, Bethesda, MD). Values are expressed as ratios of labeling intensity over background.

Statistical Analysis

We have used a total of 72 male mice in the study. Sample size was 4 to 6 brainstem sections from each of 4 mice to assess the anatomical changes, and 4 to 8 mice per group were used to assess behavioral changes. Mechanical allodynia data were analyzed by ANOVA (non-parametric). For all groups, preoperative and postoperative behaviors for intra-animal comparisons as well as group comparisons were made. Statistical significance determined by ANOVA was then further analyzed with Student-Newman-Keuls test or Student *t* test for significant pairwise comparisons. Response data are presented as mean ± SEM of the animal treatment group, with significance set at *P* < .05.

Results

Behavioral Responses

Stimulus-Evoked Mechanical Allodynia After pIONL

We applied von Frey fibers to the anterior right snout to test for mechanical allodynia in the operated mice. Ligated and sham-operated mice showed similar responses to the von Frey filament that was applied with a stimulus of 1.4 g before pIONL surgery. Mice showed significant allodynia from day 1 that lasted for more than 3 weeks after the pIONL surgery. After day 9, ligated mice began recovery toward baseline but still showed significant allodynia compared with sham mice until day 23 (Fig 1B) (*P* < .05). These results support the hypothesis that pIONL induces persistent trigeminal neuropathy.

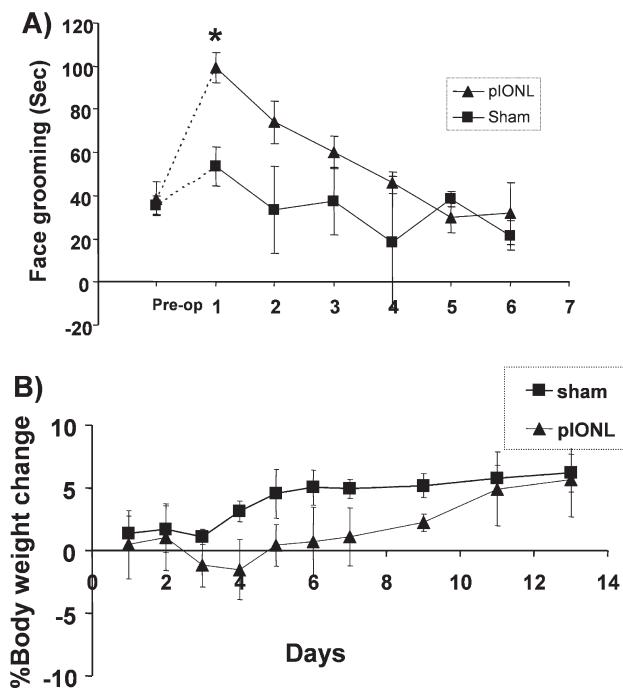


Figure 2. Change in the duration of face-grooming behaviors and body weight after partial infraorbital nerve ligation (pIONL). **A,** Total time for isolated face-grooming episodes during a 15-minute observation period was elevated for the pIONL mice only during the first day after surgery. (* $P < .05$, ANOVA followed by Student-Newman-Keuls test, $n = 4$ per group). **B,** Sham-operated mice were gaining weight by 3 days after surgery, whereas pIONL mice lost less than 5% during the first week and then started to gain weight during the second week ($n = 6$ –8 per group).

Nonevoked Behavior (Isolated Face Grooming) and Body Weight Changes After pIONL

We video recorded mouse spontaneous activity for 15 minutes each day, including once before the surgery (prestudy baseline). The face grooming duration was analyzed from the videos by an independent observer, blinded to treatment. There was a significant increase in face grooming in mice after pIONL compared with sham-operated mice 1 day after surgery (Fig 2A) ($P < .05$), but face grooming returned to normal by 3 days after surgery. We also found that during the first week after pIONL, the body weights of injured mice were slightly below pre-injury baseline (Fig 2B), but the difference did not reach statistical significance ($P > .05$) between pIONL and sham-operated mice. The body weight of operated mice increased above the pre-injury baseline during the second week after pIONL.

Neuronal Responses After pIONL Injury in Brainstem

In our study, we found that pIONL produced a marked decrease in CGRP-IR and sub P-IR and an increase in NK-1 receptor expression in the ipsilateral side of caudal medullary dorsal horn 8 days after pIONL (Fig 3A–G). NK-1 receptors localized densely in superficial lamina I–II and in deeper area lamina III–V (Fig 3C and D). Sham-oper-

ated mice showed no change in CGRP, NK-1 receptor or sub P-IR (Fig 3H–J). The decrease in CGRP-IR extended into the cervical spinal cord (C1) (Fig 3A), as expected.^{34,35}

Glial Reactions to pIONL in Brainstem

We evaluated alterations in microglia (CD11b) and astrocytes (GFAP) in caudal medulla. In the contralateral side of pIONL mice and the sham-operated mice, CD11b-IR was uniformly distributed and had modest intensity throughout caudal medulla (Fig 4A). The stained resident microglia had long, finely branched processes that extended in all directions from the cell soma (Fig 4B). In the ipsilateral side of pIONL mice, microglia had profound CD11b-IR at 1 day after pIONL and appeared to be in an activated state with enlarged cell bodies and thicker processes than on the contralateral side (Fig 4C and D). After 8 days after pIONL injury, ipsilateral CD11b-IR was reduced and was no longer different from that in the contralateral side (Fig 4E–G). The most extensive expression of activated microglia after pIONL was observed in the medial portion of superficial lamina, and microglial activation was not found in normal or sham-operated mice.

Activated astrocytes were visualized with anti-GFAP immunostaining to assess the effects of pIONL. GFAP-IR in caudal medulla was homogeneous and modest throughout the superficial lamina on the contralateral side of pIONL mice and on both sides in sham-operated mice (Fig 5A–D). However, the ipsilateral side had strong GFAP-IR 8 days after pIONL, and these cells had hypertrophic cell bodies and long processes (Fig 5E). The greatest astrocitic activation was observed in the medial portion of the superficial lamina of the ipsilateral side (Fig 5A and E). GFAP-IR was significantly increased in the ipsilateral caudal medulla compared with contralateral side and sham-operated mice 8 days after pIONL (Fig 5F) ($P < .05$).

Cellular Proliferation After pIONL (BrdU Staining) in Brainstem

We found that at 8 days after pIONL, the number of BrdU-positive cells was increased 5.6-fold in the ipsilateral side of the caudal medulla compared with contralateral side (Fig 6A and B; $P < .05$). The BrdU immunoreactive cells were preferentially located in the dorsal lamina I–III, where microglia and astrocytes were activated by pIONL. To characterize the cell types that were BrdU-positive after pIONL, we performed dual-labeling of BrdU and GFAP, BrdU and CD11b, BrdU and NeuN (neuronal marker), or BrdU and NG2 (oligodendrocytes precursor marker). A portion of BrdU-positive cells were double labeled with the microglial marker CD11b-IR (Fig 6C–E) or astrocytic marker GFAP-IR (Fig 6F–H). The majority (~70%) of the BrdU-positive cells were nestin-positive stem cells (Fig 6I–K). We did not detect BrdU-positive cells that double labeled with NG2 (Fig 6L–N) or NeuN (data not shown), suggesting that proliferation of oligodendrocytes or neurons were not evident 8 days after pIONL.

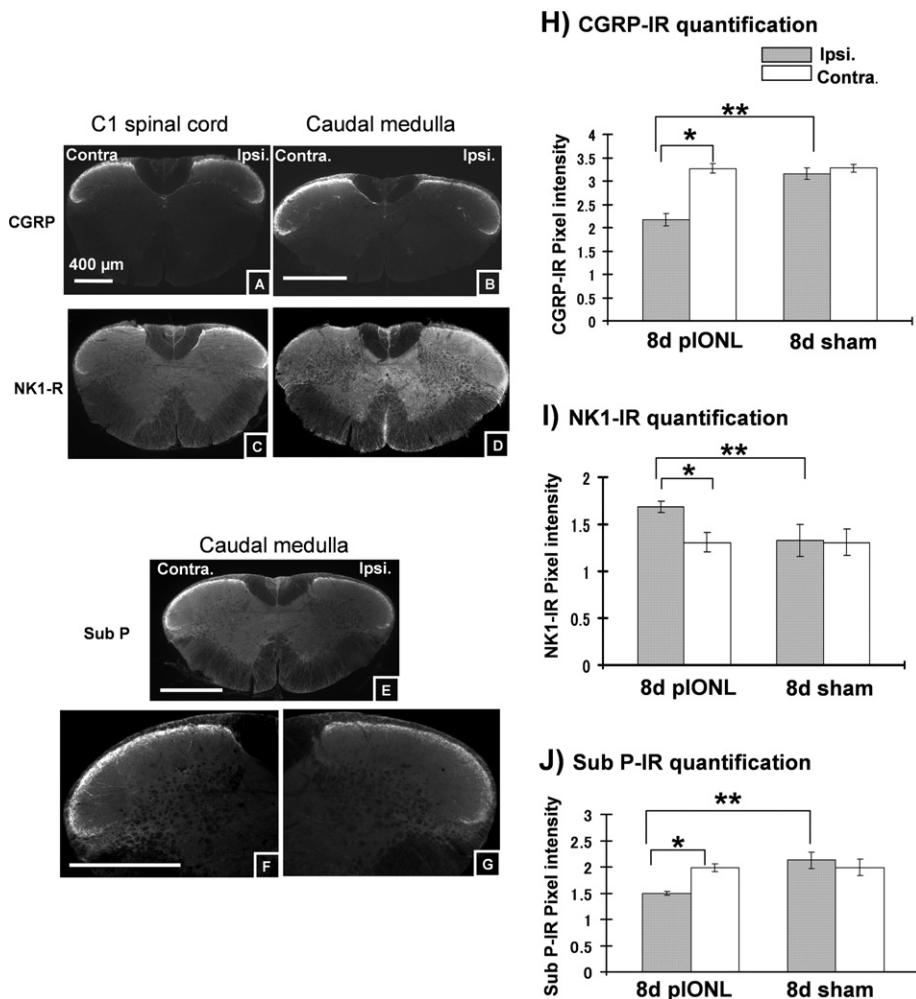


Figure 3. Neuronal expression of calcitonin gene-related peptide (CGRP)-IR, substance P (sub P)-IR, and NK1R-IR in the cervical C1 spinal cord and caudal medulla at 8 days after partial infraorbital nerve ligation (pIONL). There was a decrease of CGRP-IR (A and B) and sub P-IR (E, F, and G) and increase of NK1 receptor-IR (C and D) in the superficial dorsal horn of C1 spinal cord and caudal medulla ipsilateral to the nerve injury. A and C are cervical C1 spinal cord sections; B, D, and E are caudal medulla sections. F and G are the high magnifications of the dorsal horn in E. H, I, and J graphs showing mean \pm SEM pixel intensity of CGRP, NK1-R and sub P staining in caudal medulla lamina brainstem sections. H, image analysis of CGRP expression in ipsilateral caudal medulla after pIONL demonstrated approximately 1.5-fold lower signal intensity [2.17 ± 0.13 arbitrary units (AU); n = 20 sections from 4 animals] than that of contralateral caudal medulla (3.27 ± 0.11 AU; n = 20 sections from 4 animals, *P < .001) or sham-ligated mice (3.16 ± 0.12 AU; n = 20 sections from 4 animals, **P < .001). I, image analysis of NK1-receptor staining in ipsilateral caudal medulla after pIONL demonstrated approximately 1.26-fold higher signal intensity (1.662 ± 0.06 AU; n = 20 sections from 4 animals) than that of contralateral caudal medulla (1.309 ± 0.1 AU; n = 20 sections from 4 animals, *P < .001) or sham-ligated mice (1.33 ± 0.17 AU; n = 20 sections from 4 animals, **P < .001). J, image analysis of sub P expression in ipsilateral caudal medulla after pIONL demonstrated approximately 1.3-fold lower signal intensity (1.50 ± 0.04 AU; n = 20 sections from 4 animals) than that of contralateral caudal medulla (1.98 ± 0.07 AU; n = 20 sections from 4 animals, *P < .001) or sham-ligated mice (1.99 ± 0.16 AU; n = 20 sections from 4 animals, **P < .001). Scale bars: 400 μ m.

Anatomical Changes in Trigeminal Ganglia

We counted the number of neurons with reactive satellite cells in the TG, as shown by GFAP-IR (Fig 7). Neuronal cell body distribution in trigeminal ganglion is shown in Fig 7A: (I): Maxillary infraorbital neurons. (II): Maxillary dental neurons. (III): Mandibular neurons. We found that the number of GFAP-IR labeled satellite cells was significantly increased in the ipsilateral TG compared with contralateral side and sham-operated mice 8 day after pIONL (Fig 7B-E) ($P < .05$). The increased GFAP-IR was returning to normal by the second week (Fig 7B and F-H). Neuronal expression of ATF3, which is normally minimal,

is upregulated after peripheral nerve injury and acts as a marker of nerve injury.⁴⁸ We found that ATF3 staining was significantly increased in the ipsilateral trigeminal ganglia 8 days after pIONL ($P < .05$) (Fig 8A-E). ATF3 expression occurred within neurons in the infraorbital region of TG (Fig 8D).

Discussion

Trigeminal neuralgia is a form of neuropathic pain characterized by severe lancinating pain in orofacial regions innervated by the trigeminal nerve. Most cases of

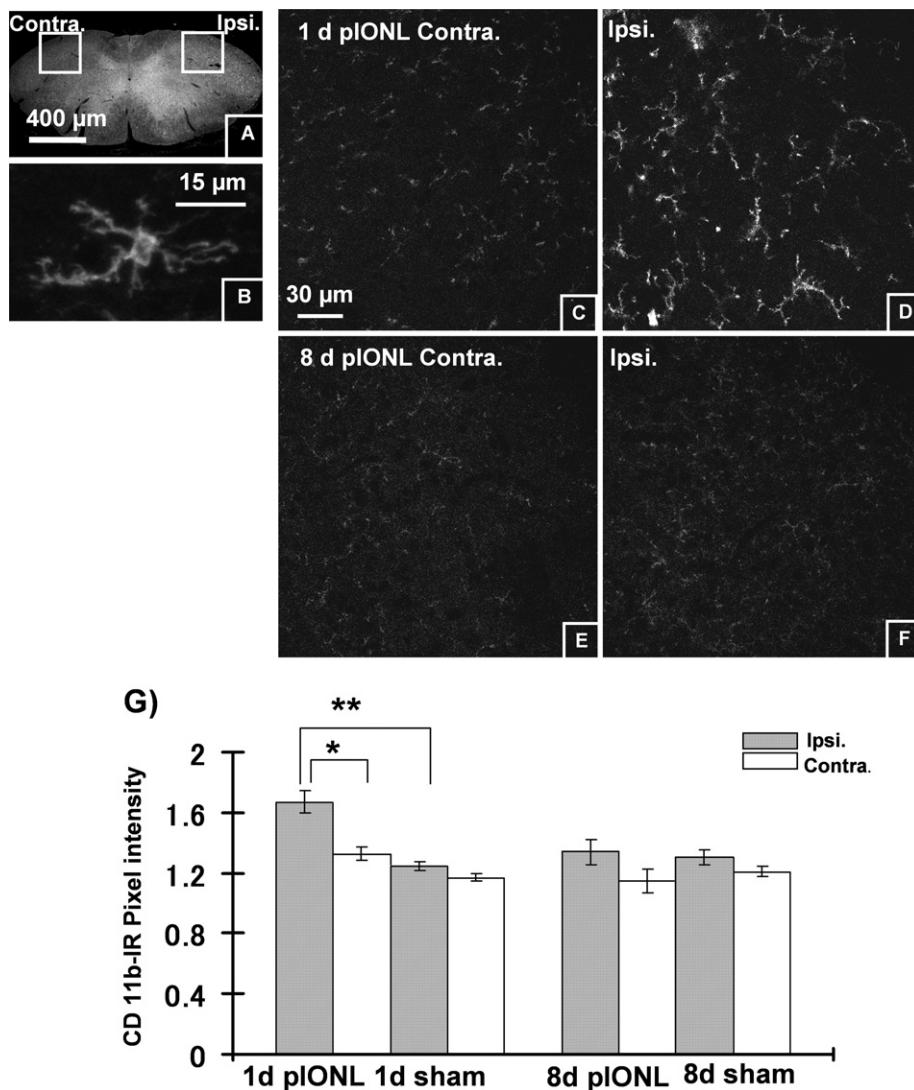


Figure 4. Microglial responses after partial infraorbital nerve ligation (pIONL). **A**, pIONL induced microglial activation (CD11b staining) in the ipsilateral side of the caudal medulla. A representative, higher-power image of a microglial cell is shown in **B**. Higher magnification images (taken from the boxes outlined in **A**) show differences in contralateral (**C**) and ipsilateral (**D**) CD11b-IR 1 day after pIONL. In contrast, CD11b-IR in the caudal medulla was not different in the contralateral (**E**) or ipsilateral (**F**) 8 days after pIONL. The microglial activation had decreased by 8 days after surgery (**E** and **F**). Quantification showing mean \pm SEM pixel intensity of CD11b staining in caudal medulla of brainstem sections (**G**). Image analysis of CD11b expression in ipsilateral caudal medulla 1 day after pIONL demonstrated approximately 1.25-fold higher signal intensity (1.67 ± 0.07 AU; $n = 16$ sections from 4 animals) than that of contralateral caudal medulla (1.32 ± 0.04 AU; $n = 16$ sections from 4 animals, $**P < .001$). In contrast, image analysis of CD 11b staining in caudal medulla 8 days after pIONL did not show a significant increase over sham mice ($P > .05$). Scale bars: **A**, 400 μm; **B**, 15 μm; **C-F**, 30 μm.

trigeminal neuralgia are caused by sensory nerve root compression.²⁶ Vos et al⁵² developed a rat model of trigeminal neuropathic pain produced by chronic constriction of the infraorbital nerve. These orofacial CCI-induced sensory changes resemble those seen in neuropathic pain of the hind limb after injury to sciatic or L5/L6 spinal nerves in rats, but the CCI surgery is prohibitively difficult in mice. In the present study, we developed and characterized a feasible mouse model of pIONL that is a combination of the partial nerve ligation (dental maxillary nerve fibers and medial ION fibers are not injured) and spared nerve injury (trigeminal mandibular and ophthalmic branches are intact) models of chronic

pain. We demonstrated that pIONL in mice produced prolonged mechanical allodynia and a transient change of grooming time. pIONL in the mouse produced persistent changes in peptide neurotransmitter and receptor expression in the caudal medulla region of the brainstem. Microglia and astrocytes were also activated in this region after pIONL injury. ATF3 staining showed that specific neurons in TG were also injured by pIONL. These results established this model as a reliable and objective model of trigeminal neuropathic pain in the mouse.

The trigeminal system shares many neuropathic features with spinal nerves but also differs in important ways.⁴³ The special features make it likely that persistent

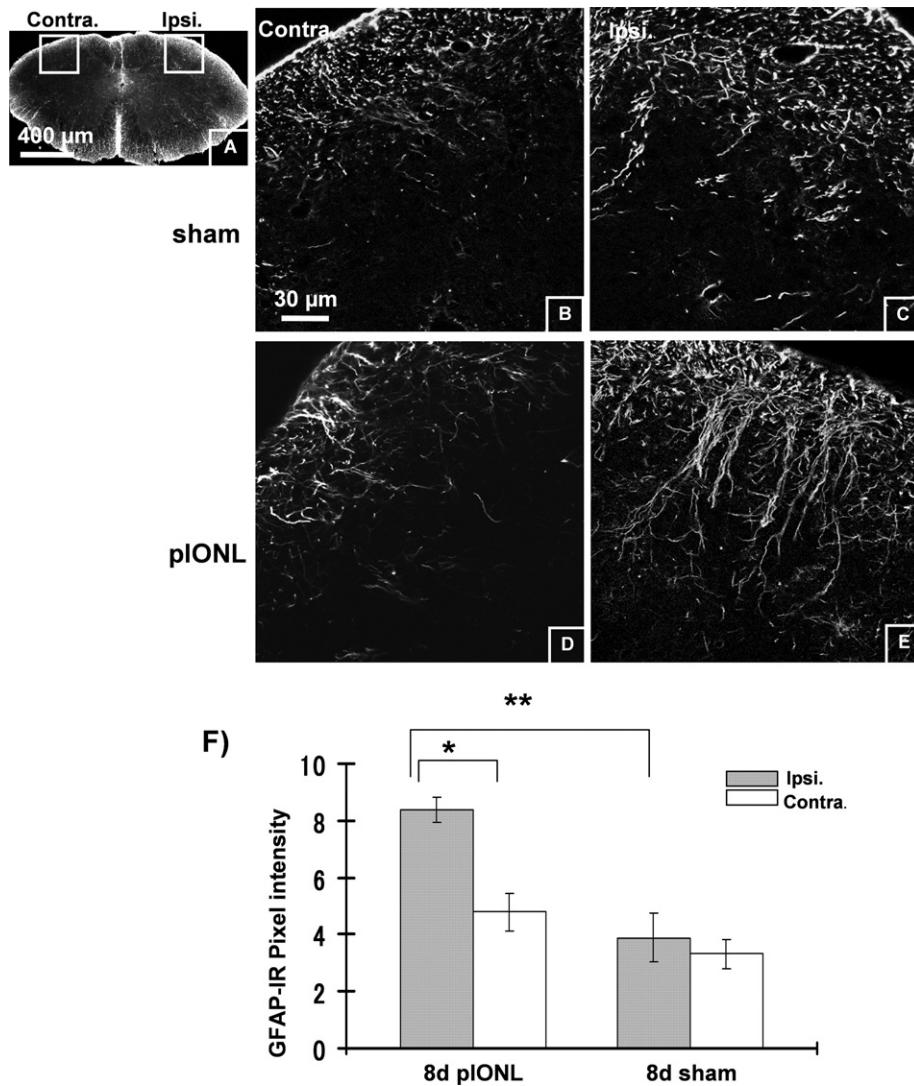


Figure 5. Astrocytic responses in caudal medulla 8 days after partial infraorbital nerve ligation (pIONL) (A). Glial fibrillary acidic protein (GFAP) staining was compared in ipsilateral and contralateral regions of the caudal medulla of sham-operated mice (B and C) and after pIONL (D and E). There was a striking increase of GFAP-IR on the ipsilateral side of caudal medulla (E) compared with contralateral side (D). The response involved hypertrophy of the ipsilateral astrocytes (E). Image analysis of GFAP expression in ipsilateral caudal medulla 8 days after pIONL (F) demonstrated approximately 2-fold higher signal intensity (8.37 ± 0.43 AU; n = 20 sections from 4 animals) than that of contralateral caudal medulla (4.78 ± 0.66 AU; n = 20 sections from 4 animals, *P < .001) or sham-ligated mice (3.88 ± 0.86 AU; n = 20 sections from 4 animals, **P < .001). Scale bars: A, 400 μm; B-E, 30 μm.

pain mechanisms in the trigeminal system may have unique features, and better animal models are needed to study the effects of trigeminal nerve injury. So far, partial trigeminal nerve injury studies have mainly used the infraorbital chronic constriction injury with loose ligatures in rats.^{52,53} Some other animal models for persistent trigeminal pain and plasticity involve peripheral inflammation,^{13,14,45,50} nerve transection,^{22,23,25,37,39,40} or chronic tooth infection.⁷ There are no reports of partial ligation of trigeminal nerves using mice. One study used complete transection of the ION at the foramen that caused large changes in neuronal and glial properties in the ganglion at 1 week later; however, behavioral data were not reported.⁹ Our present study is adapted from these preceding studies in rats and mice to give a new, reliable and easily applied partial nerve ligation of the

mouse trigeminal nerve. We found that pIONL induced persistent anatomical and behavioral changes including mechanical allodynia lasting for over 3 weeks.

The recurrent episodes of asymmetric face grooming directed to the territory of the injured nerve after chronic constriction of the infraorbital nerve have been interpreted as behavioral signs of "spontaneous" neuropathic facial pain.⁵² The results of the present study showed that mice with pIONL displayed significantly more face grooming time compared with sham-operated mice during the first day after nerve injury. These results suggest that spontaneous facial pain was only increased during the acute phase of pIONL. This is different from previous rat studies showing that CCI caused an increased face grooming lasting as long as 130 days.⁵² Therefore, when using grooming activity as a criterion

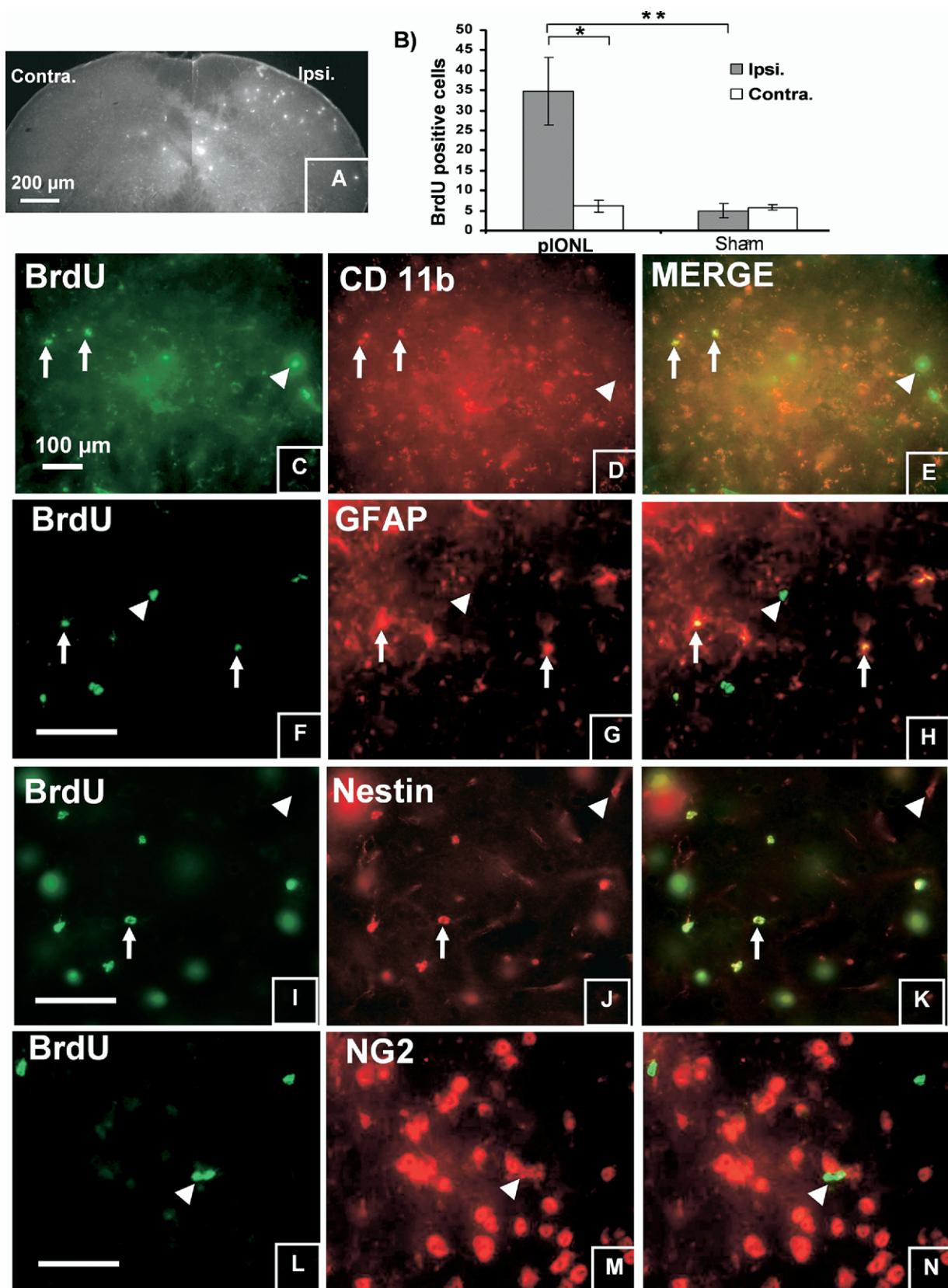


Figure 6. Cellular proliferation in caudal medulla 8 days after partial infraorbital nerve ligation (pIONL) was detected after daily bromodeoxyuridine (BrdU) administration (100 mg/kg intraperitoneally, once daily for 8 days). **A**, BrdU-positive cells were markedly increased on the ipsilateral side of the caudal medulla compared with the contralateral side. **B**, Summary graph showing mean \pm SEM BrdU-positive cells in caudal medulla of brainstem sections. Image analysis of BrdU-positive cells in ipsilateral caudal medulla 8 days after pIONL demonstrated approximately 7-fold higher BrdU-IR (34.75 ± 8.4 ; n = 8 sections from 4 animals) than that of the contralateral caudal medulla (6.12 ± 1.4 ; n = 8 sections from 4 animals, $*P < .001$) or sham-ligated mice (5 ± 1.8 ; n = 8 sections from

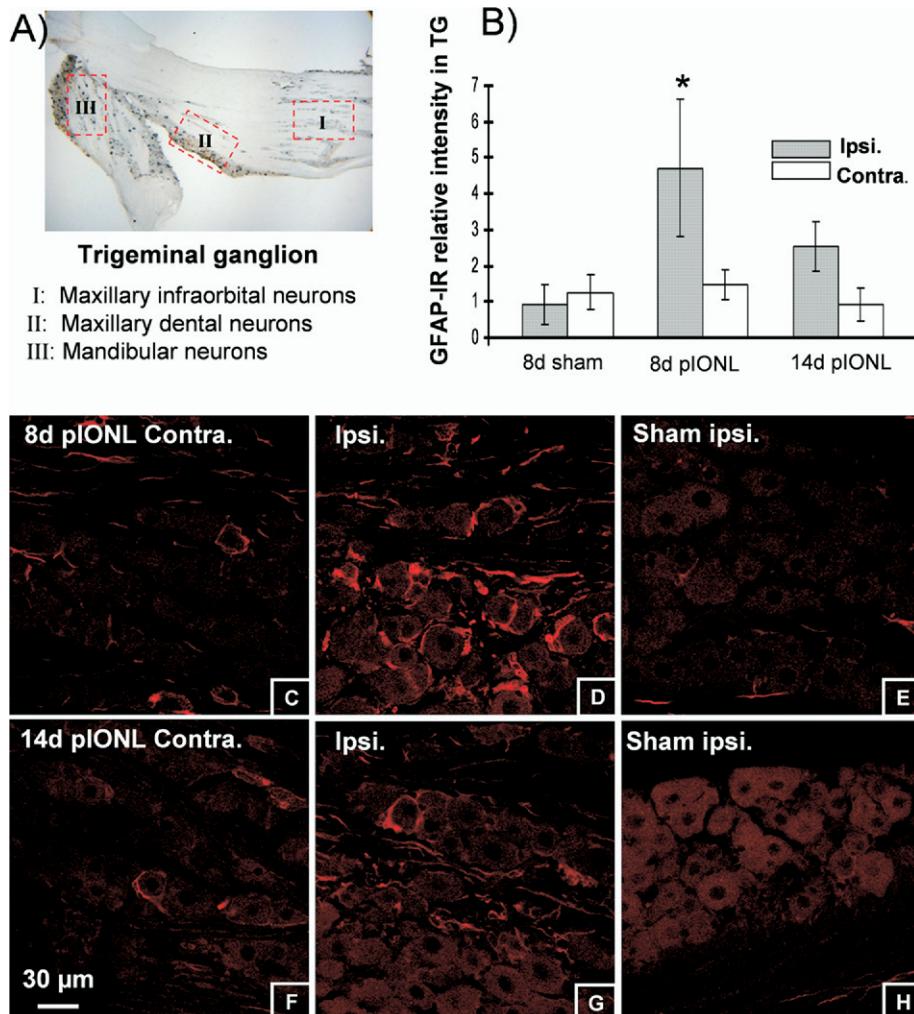


Figure 7. Glial fibrillary acidic protein (GFAP)-positive satellite cell changes in trigeminal ganglion (TG) 1 week and 2 weeks after partial infraorbital nerve ligation (pIONL). *Upper left panel:* Neuronal cell body distribution in trigeminal ganglion. *I:* Maxillary infraorbital neurons. *II:* Maxillary dental neurons. *III:* Mandibular neurons. Our quantitation was for zone (*I*) for 3 sections (20-μm thickness) per ganglion, with intervals of 60 μm between each counted section. *Upper right panel:* Quantification of satellite cell reaction in zone (*I*) of TG, **P* < .05, *n* = 4. *Lower panel:* GFAP-IR in the trigeminal ganglion of wild-type mouse 1 week and 2 weeks after pIONL. There is an apparent increase in GFAP-IR in the ipsilateral side of TG (**D**) compared with the contralateral side (**C**) or sham mice (**E**) 1 week after surgery. The GFAP-IR decreases 2 weeks after pIONL (**F** and **G**). Scale bars, C–H, 30 μm.

for facial pain, there may be important species differences in the responses to neuropathic pain.

Rats with CCI of the ION were found to have lower average daily weight gain than sham-operated animals.⁵² In contrast, Lim et al³² showed that body weight gain was not significantly decreased after ION chronic constriction injury. In our study, we found that in the first week after pIONL, the body weights of injured mice were slightly below pre-injury baseline, but the difference did not reach statistical significance between pIONL and sham-operated

mice. All groups gained weight steadily in the second week after pIONL.

Injury to a peripheral nerve not only produces profound behavioral signs of persistent pain, but it also alters peptide expression in the central nervous system (CNS). For example, sciatic nerve injury results in a decrease in CGRP, sub P and causes an increase in NK1/sub P receptor-IR in the dorsal horn of rat spinal cord.^{17,19,28} The decrease of sub P and CGRP that we observed in caudal medulla after pIONL was likely to reflect changes

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4 animals) than that of the contralateral caudal medulla (6.12 ± 1.4 ; *n* = 8 sections from 4 animals, **P* < .001) or sham-ligated mice (5 ± 1.8 ; *n* = 8 sections from 4 animals, ***P* < .001). C–E, Some of the dividing BrdU-labeled cells were also CD 11b positive microglia (arrows) and others were not CD 11b positive (arrowheads). F–H, Some of the dividing BrdU-labeled cells were glial fibrillary acidic protein (GFAP)-positive astrocytes (arrows) and others were not GFAP positive (arrowheads). I–K, Many BrdU-positive cells were obviously double-labeled with nestin, a stem cell marker (arrows), but a small fraction of BrdU-positive cells were not nestin-positive stem cells (arrowheads). L–N, BrdU-labeled cells were not colabeled by NG2, an oligodendrocyte precursor marker (L–N, arrowheads). Scale bars: A, 200 μm; C–N, 100 μm.

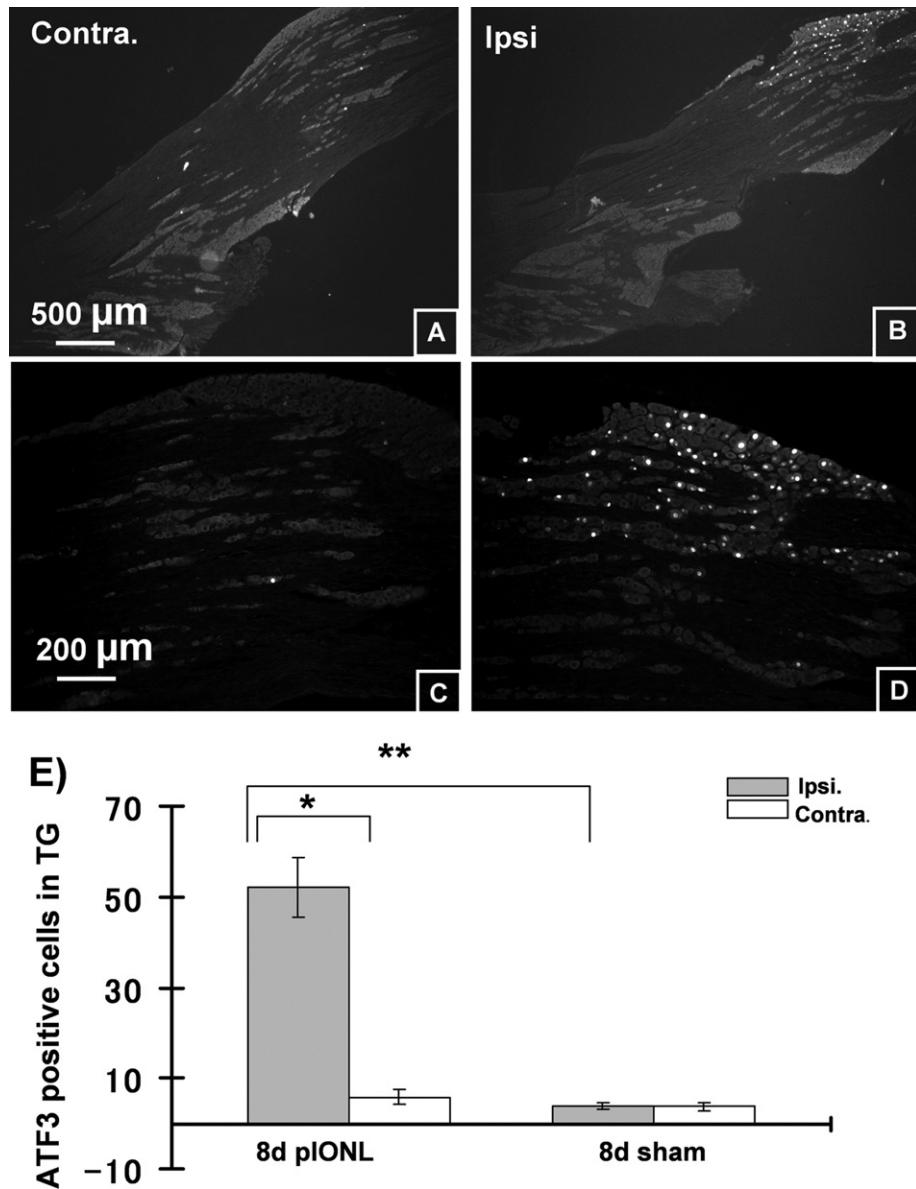


Figure 8. Immunohistochemical expression of ATF3 in trigeminal ganglia (TG) 8 days after partial infraorbital nerve ligation (pIONL). ATF3-IR increased markedly in the ipsilateral TG (B and D) compared with the contralateral TG (A and C). ATF3 expression occurred within neurons in the infraorbital region of TG (B). E, summary graph shows quantification (mean \pm SEM) of ATF3 positive cells in caudal medulla of brainstem sections. Image analysis of ATF3-positive cells in ipsilateral caudal medulla 8 days after pIONL demonstrated approximately 10-fold higher (52 ± 6.64 ; n = 8 sections from 4 animals, * $P < .001$) than that in contralateral caudal medulla (5.8 ± 1.59 ; n = 8 sections from 4 animals, ** $P < .001$). Scale bars: A and B, 500 μ m; C and D, 200 μ m.

in expression of these peptides in primary afferents. The NK-1 receptor, on which sub P acts, is expressed by most of the lamina I neurons in the spinal dorsal horn⁴⁶ and trigeminal subnucleus caudalis.³¹ The increase in NK-1 receptor expression after the pIONL suggested that this response was triggered by the injury barrage generated by the nerve ligation. Consistent with this, the sub P and CGRP system reorganization only occurred in the dorsal part of the caudal medulla, which receives primary afferent input from the trigeminal nerves. This observation is similar to the results from studies in the rat after complete transection of the sciatic nerve.^{1,17,19,28} Using the chronic sciatic nerve constriction model, Cameron et al⁹

found a significant relationship between allodynia and peptide expression levels. We also found that the anatomical and behavioral changes were correlated. These results suggest that nerve injury-induced allodynia is associated with the neurochemical reorganization of caudal medulla neurons and primary afferents.

Partial injury to peripheral nerves has been shown to induce persistent changes in nerve, ganglion, central neurons, and glial cells that are correlated with the development of pain behaviors. Microglia and astrocytes are activated by peripheral insults such as peripheral nerve injury^{18,29} and peripheral inflammation,¹⁵ spinal glial activation might be a causal factor in the pain hy-

persensitivity at the spinal level.⁵⁴ Consistent with these studies, glial activation was also induced in our pIONL model. The regions showing the greatest glial activation were in the caudal medulla. It is interesting to note that temporal changes in the activation of microglia are different from those of astrocytes; microglia were activated earlier than astrocytes by pIONL. This is consistent with results in the CNS where microglia are the initial responders to trauma, ischemia, tumors, and inflammation.³⁰ It is likely that activated microglia may subsequently lead to the activation of astrocytes after pIONL, because Minocycline (Sigma-Aldrich, St. Louis, MO), an inhibitor of microglia, was previously shown to attenuate the development of neuropathic pain after trigeminal sensory nerve injury.⁴⁰

Incorporation of BrdU after nerve ligation has revealed robust cell proliferation in the spinal cord after partial sciatic nerve ligation in mice.^{36,55} Consistent with these results, we found that astrocyte and microglia proliferation was induced by pIONL. At 8 days after pIONL, BrdU-positive cells on the ipsilateral side of the caudal medulla were increased 7-fold compared with the contralateral side in nerve-ligated mice. In addition, BrdU-positive cells were partially colocalized with GFAP and CD11b within the caudal medulla.

Previous studies have shown that injury to neurons in the maxillary division by tooth injury induced GFAP expression and satellite cell reactions around specifically injured (Di-I labeled) dental neurons and in neighboring neurons in that division as well as in mandibular regions.⁴⁴ Similarly, injury to the mandibular neurons by inferior alveolar nerve crush caused GFAP reactions in satellite cells around those cell bodies.^{2,11} Some interesting persisting neuronal or glial responses have been found in neurons and in their satellite cells in the trigeminal ganglion.³ In our study, we showed that GFAP-IR satellite cells increased 1 week after pIONL. This is consistent with previous reports that upregulated GFAP expression in satellite cells could be detected in both mandibular and maxillary divisions after inferior alveolar nerve crush.¹¹ Changes in the trigeminal ganglion cells are probably caused by the change of extracellular ionic concentrations or injury-related factors, such as growth

factors released after pIONL injury. Future studies combining intracellular recording from ganglion neurons and immunohistochemical methods may resolve these questions.

ATF3 is a member of the activating transcription factor/cAMP-responsive element binding protein family (ATF/CREB family) and is induced in response to stress signals in many different tissues.²⁰ Tsujino et al⁴⁸ first reported that ATF3 was a reliable and sensitive marker for axotomized neurons, and it has now become widely used as an indicator of nerve injury.^{4,38,49} In our study, neuronal expression of ATF3, which is normally minimal, was robustly increased only in ipsilateral side of ION region of TG, but not in contralateral side or in sham-operated mice 8 days after pIONL. This finding suggests that upregulated ATF3 in TG is caused by direct injury to ION, rather than by the surgical procedure, ATF3 expression is significantly lower in TG neurons of other regions, on the contralateral side after pIONL, or in TG of sham-ligated mice. Recent studies showed that many neurons in the L4 DRG also exhibited phenotypic plasticity of mRNA or protein expression for a variety of neurotransmitters, neurotrophic factors, ion channels and receptors after L5 SNL.^{16,21} In our experimental model, increased ATF3 expression was only seen in ION region. This is likely because of the anatomical differences between trigeminal nerve and sciatic nerve. Trigeminal nerve is surrounded by bone structure, and all branches are isolated by bone canals. Thus, there is likely less damage to adjacent neurons in our pIONL model than the sciatic nerve injury model. In summary, the pIONL model of trigeminal nerve injury is a robust and feasible tool for future studies describing the cellular and molecular mechanisms of neuropathic pain.

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