Abstract: The inferior alveolar nerve (IAN) comprises several types of sensory fibers. To clarify whether each type of primary afferent is regenerated comparably after injury, we developed a model of complete IAN transection (IANX) in mice. A retrograde tracer, fluoro-gold, injected into the mental skin was transferred to the cell bodies of a subset of isolectin B4 (IB4)-binding (non-peptidergic C) or CGRP-positive (peptidergic C) neurons at 2 weeks post-axotomy, indicating that the injured C afferents had regenerated anatomically. IANX led to a decrease of IB4-binding and CGRP immunoreactivity (IR) in the trigeminal ganglion (TG) and within the trigeminal spinal subnucleus caudalis (Vc) (i.e. terminals of the central branch of TG neurons). Two weeks after IANX, the reduction in IB4-binding activity and CGRP expression in the TG recovered to the control level; however, IB4-binding within the Vc did not, suggesting that central branch non-peptidergic neurons remained impaired. Two weeks after IANX, pinching or heat stimulus-induced extracellular signal-regulated kinase phosphorylation (pERK) was restored to the control level, but in the case of pinch stimulation the distribution pattern of pERK-IR cells was altered in the Vc. Taken together, our results support the possibility that peptidergic neurons regenerate more efficiently than non-peptidergic neurons after trigeminal nerve injury.

Keywords: axonal regeneration; trigeminal nerve; trigeminal spinal subnucleus caudalis; CGRP; IB4; ERK phosphorylation.

Introduction

Peripheral nerve injury frequently leads to somatosensory disorders, including hypoalgesia and neuropathic pain, depending on the types of axons injured, the part of the axon affected, and the severity of impairment (1). The primary afferents of the peripheral nerves are categorized into three classes, Aβ, Aδ, and C. The C afferents can be subdivided into two groups, peptidergic and non-peptidergic, based on calcitonin gene-related peptide (CGRP) expression versus isolectin B4 (IB4) binding (2,3). In the sciatic nerve, CGRP-positive and IB4-binding fibers terminate their axons in different layers in the spinal cord (lamina I and outer lamina II [IIo] versus inner lamina II [III], respectively) (4).

Recently, two groups have independently investigated whether these two neuron subtypes are required for sensing of painful stimuli. Genetic ablation in adulthood of Mrgprd-expressing sensory neurons, which correspond to a subset of IB4-binding neurons, was found to reduce behavioral sensitivity to noxious mechanical stimuli but not to heat or cold stimuli (5). In contrast, genetic ablation of CGRPα-expressing neurons reduced sensitivity to noxious heat, but did not impair mechanosensation...
Together these findings suggest that IB4-binding versus CGRP-expressing neurons predominantly convey noxious mechanosensation versus noxious heat pain, respectively.

On the other hand, it has been reported that unilateral dorsal rhizotomy of six adjacent dorsal roots induced a large decrease in peptidergic and non-peptidergic afferents on the deafferented side of the spinal cord several days after injury (7-9). Three months after surgery, the peptidergic, but not non-peptidergic, primary afferents sprouted into the center of the denervated dorsal horn (7). In addition, IB4-binding, but not IB4-nonbinding, neurons showed little neurite outgrowth after axotomy in in vitro experiments (10). These observations indicate that each C afferent has a different potential for axonal regeneration.

Trigeminal sensory neurons are pseudo-unipolar, with one axonal branch that extends to peripheral targets, and another branch forming synapses on second-order neurons in the dorsal column nuclei of the brainstem. The inferior alveolar nerve (IAN), a branch of the mandibular nerve, transmits somatic sensations, such as mechanical, thermal, and nociceptive stimuli, arising from the tooth pulp, periodontal tissue, and dental gum of the lower jaw, and mental skin to the medulla (11). Several groups, including ours, have shown that the injured IAN has an intrinsic ability to regenerate in the rat (12-14). However, it remains to be elucidated whether both C afferent types regenerate and whether regenerated axons transfer external signals to the appropriate synaptic partners in the neural circuit.

Ras/Raf/extracellular signal-regulated kinase 1/2 (ERK) signaling plays an essential role in cell proliferation, differentiation, and growth, including those occurring after injury (15). Several studies have demonstrated that various types of noxious stimuli applied to the rat hind-paw, or nerve injury, induce phosphorylation of the ERK in superficial spinal dorsal horn neurons (16-18). We have previously shown that ERK is activated in some trigeminal spinal subnucleus caudalis (Vc) neurons within 5 min of noxious stimulation of the orofacial region, and that the levels of ERK phosphorylation (pERK) change in an activity-dependent manner (19-21). These results suggest that the ERK signaling pathway plays a crucial role in pain perception, and that pERK can be utilized as a chemical marker for activated neurons involved in pain sensation and the nocifensive reflex.

In this study, we developed an IAN complete transection (IANX) model in mice and, using immunohistochemistry, evaluated regeneration of the injured axons and its correlation with ERK phosphorylation in the medulla following noxious stimulation.

Materials and Methods

Animals
Sixty-five C57BL/6 male mice (7 weeks old) were used. They were maintained at ambient temperature and humidity under a 12-h light/dark cycle with ad libitum access to food and water. The study was approved by the Animal Experimentation Committee at Nihon University (AP13D007-3, AP16D046). Experiments were performed according to the guidelines of the International Association for the Study of Pain (22).

Inferior alveolar nerve transection (IANX)
Mice were anesthetized with a combination of butorphanol tartrate (2.5 mg/kg, i.p., Meiji Seika Pharma, Tokyo, Japan), midazolam (2.0 mg/kg, i.p., Sandoz, Tokyo, Japan), and medetomidine (0.375 mg/kg, i.p., Zenoaq, Fukushima, Japan) supplemented with 2% isoflurane. A small incision was made on the lower facial skin on the left using a sharp-pointed knife. The inferior alveolar nerve was exposed in the center of the bottom edge of the mandibular angle and orbital, and then transected using a needle (IANX). The skin was then closed with 5-0 silk sutures.

Noxious stimulation
After anesthesia, noxious mechanical stimuli (10 times with a load of 60 g for 1 min, interval 30 s) or heat stimuli (5 sessions of exposure to 53°C for 1 min at 30-s intervals) were applied to the lower left lip using a clip or probe for induction of ERK phosphorylation in the Vc. After stimulation, the mice were promptly perfused as described below.

Immunohistochemistry
After survival times of 1, 3, 7, 14, and 28 days, mice were deeply anesthetized using the same procedures as those described above and perfused with 0.9% saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4). The trigeminal ganglion (TG) and the medulla and upper cervical spinal cord were removed and post-fixed in the same fixative solution for 2 days at 4°C, and then replaced in 0.1 M PB containing 20% sucrose overnight at 4°C for cryoprotection. Frozen sections were cut along the TG at 10 μm thickness, or along the coronal plane at 30 μm thickness using a cryostat (Leica CM1850; Leica Microsystems, Wetzlar, Germany), and every sixth section was used for immunofluorescence. The sections were treated in HistoVT One (Nacalai tesque, Japan) in a preheated 70°C water bath for 30 min.
After antigen retrieval, the sections were blocked with 5% normal goat serum in 0.3% Triton-X 100 for 1 h at room temperature (RT). The sections were then incubated with rabbit anti-CGRP (1:1,000; Sigma-Aldrich, USA) and rabbit anti-pERK (1:1,000; Cell Signaling Technology, Danvers, MA, USA) antibodies for 72 h at 4°C. The sections were then treated with AlexaFluor 488-conjugated goat anti-rabbit IgG (1:1,000; Invitrogen, Carlsbad, CA, USA) and AlexaFluor 568-conjugated isolectin GS-IB4 (1:1,000; Invitrogen), or AlexaFluor 568-conjugated goat anti-mouse IgG (1:1,000; Invitrogen) antibodies and AlexaFluor 488-conjugated isolectin GS-IB4 (1:1,000; Invitrogen) for 2 h at RT. Thereafter all sections were observed using a fluorescence microscope (BZ9000, Keyence, Osaka, Japan).

Fluoro-gold (FG) labeling
To confirm axonal regeneration of injured TG neurons, 5 µL of 4% FG (Fluorochrome, F-G, Denver, CO, USA) was injected into the mental skin to 3 mm left of the midline subcutaneously in IANX and sham-anesthetized animals. Three days after retrograde tracer injection, the mice were perfused as described above. FG-labeled cells were counted from 2 sections from each mouse.

Data and statistical analysis
All fluorescence images were captured with an all-in-one fluorescent microscope system with 10× or 20× objective lenses for qualitative and quantitative image analysis. The number of IB4-binding or CGRP-IR cells in the TG was counted from 3 sections per animal (n = 5). For quantifying IB4-binding or CGRP-IR axons within the Vc, the optical density of IB4-binding or CGRP-IR within the medial one-third and distal one-third of the Vc on the ipsilateral side after IANX was measured using ImageJ software (4 sections per animal, n = 5) and the relative density (medial one-third/lateral one-third) was calculated. For pERK analysis, each photographed section was imported into Neurolucida 2000 (MicroBrightField, Colchester, VT, USA) and the location of all pERK-IR cells was plotted spatially. The total number of pERK-IR cells in Vi/Vc, Vc and Vc/C2 was counted from 10 sections in each level of the medulla per animal (n = 5). Statistical analysis was performed by one-way ANOVA followed by Tukey’s test. All data are presented as mean ± SEM values. Differences at P < 0.05 were considered significant.

Results
IANX induces transient reduction of IB4-binding and CGRP expression in TG neurons
In intact animals, IB4-binding and CGRP immunoreactivity (IR) were detected in a subpopulation of neurons (Fig. 1Aa,d). Consistent with previous observations (11), overlapping of IB4-binding and CGRP-IR was rare (data not shown). The majority of both IB4-binding and CGRP-IR cells were small- or medium-sized (arrows in Fig. 1Aa,d). Values represent mean ± SEM. n = 5 in each group. *P < 0.05, **P < 0.01 (one-way ANOVA, vs naïve). Scale bar: 100 μm.
restricted to the area of the TG where cell bodies of the third branch of the trigeminal nerve are concentrated (Fig. 1Cb,e). Although not quantified, this subpopulation of FG-labeled neurons showed IB4-binding or CGRP-IR in IANX animals (Fig. 1Ca-f).

IANX differentially affects CGRP expression and IB4-binding within the Vc

In the medulla of naïve animals, IB4-binding axons were observed predominantly in lamina I and lamina II, sometimes extending into a portion of lamina III (Fig. 2Aa). By contrast, CGRP-IR axons were located superficially, i.e. in lamina I and the outer part of lamina II (IIo) (Fig. 2Ad). Three days after IANX, severe reduction of IB4-binding was observed within the inferior alveolar nerve termination zone on the injured side (Fig. 2Ab). This decrease of IB4-binding was slightly improved on day 14 (Fig. 2Ac), but had not recovered to normal levels at least up to 4 weeks post-IANX (data not shown). CGRP-IR was also greatly reduced in the Vc 3 days after IANX (Fig. 2Ae). These signals were, however, restored by 2 weeks after IANX (Fig. 2Af).

IANX modifies the distribution pattern of noxious stimulus-dependent pERK-IR neurons within the Vc

To determine whether the regenerated axons form functional synaptic connections in the medulla, we subsequently analyzed ERK activation in second-order neurons after noxious heat stimulation or pinching of the mental skin. In control animals, intense pERK-IR was observed bilaterally in a subset of neurons and their processes in the dorsomedial part of the Vi/Vc, Vc, and Vc/C2 (Figs. 3A, B, 4A, B). After mental skin
pinching, the majority of pERK-positive neurons were localized in laminae I and II, but some pERK-IR neurons were present in lamina III (Figs. 3A, 4A). Analysis of the rostrocaudal distribution showed that the pERK-IR cells were located in Vc-C2 between −1,440 and 180 µm relative to the obex, and the number of pERK-IR cells peaked at approximately −360 µm from the obex (Fig. 3C). Heat stimulation of the mental skin also rapidly induced pERK-IR in a subset of Vc neurons (Figs. 3B, 4B). Although the distribution pattern of pERK-IR cells was not distinguishable rostrocaudally between the pinching and heat stimulation groups, the number of pERK-IR neurons responding to noxious heat stimulation always appeared to be lower than those responding to mechanical stimulation (Fig. 3C, D).

Three days after IANX, ERK-IR cells were drastically decreased in the medulla (Fig. 3C, D), and the level of ERK activation in individual cells was also reduced (data not shown). Noxious stimulus-dependent ERK-IR cells reappeared 2 weeks after IANX, but the distribution of pERK-IR cells was different from that in the control (Fig. 3C, D). The rostrocaudal peak number of pERK-IR cells moved from −360 µm relative to the obex in naïve animals to −180 µm (i.e. more rostral) in the IANX group (Fig. 3C). Intriguingly, in the IANX animals, pERK-positive cells were densely packed within the dorsolateral part of the Vc (at −360 µm from the obex, Fig. 3A, C, Fig. 4A). The location of the accumulated pERK signals bordered on the edges of the IB4-binding-positive areas, suggesting that ERK phosphorylation did not occur in the IB4-negative area of the Vc (Fig. 4A).

Importantly, at 2 weeks after IANX, pERK signals were never detected in IANX animals when no heat or pinching stimulation was performed, indicating that pERK activation was not induced by IANX itself (data not shown). On the other hand, heat stimulus-dependent pERK-IR was detected in the region negative for IB4-binding at 2 weeks after IANX (Fig. 4B).

**Discussion**

**CGRP-IR and IB4-binding TG neurons in IAN regeneration**

Unmyelinated C fibers, which transmit an array of somatosensory information, are classified into two groups: peptidergic and non-peptidergic (3). In the present study, the retrograde tracer FG was injected into the mental skin and subsequently observed in a small subset of both CGRP-IR and IB4-binding neurons at 2 weeks after IANX. At least some axons of both peptidergic and non-peptidergic neurons in the injured peripheral branch had re-innervated their appropriate receptive fields within this period.

IANX induced a reduction of CGRP-IR and the IB4-binding signal in the cell bodies of TG neurons. The signal recovered to control levels by 2 weeks after axonal injury. Similarly, CGRP expression was also restored in the Vc, i.e. the central axon terminals of TG neurons, at 2 weeks after IANX. In contrast, IB4-binding in the Vc was still significantly lower than in the control groups. These results indicated that peptidergic C fibers are able to regenerate more efficiently than non-peptidergic C fibers.
IB4, a glycoprotein isolated from *Griffonia simplicifolia*, has been used as a marker of non-peptidergic neurons by many researchers (10,23). Although the molecules that bind IB4 in sensory neurons are still unknown, it is postulated that injury to peripheral axonal branches causes TG neurons to modify the expression levels of unidentified IB4-interacting molecules and/or proteins involved in intracellular transport, which would in turn influence axonal transport in the central branch of the injured neuron, as well as the peripheral branch itself.

**Interaction between CGRP-IR or IB4-binding central axons and pERK Vc neurons**

Noxious perception, including mechanical, heat, and cold pain, is considered to be conveyed by differential activation of several distinct types of neurons (1). On the basis of a genetically precise ablation strategy, it has recently been proposed that unmyelinated IB4-binding versus CGRP-expressing neurons contribute predominantly to nocuous mechanical versus noxious heat sensation, respectively (5,6). Consistent with this view, we found in this study that ERK phosphorylation in response to a pinch stimulus was evident in neurons within the IB4-binding-positive area of the Vc in sham-treated mice. Pinching-induced ERK phosphorylation had recovered in the Vc by 2 weeks after IANX, and pERK was observed only in the region of the Vc where IB4-binding had recovered in the treated mice. Interestingly, the density of pERK signals was increased at the edge of the IB4-binding region. It has been reported that peripheral nerve injury is involved in synaptic plasticity, degeneration, sprouting, or regeneration of central axon terminals (24,25). The pattern of change of pinch stimulus-dependent ERK phosphorylation in the Vc may reflect IANX-induced synaptic modification between sensory neurons and second-order neurons. Based on our findings, it is presumed that most cases of non-peptidergic axonal injury are not repaired completely, and that some of the repaired axons show a change in the number of their synaptic connections and/or modification of their synaptic partners after IANX.

By contrast, the distribution of noxious heat-dependent ERK phosphorylation in Vc neurons was similar between the IANX and sham groups, and CGRP expression in the central axon terminals of TG neurons recovered within 2 weeks after IANX. This suggests that CGRP-positive TG neurons are responsible for noxious heat-dependent ERK activation in a subset of Vc neurons, and that peptidergic neurons regenerate more efficiently than non-peptidergic neurons after trigeminal nerve injury.

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**Conflict of interest**

The authors have no conflict of interest to declare.

**References**

1. Woolf CJ, Salter MW (2000) Neuronal plasticity: increasing the gain in pain. Science 288, 1765-1769.
2. Hunt SP, Mantyh PW (2001) The molecular dynamics of pain control. Nat Rev Neurosci 2, 83-91.
3. Peirs C, Seal RP (2016) Neural circuits for pain: recent advances and current views. Science 354, 578-584.
4. Lai HC, Seal RP, Johnson JE (2016) Making sense out of spinal cord somatosensory development. Development 143, 3434-3448.
5. Cavanaugh DJ, Lee H, Lo L, Shields SD, Zylka MJ, Basbaum AI et al. (2009) Distinct subsets of unmyelinated primary sensory fibers mediate behavioral responses to noxious thermal and mechanical stimuli. Proc Natl Acad Sci U S A 106, 9075-9080.
6. McCoy ES, Taylor-Blake B, Street SE, Pribisko AL, Zheng J, Zylka MJ (2013) Peptidergic CGRPα primary sensory neurons encode heat and itch and tonically suppress sensitivity to cold. Neuron 78, 138-151.
7. Runyan SA, Roy RR, Zhong H, Phelps PE (2007) L1 cell adhesion molecule is not required for small-diameter primary afferent sprouting after deafferentation. Neuroscience 150, 859-869.
8. Shi TJ, Xiang Q, Zhang MD, Tortoriello G, Hammarberg H, Mulder J et al. (2012) Secretagogin is expressed in sensory CGRP neurons and in spinal cord of mouse and complements other calcium-binding proteins, with a note on rat and human. Mol Pain 8, 80.
9. Miskimon M, Han S, Lee JJ, Ringkamp M, Wilson MA, Petralia RS et al. (2014) Selective expression of Narp in primary nociceptive neurons: role in microglia/macrophage activation following nerve injury. J Neuroimmunol 274, 86-95.
10. Leclere PG, Norman E, Groutsi F, Coffin R, Mayer U, Pizzey J et al. (2007) Impaired axonal regeneration by isolectin B4-binding dorsal root ganglion neurons in vitro. J Neurosci 27, 1190-1199.
11. Bae JY, Kim JH, Cho YS, Mah W, Bae YC (2015) Quantitative analysis of afferents expressing substance P, calcitonin gene-related peptide, isolectin B4, neurofilament 200, and Peripherin in the sensory root of the rat trigeminal ganglion. J Comp Neurol 523, 126-138.
12. Fristad I, Heyeraas KJ, Kvinsland IH (1996) Neuropeptide Y expression in the trigeminal ganglion and mandibular division of the trigeminal nerve after inferior alveolar nerve
13. Imai T, Atsumi Y, Matsumoto K, Yura Y, Wakisaka S (2003) Regeneration of periodontal Ruffini endings of rat lower incisors following nerve cross-anastomosis with mental nerve. Br J Oral Maxillofac Surg 41, 276-286.

14. Tsuboi Y, Honda K, Bae YC, Shinoda M, Kondo M, Katagiri A et al. (2015) Morphological and functional changes in regenerated primary afferent fibres following mental and inferior alveolar nerve transection. Eur J Pain 19, 1258-1266.

15. Johnson GL, Lapadat R (2002) Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. Science 298, 1911-1912.

16. Ji RR, Baba H, Brenner GJ, Woolf CJ (1999) Nociceptive-specific activation of ERK in spinal neurons contributes to pain hypersensitivity. Nat Neurosci 2, 1114-1119.

17. Polgár E, Campbell AD, Machintyre LM, Watanabe M, Todd AJ (2007) Phosphorylation of ERK in neurokinin 1 receptor-expressing neurons in laminae III and IV of the rat spinal dorsal horn following noxious stimulation. Mol Pain 3, 4.

18. Gao YJ, Ji RR (2009) c-Fos and pERK, which is a better marker for neuronal activation and central sensitization after noxious stimulation and tissue injury? Open Pain J 2, 11-17.

19. Shimizu K, Asano M, Kitagawa J, Ogiso B, Ren K, Oki H et al. (2006) Phosphorylation of extracellular signal-regulated kinase in medullary and upper cervical cord neurons following noxious tooth pulp stimulation. Brain Res 1072, 99-109.

20. Noma N, Tsuboi Y, Kondo M, Matsumoto M, Sessle BJ, Kitagawa J et al. (2008) Organization of pERK-immunoreactive cells in trigeminal spinal nucleus caudalis and upper cervical cord following capsaicin injection into oral and craniofacial regions in rats. J Comp Neurol 507, 1428-1440.

21. Hasegawa M, Kondo M, Suzuki I, Shimizu N, Sessle BJ, Iwata K (2012) ERK is involved in tooth-pressure-induced Fos expression in Vc neurons. J Dent Res 91, 1141-1146.

22. Zimmermann M (1983) Ethical guidelines for investigations of experimental pain in conscious animals. Pain 16, 109-110.

23. Wu YJ, La Pierre DP, Wu J, Yee AJ, Yang BB (2005) The interaction of versican with its binding partners. Cell Res 15, 483-494.

24. Kapadia SE, LaMotte CC (1987) Deafferentation-induced alterations in the rat dorsal horn: I. Comparison of peripheral nerve injury vs. rhizotomy effects on presynaptic, postsynaptic, and glial processes. J Comp Neurol 266, 183-197.

25. Woolf CJ, Shortland P, Coggeshall RE (1992) Peripheral nerve injury triggers central sprouting of myelinated afferents. Nature 355, 75-78.