Modulation of Trigeminal Spinal Subnucleus Caudalis Neuronal Activity Following Regeneration of Transected Inferior Alveolar Nerve in Rats

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Saito K, Hitomi S, Suzuki I, Masuda Y, Kitagawa J, Tsuboi Y, Kondo M, Sessle BJ, Iwata K. Modulation of trigeminal spinal subnucleus caudalis neuronal activity following regeneration of transected inferior alveolar nerve in rats. J Neurophysiol 99: 2251–2263, 2008. First published March 12, 2008; doi:10.1152/jn.00794.2007. Modulation of trigeminal spinal subnucleus caudalis neuronal activity following regeneration of transected inferior alveolar nerve in rats. To clarify the neuronal mechanisms of abnormal pain in the face innervated by the regenerated inferior alveolar nerve (IAN), nociceptive behavior, trigeminal ganglion neuronal labeling following Fluorogold (FG) injection into the mental skin, and trigeminal spinal subnucleus caudalis (Vc) neuronal properties were examined in rats with IAN transection. The mechanical escape threshold was significantly higher at 3 days and lower at 14 days after IAN transection, whereas head withdrawal latency to heat was significantly longer at 3, 14, and 60 days after IAN transection. The number of FG-labeled ganglion neurons was significantly reduced at 3 days after IAN transection but increased at 14 and 60 days. The number of wide dynamic range (WDR) neurons with background (BG) activity was significantly higher at 14 and 60 days after IAN transection compared with naïve rats, and the number of WDR and low-threshold mechanoreceptive (LTM) neurons with irregularly bursting BG activity was increased at these two time points. Mechanically evoked responses were significantly larger in WDR and LTM neurons 14 days after IAN transection compared with naïve rats. Heat- and cold-evoked responses in WDR neurons were significantly lower at 14 days after transection compared with naïve rats. Mechanoreceptive fields were also significantly larger in WDR and LTM neurons at 14 and 60 days after IAN transection. These findings suggest that these alterations may be involved in the development of mechanical allodynia in the cutaneous region innervated by the regenerated IAN.

INTRODUCTION

Trigeminal neuropathic pain conditions are well known to be difficult to diagnose and treat and, for most, their etiology and pathogenesis are still unclear (Bennett 2004; Forssell et al. 2007; Jääskeläinen 2004; Robinson et al. 2004; Truelove 2004; Vickers et al. 1998). Some trigeminal neuropathic pain behavioral models have been developed in rats to clarify the neural mechanisms underlying these neuropathic pain conditions (Imamura et al. 1997; Iwata et al. 2001; Robinson et al. 2004; Vos et al. 1994), but little is known about the peripheral and central neuronal changes that occur after trigeminal nerve injury. There are, however, a number of reports using spinal neuropathic pain models in which axotomy causes a variety of substantial changes in peripheral nerves and ganglion cells, including changes in expression of ion channels, neuropeptides, and intracellular molecules, which are thought to be involved in modulating the excitability of peripheral afferent fibers. These changes may be associated with changes in the excitability of neurons in the CNS (Henry et al. 2006, 2007; Ma et al. 2003; McCallum et al. 2006; Shortland et al. 2006; Woolf and Ma 2007).

In many of these and other nerve injury models, the sciatic nerve has been used, but this nerve includes motor nerves as well as sensory nerves, and it is very important to study the effect of pure sensory nerve lesion on nociception, without motor nerve damage, to clarify the underlying mechanisms of the sensory nerve injury-induced pain. The inferior alveolar nerve (IAN) branch of the trigeminal nerve is such a nerve and it has been reported that the IAN transection causes an abnormal nociceptive behavior and an increase in nociceptive neuronal excitability in the trigeminal spinal subnucleus caudalis (Vc) as well as primary afferent neurons (Iwata et al. 2001; Nomura et al. 2002; Tsuboi et al. 2004). Spontaneous neuronal activity, responses evoked by mechanical stimulation of regions adjacent to the injured IAN [e.g., the upper lip whisker pad innervated by the infraorbital nerve (ION)], and mechano-receptive field (RF) size were significantly increased in Vc neurons after IAN transection (Iwata et al. 2001). Trigeminal primary afferent fibers also increased their activity after IAN transection (Tsuboi et al. 2004). These data suggest that IAN injury produces an increased excitability of uninjured ION afferents and of Vc neurons receiving afferent inputs from the ION-innervated region, and suggest that these changes may underlie the behavioral allodynia in the ION-innervated region that can be evoked by noxious mechanical stimulation in this IAN transection model.

It is also known that transected nerves may regenerate following nerve injury (Imai et al. 2003; Jungnickel et al. 2006; Lozeron et al. 2004; Lundborg and Rosen 2007; Puigdellivol-Sanchez et al. 2006; Rodger et al. 2006). Anatomical studies have reported that the injured IAN begins to reinnervate...
peripheral structures within 7 days after nerve injury in rats (Atsumi et al. 2000; Harada et al. 2003; Youn et al. 1997). It was also reported that the region innervated by the injured nerve may be reinnervated by the nerves adjacent to the injured nerve (Robinson et al. 2004). Clinical studies have also reported that the region innervated by the injured nerve may be reinnervated by the nerves adjacent to the injured nerve (Robinson et al. 2004). Clinical studies have also reported that the area reinnervated by the injured nerve may be associated with abnormal pain and nonpain sensations long after the nerve injury and that the abnormal pain sensation is often difficult to treat (Carroll 2007; Kupers and Kehlet 2006; Mackey and Feinberg 2007; Priest and Kaczorowski 2007).

Since it is very important to understand the mechanisms underlying these abnormal pain states to develop improved treatment approaches, nocifensive behavior and single neuronal activity in Vc were analyzed in rats with IAN transection and a Fluorogold (FG) tracing study of trigeminal ganglion (TG) neurons was also conducted for evidence suggesting reinnervation by transected IAN afferents.

METHODS

This study was approved by the Animal Experimentation Committee at Nihon University School of Dentistry and procedures were performed according to the guidelines of the International Association for the Study of Pain (Zimmermann 1983). A total of 151 male Sprague–Dawley rats weighing 250–350 g were used in this study (IAN-transected rats: mechanical nocifensive behavior, n = 5, heat nocifensive behavior, n = 15, FG immunohistochemistry, n = 15, Vc neuronal recording, n = 43; sham-operated rats: mechanical nocifensive behavior, n = 5, heat nocifensive behavior, n = 15; naïve rats: mechanical nocifensive behavior, n = 10, heat nocifensive behavior, n = 5, FG immunohistochemistry, n = 5, Vc neuronal recording, n = 33).

Inferior alveolar nerve transection

Rats were initially anesthetized with sodium pentobarbital [50 mg/kg, administered intraperitoneally (ip)] and then the left IAN was transected. For the IAN transection, rats were placed on a warm mat and a small incision was made on the surface of the facial skin over the masseter muscle and tissue dissected to expose the surface of the alveolar bone. The bone covering the IAN was removed and the exposed IAN was lifted and transected at just above the angle of the mandible and placed back in the mandibular canal without any discernable gap between the IAN cut ends (Fig. 1A). Rats with a similar facial skin dissection and IAN exposure (but no IAN transection) were used as the sham-operated rats (n = 20). After surgery, penicillin G potassium (20,000 units, administered intramuscularly) was injected to prevent infection and the rats were allowed to recover.

Behavioral testing

Rats were trained daily to stay in a plastic cage for 20 min and drink water through a hole made in the wall of the plastic cage. For training...
related to the mechanical escape threshold measurement, a series of von Frey filaments was used for mechanical stimulation of the mental skin. After 1 wk of training, rats became capable of receiving the mechanical stimuli during drinking, with their perioral region protruding through the hole for 20 min. Before the start of the training procedure, water was restricted to 50 ml/day for 2 days. In daily sessions, water was used as a reward to train rats to stay in the plastic cage and to drink water through the hole during noxious stimulation of the mental skin supplied by the mental nerve. The criterion performance was when the rats could keep drinking water for 20 min without escape from noxious mechanical stimulation applied by the von Frey hair to the mental skin. Since each rat had a different escape threshold to mechanical stimulation, the maximum stimulus intensity differed between rats before training (15–60 g). All rats showed escape behavior when a 60-g stimulus was applied to the mental skin, and so 60 g was the strongest stimulus intensity used for the nociceptive behavioral test. The daily training sessions took place until criterion performance was reached. At this time, IAN transection was performed. The threshold for escape behavior to mechanical stimulation of the mental skin was then measured before and after 3, 14, and 60 days after IAN transection. The IAN supplies mandibular teeth and mucosa as well as the mental skin, but technical limitations prevented us from testing intraoral structures.

It is very difficult to apply thermal stimulation to the mental skin in awake rats because the mental skin region is located under the lower jaw. Thus we used lightly anesthetized rats to obtain behavioral measures for heat stimulation of the mental skin. For measurement of head withdrawal latency to heat stimulation of the mental skin, rats were lightly anesthetized with urethane (1.6 g/kg, ip). Bipolar enamel-coated silver-wire electrodes were placed in the splenius capitis muscle for electromyographic (EMG) recording (interelectrode distance: 5–6 mm). A heat stimulus (34°C) was applied to the mental skin through a contact heat probe (5 mm in diameter) and the head withdrawal latency was measured from the onsets of the heat stimulus and neck EMG activity (cutoff latency = 30 s). We used rats with cut facial skin and nerve exposure as the sham-operated rats (n = 5).

FG tracing

Twenty rats were used for the FG tracing study (naïve: n = 5; 3 days after transection: n = 5; 14 days after transection: n = 5; 60 days after transection: n = 5). Rats were anesthetized with sodium pentobarbital (50 mg/kg, ip) and 10 μl of 4% FG (Fluorochrome, Vysis, Richmond, UK) was subcutaneously injected into the mental skin (1.5–2.0 mm lateral and 1.0–1.5 mm below the edge of the lower lip and at a depth of about 5 mm from the surface. One day after the FG injection, rats were deeply anesthetized with the same anesthetic and perfused with 200 ml 0.9% saline followed by 500 ml of 4% paraformaldehyde. The trigeminal ganglion (TG) was removed and postfixed in the same fixative for 2 days and the tissue was then paraformaldehyde. The trigeminal ganglion (TG) was removed and FG tracing performed. The threshold for escape behavior to mechanical stimulation of the mental skin was then measured before and after 3, 14, and 60 days after IAN transection. The IAN supplies mandibular teeth and mucosa as well as the mental skin, but technical limitations prevented us from testing intraoral structures.

Animal preparation for Vc neuronal recording

Rats at 14 and 60 days after IAN transection (n = 43) and naïve rats (n = 33) were used for an acute recording experiment. The experimental conditions were similar for both groups of rats. Rats were initially anesthetized with sodium pentobarbital (50 mg/kg, ip) and the trachea and right femoral vein were cannulated to allow artificial respiration and intravenous administration of drugs. Anesthesia was maintained with halothane (2–3%) mixed with oxygen during surgery. Rats were mounted in a stereotaxic frame, the medulla was exposed by a laminectomy, and a mineral oil pool was made with the skin flaps surrounding the laminectomy. A head holder was rigidly secured to the skull by stainless steel screws and dental acrylic resin; stereotaxic ear bars and nose holder were removed. This setup allowed convenient access to the orofacial region to examine neuronal RFs.

After surgery, anesthesia was maintained throughout the experiment by continuous inhalation of halothane (1–2%) mixed with oxygen. During each recording session, the rats were immobilized with pancuronium bromide (1 mg·kg⁻¹·h⁻¹, iv) and artificially ventilated. The expired CO₂ concentration was monitored and maintained between 3.0 and 4.0%. Rectal temperature was maintained at 37–38°C by a thermostatically controlled heating pad (FHC, Bowdoin, ME) and the animal’s electrocardiogram was monitored.

Vc neuronal recording and stimulation

In IAN-transected rats and naïve control rats, enamel-coated tungsten microelectrodes (impedance = 10–12 MΩ, 1,000 Hz; FHC) were perpendicularly inserted to the medullary surface and were advanced in 2-μm steps into the Vc about 2 mm caudal to the obex. Mechanical stimulation (pressure or brush) of the mental skin was used as the search stimulus for evoked activities of Vc neurons. When a single neuron was isolated, the responses to mechanical stimulation of the facial skin were carefully examined and the RF was mapped. Only cutaneous RFs innervated by the third branch of the trigeminal nerve were mapped in the present study.

Mechanical stimuli were applied to the most sensitive region of the RF. Mechanical stimuli consisted of brushing with a camel-hair brush, graded pressure (1–60 g) produced by von Frey filaments, and pinch produced by a small arterial clip. To avoid sensitization due to repeated stimulation, noxious mechanical stimuli were applied to only a small part of the RF in each neuron. If there was overlap in the nonnoxious RFs of the first and second encountered nociceptive neurons, the second neuron was not included in the analysis. Each neuron was classified as large cells (>600 μm²) and small cells (<400 μm²).
in diameter) were used as the mechanical stimuli to determine the neuronal RF. Pinch stimuli were used to determine whether neuronal firing frequency increased above that induced by the low-threshold mechanical stimuli. If a neuron showed no responses to brushing, a noxious pinch was applied to determine whether it was a NS neuron. The border of the pinch RFs of NS neurons was defined by use of the metal rod for pressure. The tactile RFs of WDR and LTM neurons were drawn to scale on standard diagrams of a rat face. The RF area was calculated using image analysis software (National Institutes of Health image 1.61). Since it is difficult to accurately determine facial RF size because of the curvature of the face, RF sizes were likely underestimated in this study.

When a WDR neuron or NS neuron was identified, hot and cool stimuli were also applied by a thermal probe to the most sensitive part of the RF. The tip of the thermal probe was 5 mm in diameter and the rate of temperature change was set at 10°C/s, as reported in our previous study (Iwata et al. 1990). Before application of the thermal stimulus to the RF, the surface temperature was adapted to 38°C for 180 s. Skin heating ranged from 44 to 50°C and lasted for 10 s. Cold stimuli consisted of cooling of the skin to 10–30°C. The thermal stimuli were applied to the RF at 190-s intervals (adaptation time: 180 s, stimulus time: 10 s) to avoid sensitization of peripheral nociceptors.

Neuronal responses were recorded and stored in a microcomputer and firing frequency was analyzed off-line. Only one lesion was made in each rat after recording of the response properties of the Vc neurons. Lesions were made at the recording site by passing DC of 20 µA for 10 s.

Histology

At the end of each Vc recording experiment, the rat was overdosed with sodium pentobarbital and killed. The brain was removed and placed in cold fixative for a few days, then transferred to cold phosphate-buffered 30% sucrose for 48 h. Serial sections (50-µm-thick) were cut along the path of the microelectrode penetration. The sections were counterstained with thionin for identification of recording sites. The recording sites where exact locations were identified after histological examination were drawn at ×400 magnification with Neuro-lucida software (MicroBrightField, Colchester, UK).

Data analysis

The waveform of single or multiple neuronal activities was analyzed off-line. The waveform of each neuron was identified using Spike2 software (CED, Cambridge, UK). Peristimulus time histograms (bin width = 1 s) were generated in response to each stimulus. Background (BG) activities were first recorded for 10 s before application of the mechanical or thermal stimulus and they were subtracted from the neuronal responses during analysis. The interstimulus intervals (ISIs) of BG activity for 60 s in Vc neurons with high-frequency BG activity (>5 Hz) were analyzed according to the methodology described by Kaneoke and Viteck (1996). First, the mean ISI was calculated for the 60-s recording time immediately before the application of the mechanical stimuli to the mental skin. Then the 60 s was subdivided into time periods each of which corresponded to the mean ISI, and the number of spikes within each period was calculated. The number of periods with the same number of spikes was plotted as a discharge density histogram.

The mean firing frequency (spikes/s) during mechanical or thermal stimulation was calculated. Stimulus–response (S-R) functions of each Vc neuron were obtained in response to the mechanical (brush, graded pressure, or pinch) or thermal (heat: 44–50°C; cold: 10–30°C) stimuli. The mechanical or thermal stimulation of the RF was considered to have induced an effect when the peak firing frequency at 5 s after mechanical and 10 s after thermal stimulation (one trial of heat and cold stimulation for each neuron with 180-s intervals between mechanical and thermal stimuli) differed from the mean BG discharge rate by more than ±2SD.
Statistical analysis

Results are presented as means ± SE. Statistical analysis was performed by ANOVA followed by Dunnett’s test for the behavioral data, FG labeling of TG neurons, and Vc neuronal RF sizes. One-way ANOVA with Tukey test was also used for evoked Vc neuronal responses and BG activities. Differences were considered significant at P < 0.05.

RESULTS

Nocifensive behavior to mechanical or heat stimulation of the mental skin

During mechanical stimulation of the mental skin region, vocalization and autotomy were never observed in rats before or after left IAN transection. We could not observe any motor abnormalities in the orofacial regions in the IAN-transected rats. On the other hand, severe motor deficits are frequently observed in rats with sciatic nerve injury. There were also no significant differences in body weight between sham and IAN rats (sham and IAN, 3 days: 240.8 ± 4.57 and 246.4 ± 3.98 g; 14 days: 297.4 ± 3.80 and 295.2 ± 7.74 g; 60 days: 429.2 ± 0.86 and 422.4 ± 3.39 g; n = 5 in each group). The mean escape threshold values were 16.2 ± 2.6 g on the ipsilateral side of the face and 17.2 ± 2.2 g on the contralateral side before IAN transection (n = 5). The mean threshold value was significantly increased at 3 days after IAN transection and reduced at 14 days, and had returned to the preoperative level by 60 days on the side ipsilateral to the IAN transection, as illustrated in Fig. 1B (ipsilateral side to IAN treatment, 3 days: 53.2 ± 6.8 g, 14 days: 6.8 ± 1.4 g, 60 days: 30.0 ± 12.2 g; contralateral side to IAN treatment, 3 days: 18.4 ± 3.2 g, 14 days: 25.2 ± 9.1 g, 60 days: 32.0 ± 11.5 g; n = 5 in each day). Since the mechanical escape threshold was well above the normal noxious threshold intensity at 3 days after IAN transection, this suggests that the transected IAN had not regenerated in this time period. Head withdrawal latency to heat stimulation of the mental skin was also measured from the neck EMG activity in the rats with IAN transection (Fig. 1C) and was significantly longer in the IAN-transected rats at 3, 14, and 60 days after the transection compared with naïve rats (Fig. 1D). Thus we did not record in Vc of IAN-transected rats at 3 days after transection. We also measured the escape threshold to mechanical and escape latency to heat stimulation of the face in sham-operated rats and no significant differences in escape threshold and escape latency were observed between sham-operated rats and naïve rats. Therefore single neuronal recording experiments were carried out in control rats not having any surgical treatment.

Distribution and size of FG-labeled TG neurons

Large numbers of FG-labeled TG neurons were observed in naïve rats, as illustrated in Fig. 2A. FG-labeled neurons were localized in the root of the third branch of the trigeminal nerve (Fig. 2, A and B). No labeled neurons in the TG were observed at 3 days after IAN transection (Fig. 2C). Large numbers of FG-labeled neurons were apparent in the TG at 14 and 60 days after IAN transection (Fig. 2, D and E). However, the mean number of FG-labeled neurons at 60 days after transection was significantly smaller compared with that of naïve rats (Fig. 2F). We also could not observe any FG-labeled TG neurons in the root of the second branch of the trigeminal nerve at 14 and 60 days after IAN transection.

The area of FG-labeled TG neurons was also measured in the rats with IAN transection. The number of small-sized TG neurons was significantly decreased at 14 and 60 days after IAN transection compared with naïve rats (see Fig. 3).
Spatial arrangement of neurons in Vc

The activity of a total of 119 nociceptive and LTM neurons was recorded from Vc (naïve rats: n = 45, 14 days after IAN transection: n = 32, 60 days after IAN transection: n = 42) and electrophysiological properties of the LTM, WDR, and NS neurons were analyzed in detail (Table 1). The histologically verified locations of neurons were plotted on schematic illustrations of Vc, as illustrated in Fig. 4. Since we focused on analyzing neurons with mandibular RFs (see METHODS), it was not surprising that all analyzed neurons were located in the dorsal portion of the Vc, where Vc neurons with mandibular RFs are concentrated (Hu et al. 2005; Noma et al. 2008; Strassman et al. 1993). LTM, WDR, and NS neurons were distributed in both the superficial (laminae I–II) and deep laminae (laminae III–IV) of the Vc in naïve and IAN-transected rats, and there was no apparent difference in the distribution of neurons in Vc between naïve and IAN-transected rats.

BG activity of Vc neurons

Examples of the mean BG activity of each type of neuron at different time points after IAN transection are shown in Fig. 5. The burst discharge and irregular firing patterns were defined from the spike-density histograms. BG activity with regular firing showed one peak (Fig. 5Bb) and BG activity with burst firing had more than two peaks of discharge densities (Fig. 5Bd). WDR neurons showed a significant increase in BG firing at 14 days after IAN transection and BG activity returned to naïve levels at 60 days, whereas NS neurons showed very low frequency BG activity or no firing after IAN transection (Table 2). The BG activity of all WDR, NS, and LTM neurons from naïve rats was classified as regular firing, but at 14 and 60 days after IAN transection >50% of the WDR and LTM neurons showed irregularly bursting BG activity (Table 2). The mean number of bursts was also calculated in WDR (14 days: 3.0 ± 0.6; 60 days: 2.7 ± 0.5) and LTM neurons (14 days: 4.1 ± 0.7; 60 days: 4.5 ± 0.5) with burst firing, and revealed no obvious differences between the two different postoperative times. There were also no differences in the number of bursts in such neurons in naïve and IAN rats.

Evoked responses of Vc neurons

The mechanically evoked responses of WDR neurons in naïve and IAN-transected rats are illustrated in Fig. 6A. Typical responses of two WDR neurons recorded from naïve rats are shown in Fig. 6A, a1 and a2. These neurons were located at sites very close to each other within Vc. These neurons showed similar responses to mechanical stimulation, suggesting that preceding stimulation sequences applied in the study of the first neuron did not affect the responses subsequently recorded in the other neuron. The mean number of neurons recorded from each group of rats was not significantly different (naïve: 2.82 ± 0.24, n = 33; 14 days: 2.85 ± 0.27, n = 20; 60 days: 2.65 ± 0.2, n = 23). All WDR neurons showed a gradual increase in firing frequency as the mechanical stimulus intensity applied to the RF was progressively increased. The largest mechanically evoked responses occurred with pinching of the RF in WDR and NS neurons. The S-R functions for graded mechanical stimulation revealed that the mean evoked response of WDR neurons was significantly higher in rats 14 days after IAN transection compared with naïve rats, but by 60 days had returned to levels comparable to those documented in naïve rats (Fig. 6Ba). On the other hand, NS neurons did not show any changes in mechanically evoked responses after IAN transection (Fig. 6Bb). In LTM neurons, their mechanically evoked responses significantly increased after IAN transection but had returned to naïve levels by 60 days after transection (Fig. 6Bc).

The incidences of thermally evoked responses in LTM, WDR, and NS neurons are shown in Table 1. Typical responses of WDR neurons following heating or cooling of the RFs are shown in Fig. 7, A and B, respectively. No such responses were detected in the LTM neurons and, since only a relatively small number of NS neurons responded to thermal stimuli in IAN-treated rats, S-R functions of graded heating (Fig. 7C) and cooling stimuli (Fig. 7D) were constructed only for WDR neurons. Heat-responsive WDR neurons recorded from the naïve and IAN-transected rats increased in their firing frequency following increases in stimulus temperature (Fig. 7C) and cold-responsive neurons increased their firing following decreases in stimulus temperature (Fig. 7D). Significant depression of responses to 50°C stimulation in heat-responsive WDR neurons, and to 10°C stimulation in cold-responsive WDR neurons, were observed in IAN-transected rats at 14 days posttranssection compared with WDR neurons in naïve rats (Fig. 7, C and D).

Mechanoreceptive field (RF) size

We investigated the RF size of Vc neurons only with the RF innervated by the third branch of the trigeminal nerve. Examples of RFs for each neuron type are illustrated in Fig. 8A. WDR neurons had a large RF compared with that of LTM neurons in naïve rats, and RF size was significantly larger in all types of neurons at 14 days after IAN transection compared with naïve rats (Fig. 8, B, C, and D). The RF expansion was apparent in WDR and LTM neurons at 14 and 60 days after IAN transection.

### Table 1. Incidence of WDR, NS, and LTM neurons recorded from Vc

<table>
<thead>
<tr>
<th>Time</th>
<th>WDR</th>
<th>NS</th>
<th>LTM</th>
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<tr>
<td></td>
<td>M + H</td>
<td>M + C</td>
<td>M + H + C</td>
</tr>
<tr>
<td>naïve</td>
<td>6</td>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td>14 days</td>
<td>3</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>60 days</td>
<td>18</td>
<td>1</td>
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M, Mechanical; M + H, Mechanical + Heat; M + C, Mechanical + Cool; M + H + C, Mechanical + Heat + Cool. Total 119.
DISCUSSION

This study has documented that IAN transection produces a transient decrease in TG mandibular afferent neurons that is associated with a reduced nocifensive behavior to mechanical and heat stimulation of the mandibular skin at 3 days, but at 14 days after the IAN transection, there was enhanced nocifensive behavior to mechanical stimulation of the mental skin. These changes were also associated with alterations in the RF and response properties of Vc neurons that were especially apparent in WDR and LTM neurons as long as 60 days after IAN transection. These findings indicate that regeneration of the injured IAN may be associated with abnormal neuronal discharges and changes in mechanically evoked responses in both LTM and WDR Vc neurons, and suggest that these alterations may be involved in the development of mechanical allodynia in the cutaneous region reinnervated by the regenerated IAN.

Identification of regenerated IAN

We observed a substantial number of FG-labeled neurons in TG at 14 and 60 days after IAN transection but not at 3 days after IAN transection. The number of FG-labeled neurons detected at both 14 and 60 days after IAN transection was significantly lower compared with naive rats. We also observed that the number of small-sized TG neurons was decreased at 14 and 60 days after IAN transaction, indicating that the FG-labeled TG neurons are the result of FG transportation from the mental skin to the TG neurons. This also indicates that the transected IAN was undergoing regeneration at 14 days after transection and that the regeneration of large-diameter IAN afferents may be more advanced compared with small-diameter IAN afferents. We also could not observe any TG neurons in the root of the second branch of the trigeminal nerve labeled with FG at 14 and 60 days after IAN transection, suggesting that these neurons had not branched to innervate the deafferented mandibular region.

Nocifensive behavior

It has been reported that spinal nerve axotomy causes a marked increase in excitability of primary afferent neurons (Ma et al. 2003; Woolf and Ma 2007). The hyperexcitability of peripheral nerves is also thought to have effects within the CNS, affecting the dorsal horn (DH) neuronal activity and resulting in central sensitization of the DH neurons (Xu et al. 1995). Peripheral nerve transection is also known to enhance nocifensive behavior to mechanical and heat stimulation of the area innervated by the spared neurons adjacent to the transected nerves (Decosterd and Woolf 2000; Fukuoka et al. 2001). The lowering of the mechanical escape threshold and shortening of the heat escape latency in this model lasted >50 days. Thus sciatic nerve transection induces decrements of paw withdrawal threshold that can persist for a long period.

In the trigeminal system, it has been reported that ION ligation causes a variety of behavioral abnormalities in rats that includes face-grooming behavior and a reduction in escape
threshold to mechanical and/or heat stimulation of the face and that are thought to be involved in neuropathic pain (Imamura et al. 1997; Vos et al. 1994). Like the ION, the IAN is a pure sensory nerve and it innervates alveolar bone, mental skin, and lower lip. We could not observe any orofacial motor abnormalities in the IAN-transected rats. Food intake was also not changed after the IAN transection. On the other hand, severe motor deficits are frequently observed in rats with sciatic nerve injury. In view of these findings, the IAN is suggested to be an adequate target nerve to study sensory abnormalities after nerve injury.

We previously reported that IAN transection causes a significant decrease in the escape threshold to mechanical stimulation of the whisker pad skin innervated by the uninjured ION, which may reflect a secondary hyperalgesia induced by the spared neurons adjacent to the injured nerve (Iwata et al. 2001). Although we did not test the whisker pad region in the present study, it is thus likely that the whisker pad region was also hypersensitive to mechanical stimulation. In the present study, escape threshold to mechanical stimulation of the mental skin was significantly higher at 3 days after IAN transection, but significantly lower at 14 days and had returned to preoperative levels by 60 days after the IAN transection (Fig. 1B). On the other hand, the onset latency of head withdrawal to heat stimulation of the mental skin was significantly prolonged at 3, 14, and 60 days after IAN transection compared with naïve rats (Fig. 1D). It has been reported that Schwann cells play an important role in regeneration of injured nerves during the regeneration process and that injured unmyelinated fibers take a long time to repair compared with myelinated fibers (Bhatheja and Field 2006; Lozeron et al. 2004). These previous results together with our behavioral and TG morphological data suggest that the decrease in the number of small-diameter TG neurons may contribute to the reduced thermal responsiveness in rats in which the IAN is regenerating compared with naïve rats.

### Vc neuronal BG activity

It has been reported that peripheral nerve injury causes a marked increase in BG activity in peripheral neurons and in

| TABLE 2. Mean background activity and incidence of neurons with background activity |
|---------------------------------|-------|-------|-------|
|                                | WDR   | NS    | LTM   |
| Naïve                          | 0.15 ± 0.04 (20) | 0.17 ± 0.07 (13) | 4.05 ± 1.88 (12) |
| 14 days                        | 7.55 ± 2.44 (13) | 0.0 (7) | 5.74 ± 1.65 (12) |
| 60 days                        | 1.34 ± 0.57 (29) | 0.0 (5) | 2.40 ± 1.01 (8)  |
|                                | 100% (13/13) | 100% (8/8) | 100% (8/8) |
|                                | 58.3% (7/12) | — | 36.4% (4/11) |
|                                | 50% (7/14) | — | 66.7% (4/6) |
|                                | 41.7% (5/12) | — | 63.6% (7/11) |
|                                | 50% (7/14) | — | 33.3% (2/6) |

FIG. 5. Comparison of mean background (BG) activity of WDR, NS, and LTM neurons. The data are collected from the BG activity before the first stimulus applied to the mechanoreceptive fields (RFs). A: sequential histograms of BG activity of WDR, NS, and LTM neurons from naïve rats (a) and rats at 14 days (b) and 60 days (c) after IAN transection. B: typical example of BG activity with regular firing (a, b) and burst firing (c, d); b and d are discharge density histograms (see METHODS). C: mean BG activity of each type of neuron. ***P < 0.001.

It has been reported that peripheral nerve injury causes a marked increase in BG activity in peripheral neurons and in...
CNS neurons (Abdulla and Smith 2001; Biella et al. 2003; Gautron et al. 1990; Guilbaud et al. 1990; Iwata et al. 2001; Kajander and Bennett 1992; Laird and Bennett 1992, 1993; Miki et al. 1998b, 2000; Palecek et al. 1992a). A high level of BG activity of primary afferent neurons, which is considered to be a good indicator of the excitability of neurons, can last for a long period after nerve injury and is also thought to reflect the changes in the intracellular transduction cascade involved in the production of a variety of neuromodulators such as neuropeptides and also may affect the excitability of neurons in the CNS (Kajander and Bennett 1992; Sotgiu and Biella 2000; Tal and Eliav 1996; Wall and Devor 1983).

It has been reported that the high BG activity of WDR and LTM neurons with RFs in the ION-innervated area returns to preoperative levels at 60 days after IAN injury (Iwata et al. 2001). We also observed significant increases in the BG activity of Vc neurons at 14 and 60 days after IAN transaction; moreover, the majority of WDR and LTM neurons that showed BG activity with regular or burst firing has also been reported in primary afferent neurons after sciatic nerve or IAN transection (Ma et al. 2003; Robinson et al. 2004). It is likely that the abnormal activity in primary afferent neurons affects the excitability of the second-order neurons in the CNS (Xu et al. 1995), and so the regeneration of IAN fibers may be involved in the induction of BG activity with burst firings in Vc neurons that were demonstrated in the present study.

Vc neuronal evoked responses

Many nociceptive neurons obtained in this study responded to mechanical, cooling, and/or heating stimuli, which is consistent with features described previously for nociceptive lamina 1 neurons (e.g., Craig et al. 2001). Although this modality feature to mechanical and thermal stimuli was not different between naïve and IAN rats, suggesting that the convergence of mechanosensitive and thermosensitive afferents in Vc nociceptive neurons may not be changed with IAN regeneration, nonnoxious and noxious mechanically evoked response magnitudes of WDR neurons were significantly increased 14 days after IAN transection and by 60 days had returned to levels apparent in naïve animals. The firing frequency of nociceptive neurons directly contributes to the intensity of evoked pain following peripheral stimulation (Orstavik et al. 2003; Robinson et al. 1983; Schmelz et al. 1995) and peripheral nerve injury can cause a significant increase in mechanically evoked responses in primary afferent and second-order neurons (Chiang et al. 2005; Dubner and Bennett 1983; Kajander and Bennett 1992; Kim and Chung 1992; Laird and Bennett 1992, 1993; Ma et al. 2003; Miki et al. 1998b; Palecek et al. 1992a,b; Pitcher et al. 1999; Robinson et al. 2004; Tal and Eliav 1996). Interestingly, we also observed a significant increase in mechanically evoked responses in LTM neurons at 14 days after IAN transection that by 60 days had returned to levels observed in naïve animals. It has been
reported that the physiological properties of myelinated primary afferent nerve fibers and Vc WDR neurons are predominantly affected by the IAN injury (Iwata et al. 2001; Tsuboi et al. 2004). The A\(\delta\) fibers in the ION become more excitable following ION injury (Kitagawa et al. 2006) and A fibers in the IAN significantly increase their spontaneous activity after IAN injury and show large responses to substance P application (Robinson et al. 2004; Smith et al. 2005). It is possible that regenerated A\(\delta\) fibers are involved in the enhancement of mechanically evoked responses of WDR and LTM neurons in Vc.

In contrast to the changes in the mechanically evoked responses, heat- and cold-evoked responses of Vc neurons were significantly depressed at 14 days after IAN transection. It has been reported that thermal sensory information is predominantly conveyed by C-fibers (Price et al. 1976; Robinson et al. 1983). It has been reported that the rate of heating of the skin affects primary afferent fiber responses, with a slow rate of heating primarily involving C-fiber activation and a fast rate involving A\(\delta\)-fiber activation (Yeomans et al. 1996). The heating rate used in this study (10\(^{\circ}\)C/s) was quite fast, suggesting that the heat-responsive units that we observed may have received mainly A\(\delta\)-fiber heat-sensitive afferents. Nerve-supporting structures such as the myelin sheath are considered to be involved in regeneration of nerve fibers after nerve injury by guiding nerve sprouting from the injured site to the reinnervated regions (Inoue et al. 2004). Therefore myelinated fibers and unmyelinated fibers may have different regenerative abilities after nerve injury, suggesting that the functional properties of regenerated C-fibers in the IAN may be different from those in naïve IAN C-fibers. It has also been reported that sciatic nerve fibers may show phenotypic changes after chronic construction injury (Miki et al. 1998a; Noguchi et al. 1994, 1995). A-fibers become able to produce substance P and calcitonin gene-related peptide (CGRP) after nerve injury (Noguchi et al. 1995), and so it is probable that the regenerated IAN A-fibers may be able to produce substance P and CGRP and induces an increase in Vc neuronal activity, whereas the regenerated C-fibers may have less ability to respond to thermal stimuli.

**Vc neuronal RF properties**

It has been documented that the analysis of RF size is a good strategy to determine changes in neuronal networks in the CNS following a variety of peripheral nerve injuries (Dubner and Bennett 1983; Iwata et al. 1999, 2001; Laird and Bennett 1993;
We observed significant increases in the RF size of WDR and LTM neurons at 14 and 60 days after IAN transection; NS neurons did not show any change in RF size after IAN transection. It is thought that reinnervation of peripheral nerves adjacent to injured nerves may be involved in the expansion of the RF following nerve injury (Robinson et al. 2004). Since we examined only neuronal RFs restricted to the mental skin region innervated by the transected IAN, it is therefore very likely that the RFs that we documented indeed involved cutaneous areas reinnervated by the regenerated IAN. Several studies have reported a significant RF expansion induced after peripheral nerve injury, apparently involving neuroplastic changes in the peripheral and CNS following nerve injury (Dubner and Bennett 1983; Iwata et al. 2001; Laird and Bennett 1993; Laird and Cervero 1989; Xu et al. 1995). These findings suggest that neuroplastic changes in Vc as well as peripheral reinnervation may underlie the RF changes documented in the present study.

We observed a transient increase in the excitability of WDR and LTM neurons to mechanical stimulation of the mental skin at 14 days after IAN transection, whereas RF size had not returned to the preoperative level by 60 days after IAN transection. These findings suggest that the long-lasting RF expansion observed at 60 days after IAN transection may reflect a persistent reorganization of spatial coding properties of these Vc neurons that may not be reflected in nocifensive behavioral changes since the mechanically evoked nocifensive behavior, and Vc neuronal responses to mechanical stimulation, had returned to naïve levels by 14 days following IAN transection.

**Concluding remarks**

The present study has demonstrated that the regenerated IAN has a potent effect on brain stem neural networks in addition to its role in reinnervation of peripheral structures. The increases in excitability of WDR and LTM neurons receiving afferent inputs from the reinnervated IAN were long-lasting and were associated with changes in nocifensive behavior. These findings suggest that the neuroplastic changes in Vc WDR and LTM neurons may be involved in the development of abnormal pain following trigeminal nerve injury.
REFERENCES


