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The relationship between changes in the expression of growth associated protein–43 and functional recovery of the injured inferior alveolar nerve following transection without repair in adult rats

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ABSTRACT

Objective: The objective of this study was to analyze the changes in the expression of growth associated protein–43 (GAP-43) in trigeminal ganglions (TGs) and in the distal stumps of transected inferior alveolar nerves (IANs), and to clarify the relationship between these changes and functional recovery of the transected IAN without repair using a rat IAN axotomy model.

Material and methods: Following transection, GAP-43 expression was measured at multiple time points. The functional recovery of the transected IAN was evaluated based on the compound muscle action potentials recorded from the digastric muscle.

Results: GAP-43 expression in TGs was significantly higher at 2, 7, 14, 28, and 56 days following IAN transection compared to that in samples from sham-operated rats ($p < 0.0005$, $p < 0.0005$, $p < 0.0005$, $p = 0.007$, and $p = 0.023$, respectively). GAP-43 expression in the distal stumps of transected IANs was significantly higher at 2, 7, 14, and 28 days following IAN transection compared to that in samples taken from sham rats ($p < 0.0005$, $p < 0.0005$, $p < 0.0005$, and $p = 0.009$, respectively). GAP-43 expression in the distal stumps of transected IANs returned nearly to sham levels by day 56 following IAN transection. On days 7, 14, 28, and 56 following transection, the amplitude of the compound muscle action potential gradually increased, the latency gradually decreased, and the duration gradually increased. The amplitude, latency, and duration of the compound muscle action potentials nearly returned to sham levels on post-transection day 56.

Conclusions: Time-dependent changes in the expression of GAP-43 in both TGs and distal stumps of transected IANs without repair are synchronously consistent with the regeneration and functional recovery of the transected IAN. The recovery of the amplitude, latency, and duration of the compound muscle action potentials indicates increased myelination and increased axon density of the regenerated nerve fibers.

1. Introduction

The inferior alveolar nerve (IAN) is part of the mandibular division of the trigeminal nerve and lies within a bony canal in the mandible in close proximity to the root tips of the mandibular molar teeth. The majority of the sensory neurons of the IAN are localized to the trigeminal ganglion (TG). The IAN can be damaged during the removal of the third molars or as a result of orthognathic surgery or mandibular fractures (Rustemeyer and Gregersen, 2012; Qinyong et al., 2014; Hillerup, 2008). Hillerup reported a significant spontaneous recovery in 66% of iatrogenic IAN injuries associated with the removal of the third molars (Hillerup, 2008). A review article showed 96%–98% sensory recovery of iatrogenic IAN injuries after bilateral sagittal split osteotomy (Antonarakis and Christou,

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2012). The incidence of post-traumatic IAN neurosensory dysfunction was reported to be 46% for mandibular fractures, and the incidence of persistent IAN sensory dysfunction following surgical treatment was reported to be 7.7% (Schultze-Mosgau et al., 1999).

Previous experimental studies have examined the regeneration of injured IANs and the reinnervation of the teeth after IAN ligation, crushing, and transection injury (Elcock et al., 2001a, 2001b; Long et al., 1998; Berger and Byers, 1983; Fristad et al., 1995; Iijima et al., 2003; Teramoto et al., 2013). The results of our previous study suggest that axon guidance cues such as Slit1/Robo2 signaling contribute to the regulation of the regeneration of the transected IAN without repair (Ceber et al., 2015). However, the microenvironmental mechanisms underlying the spontaneous regeneration and functional recovery of the transected IAN without repair are still largely unknown. Improving our understanding of the mechanisms regulating IAN regeneration is critical to the development of new therapeutic strategies aimed at accelerating the regeneration and functional recovery of injured peripheral nerves.

Peripheral nerve injury induces profound structural, biochemical, and physiological changes in both primary sensory neurons and peripheral nerves (Marchena et al., 1998; Bloechlinger et al., 2004; Teramoto et al., 2013). A key component of successful axonal regeneration in adult neurons is the induction of the neuron's regenerative competence (Chong et al., 1994; Teramoto et al., 2013). Growth associated protein–43 (GAP-43) expression is correlated with neuronal regenerative competence after axotomy (Yuan et al., 2009). The expression of GAP-43 in primary neurons increases as a result of neuronal damage, and this increase is terminated when the regeneration process is complete (Teramoto et al., 2013). Moreover, in the chronically denervated distal stump of the transected rat sciatic nerve, maximal GAP-43 expression was found in the absence of regenerating axons 4 weeks after transection (Curtis et al., 1992). However, longer-term GAP-43 expression has not been investigated.

Previous studies have shown a rapid increase in GAP-43 mRNA levels in cell bodies and proximal axon segments in motor neurons after axotomy (Palacios et al., 1994; Tetzlaff et al., 1991). A recent study has shown that IAN transection causes changes in GAP-43 expression in the TG (Teramoto et al., 2013). However, changes in the expression of GAP-43 in the distal stumps of transected, unrepaired peripheral nerves have not been evaluated. The regenerating proximal stumps of injured peripheral nerves elongate if they find favorable terrain. In the absence of a guiding structure such as a distal nerve stump, regenerating axons cannot elongate. Thus, determining whether there are changes in the expression of GAP-43 in the distal stumps of injured peripheral nerves may be important.

The objective of this study was to analyze changes in the expression patterns of GAP-43 both in TGs and in the distal stumps of transected IANs. We evaluated the relationship between these changes and the spontaneous functional recovery of the transected IAN without repair using a rat IAN transection model. The functional recovery of the transected IAN was evaluated using the compound muscle action potentials (CMAPs) recorded from the digastric muscle.

2. Material and methods

2.1. Animal surgery and tissue preparation

The experimental protocol used in this study was reviewed and approved by the local animal ethics committee in accordance with the National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals. A total of 74 male Sprague–Dawley rats weighing 250–300 g were used. For immunohistochemical analysis, 42 rats were anesthetized intraperitoneally with sodium pentobarbital (50 mg/kg). This surgical procedure was described in our previous study (Ceber et al., 2015). To expose each rat's right IAN, an extraoral horizontal incision approximately 10 mm in length was made in the skin covering the masseter muscle; the masseter muscle was partially separated, and the alveolar bone was exposed. The trajectory of the IAN was clearly identified through the transparency of the covering bone medial to the bony prominence in the body of the mandible. A small (approximately 0.5 × 0.5 cm) window in the outer part of the external cortex overlying the mandibular canal just above the angle of the mandible was removed slightly inferior to the prominence using a slowly rotating round dental bur under isotonic saline rinse until the nerve was visible and could be separated from its surrounding blood vessels. To preserve the integrity of the mandibular cortex medially and to prevent the complete penetration of both medial and lateral bony cortices, which are thin in the area distal to the bony prominence, the bone window was removed just mesial (anterior) to the bony prominence (Kassab et al., 2013). The inferior, superior, and medial circumference of the mandibular canal was preserved as a natural conduit for the regenerating axons. Using an operating microscope (Zeiss SV6/11; Carl Zeiss, Oberkochen, Germany), the exposed nerve was manipulated with fine microsurgical forceps at a magnification of ×16 to ×25. The IAN was carefully separated from the surrounding blood vessels and lifted from the mandibular canal, without any externally visible injury, by curved, angulated, thin glass rods. Then, the IAN was transected completely with a micro-scissors, without any further damage, using a meticulous and pressureless cutting technique. The cut ends of the transected IAN were immediately brought back into direct contact with each other and to their original position in the mandibular canal without any discernible gap between the cut ends and without any rotational error. The muscle and skin were then closed and sutured. The rats were allowed to recover and to survived for 2, 7, 14, 28, or 56 days (n = 7 for each period). Rats that did not undergo IAN transection were used as a sham treated group (n = 7); in this group, the skin was incised, the masseter separated, and the mandibular canal fenestrated. All rats were given the antibiotic penicillin G procaine (Phoenix Pharmaceutical, Inc., St. Joseph, MO) the day before, the day of, and the day after surgery (30,000 U/250 kg). After transection, rats were fed powdered food. Transected rats at 2, 7, 14, 28, and 56 days following transection and sham-treated rats at day 0 (n = 7 per group) were sacrificed. Rats were sacrificed using an overdose of sodium pentobarbital (>50 mg/kg, intraperitoneally). The TG and hemimandible of the transection site were quickly harvested and immediately flash-frozen in dry ice in preparation for immunohistochemical analysis. Hard tissues from the right sides of the harvested hemimandibles were decalcified for 3 weeks using 10% ethylenediaminetetraacetic acid (EDTA) in 0.1 M phosphate buffer. The right sides of both the TGs and decalcified hemimandibles were stored at −80 °C until sectioning.

For quantitative analysis of the CMAPs, unilateral axotomy of the right IAN was performed in 32 adult rats. CMAPs recorded from the digastric muscle were measured in sham-operated rats (n = 4) and on days 7, 14, 28, and 56 following the IAN transectionsq (n = 7 per time point).

2.2. Immunohistochemistry of the TG and hemimandible

The TG tissue that was collected for immunohistochemical study was frozen in dry ice and cut into 20-μm sections using a cryostat (Leica Instruments, Wetzlar, Germany). To ensure the examination of samples from the distal IAN stump, the hemimandible distal to the removed bone window was sectioned longitudinally,

and the harvested distal portions of the hemimandibles were decalcified. The decalcified hemimandibles were prefixed in 10% formaldehyde solution, decalcified with 10% EDTA in 0.1 M phosphate buffer for 3 weeks and embedded in paraffin blocks. Sections from the distal IAN stump (5 μm) were cut using a microtome and deparaffinized. TG and distal IAN stump sections were fixed in 4% paraformaldehyde in 0.1 M phosphate-buffered saline solution, rinsed, pretreated for antigen retrieval with 0.1 M phosphate-buffered saline containing 1% sodium dodecyl sulfate, rinsed, and immersed for 1 h in 0.1 M phosphate-buffered saline containing 0.3% Triton X–100 and 10% normal donkey or 10% normal goat serum. The sections were incubated overnight at 4 °C with polyclonal rabbit anti-GAP43 (#NB300-143SS; Novus Biologicals, USA), monoclonal mouse anti-S100α/β (#sc130110; Santa Cruz) and monoclonal mouse anti-glial fibrillary acidic protein (GFAP) (#3670; Cell Signaling) (diluted 1:100 in 0.1 M phosphate-buffered saline). These antibodies were detected with Alexa Fluor 633 goat anti-mouse (#A21050; Invitrogen), Alexa Fluor 555 goat anti-rabbit (A21428; Invitrogen), and Alexa Fluor 488 goat anti-rabbit (A11008; Invitrogen) secondary antibodies, respectively. Tissue sections were counterstained with 40–6-diamidino–2-phenylindole (DAPI) and were evaluated under a fluorescence microscope (Nikon Eclipse N*i*) connected to a CCD camera (Nikon DS-Fi1c). To quantify GAP-43 expression, images (original magnification ×400) of two defined regions of interest per tissue, each measuring 62.500 μm^2, were obtained (Nikon Eclipse Ni), and the intensity of fluorescence was analyzed using Metamorph Image Analysis software version 6.3 (Molecular Devices, Sunnyvale, CA). Five sections from each animal were processed. The mean fluorescence intensity values were calculated using data from the five sections that were analyzed. Images were analyzed by an independent assistant who was blinded to the conditions of the study.

2.3. Electrophysiological analysis of CMAPs

To evaluate the functional recovery of the transected IAN, CMAPs recorded from the digastric muscle were measured before surgery (sham-operated, n = 4) and 7, 14, 28, and 56 days following the IAN transection (n = 7 per time point). Each rat was deeply anesthetized, and the trachea was cannulated for respiration. Continuous inhalation of 0.5%–1.0% halothane (Fluothane; Astra Zeneca, Turkey) was used to maintain a stable depth of anesthesia. Next, the right mandibular canal was opened again, and a pair of silver wire electrodes (interelectrode distance = 0.5 mm; diameter = 0.2 mm) was inserted into the right mandibular canal (0.5 mm proximal or distal from the transection site) to electrically stimulate the right IAN. The electrodes were fixed to the adjacent mandibular bone with a light-cured dental resin. A hook-type enamel steel wire electrode was implanted into the digastric muscle along the direction of the muscle fibers to record its evoked electromyographic (EMG) activity. The distal and proximal stumps of the IAN adjacent to the transection site were electrically stimulated, and the evoked EMG activity was recorded from the digastric muscle. Stimulation pulses at a supramaximal level (duration = 0.2 ms; intensity = 2 mA) were delivered to the nerves for a total of 20 repetitions of each resulting signal at 0.2 Hz by means of a stimulus isolator (DS4; Biphasic Stimulus Isolator, Warner Instruments, USA). The signals were passed through band-pass filters of 100 to 2000 Hz, with the automatic artifact reject activated during the test. After the electrophysiological recordings, the rats were sacrificed using an overdose of sodium pentobarbital. The digastric muscle was dissected to confirm the location of the electrodes.

All signals were stored on a personal computer at a sampling rate of 5000 s^{-1} and were later analyzed offline using Spike2 software for Windows, version 7.05 (Cambridge Electronic Design Limited, Cambridge, UK). The amplitude, duration and latency were measured as parameters of the CMAPs. The amplitude was measured as the difference in voltage between the maximum and baseline CMAP amplitudes. The latency was measured as the time interval between the stimulus and the onset. The duration was calculated as the time between the two points where the baseline was crossed by the rising and declining CMAP curves. The mean values of these parameters are reported as averages of the reflex responses after 20 consecutive stimuli in each rat at each time point.

2.4. Statistical analysis

All data were analyzed using PASW Statistics 18 for Windows statistical software (SPSS Inc., Chicago, IL, USA). Significant differences between time points were determined using the Kruskal–Wallis test. All data were reported as the mean ± SD; p values of less than 0.05 were considered statistically significant.

3. Results

3.1. GAP-43 expression in the trigeminal ganglion

Immunostaining revealed that GAP-43 expression in the TGs was significantly higher at 2, 7, 14, 28, and 56 days following IAN transection compared to the samples from sham-operated rats ($p < 0.0005$, $p < 0.0005$, $p < 0.0005$, $p = 0.007$, and $p = 0.023$, respectively) (Fig. 1a). GAP-43 was expressed at low intensity in the TGs of sham rats. Significantly increased GAP-43 expression was observed in the TGs 2 days post-transection. At day 7 post-transection, the expression intensity gradually began to decrease but did not return to sham levels by day 56 following transection. To further determine whether the upregulation of GAP-43 was attributable to satellite glial cells, double immunostaining of GAP-43 either with S-100 or GFAP, both of which are markers of satellite glial cells and Schwann cells in TG, was performed. As shown in Fig. 1b, c, and d, GAP-43– immunostained cells were co-localized with satellite glial cells surrounding the neurons, indicating that IAN transection activates satellite glial cells in TG to release GAP-43.

3.2. GAP-43 expression in the distal IAN stump

Immunostaining revealed that GAP-43 expression in the distal stumps of transected IANs was significantly higher at 2, 7, 14, and 28 days following IAN transection compared to the samples taken from sham-operated rats ($^{***}p < 0.0005$, $^{***}p < 0.0005$, $^{***}p < 0.0005$, and $^{**}p = 0.009$, respectively) (Fig. 2a). GAP-43 was expressed at low intensity in the IANs of sham rats. Significantly increased GAP-43 expression was measured in the distal stumps of transected IANs 2 days post-transection. At day 7 post-transection, GAP-43 expression gradually began to decrease and nearly returned to sham levels by day 56 following IAN transection. Double immunostaining revealed that GAP-43 and S100 were co-localized in some non-neuronal cells including reactive Schwann cells in the distal stumps of transected IANs, which underwent Wallerian degeneration on day 2 following axotomy (Fig. 2b, c, and d). It can be inferred that IAN transection activates Schwann cells in the distal stumps of transected IANs to release GAP-43.

3.3. CMAP recordings

Figs. 3–5 show the time-dependent changes in the amplitude, latency, and duration of the CMAP corresponding to distal and proximal stimulation after IAN transection. With distal stimulation,

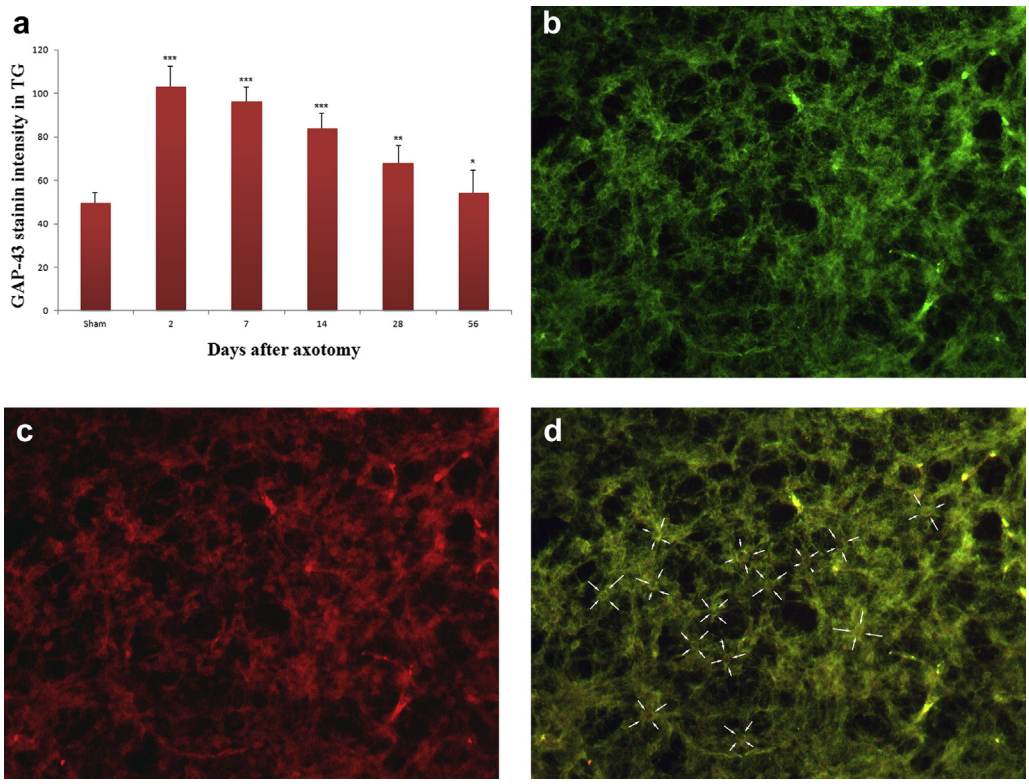

Fig. 1. (a) The fluorescence intensity of GAP-43 in TGs was significantly elevated at 2, 7, 14, 28, and 56 days following IAN axotomy compared to levels insham-operated rats (***$p < 0.0005$, ***$p < 0.0005$, ***$p < 0.0005$, **$p = 0.007$, and *$p = 0.023$, respectively); (b) GAP-43 immunofluorescence staining of injured TG; (c) GFAP immunofluorescence staining of injured TG; (d) double immunofluorescence staining shows that GAP-43 and GFAP were colocalized in satellite glial cells of the TG; arrows indicate double staining. Scale bar = 50 μm.

on days 7, 14, 28, and 56 following transection, the amplitude of the CMAP (sham, 2.550 ± 0.623 mV; day 7, 0.491 ± 0.125 mV; day 14, 0.801 ± 0.245 mV; day 28, 1.447 ± 0.345 mV; and day 56, 2.351 ± 0.533 mV) gradually increased, the latency (sham, 1.504 ± 0.375 ms; day 7, 5.123 ± 1.158 ms; day 14, 4.413 ± 0.975 ms; day 28, 3.307 ± 0.825 ms; and day 56, 1.684 ± 0.412 ms) gradually decreased, and the duration (sham, 2.741 ± 0.656 ms; day 7, 1.231 ± 0.285 ms; day 14, 1.444 ± 0.314 ms; day 28, 1.830 ± 0.356 ms; and day 56, 2.638 ± 0.573 ms) gradually increased. With distal stimulation, on day 56 after axotomy, compared with the sham group, the amplitude was slightly lower (2.351 ± 0.533 mV vs. 2.550 ± 0.623 mV), the duration was slightly shortened (2.638 ± 0.573 ms vs. 2.741 ± 0.656 ms), and the latency was slightly extended (1.684 ± 0.412 ms vs. 1.504 ± 0.375 ms). With proximal stimulation, on days 7, 14, 28, and 56 following axotomy, the amplitude of the CMAP (sham, 2.456 ± 0.575 mV; day 7, 0.385 ± 0.112 mV; day 14, 0.755 ± 0.217 mV; day 28, 1.386 ± 0.286 mV; and day 56, 2.295 ± 0.485 mV) gradually increased, the latency (sham, 1.485 ± 0.355 ms; day 7, 5.236 ± 1.146 ms; day 14, 4.683 ± 0.845 ms; day 28, 3.275 ± 0.765 ms; and day 56, 1.592 ± 0.376 ms) gradually decreased, and the duration (sham, 2.675 ± 0.583 ms; day 7, 0.675 ± 0.233 ms; day 14, 1.078 ± 0.294 ms; day 28, 1.525 ± 0.315 ms; and day 56, 2.527 ± 0.525 ms) gradually increased. With proximal stimulation, on day 56 after axotomy, compared with the sham group, the amplitude was slightly diminished (2.295 ± 0.485 mV vs. 2.456 ± 0.575 mV), the duration slightly shortened (2.527 ± 0.525 ms vs. 2.675 ± 0.583 ms), and the latency slightly extended (1.592 ± 0.376 ms vs. 1.485 ± 0.355 ms). No significant difference was found between the distal and proximal stimulation results (amplitude, $p = 0.083$; latency, $p = 0.12$; duration, $p = 0.075$). Typical waveforms of CMAPs recorded from the digastric muscle are described in Fig. 5.

4. Discussion

Although the peripheral nervous system has the ability to regenerate injured axons leading to functional recovery, transection injuries typically require microneural repair. Previous experimental studies have examined the regeneration of injured IANs and the reinnervation of the teeth after IAN transection injury (Berger and Byers, 1983; Fristad et al., 1995; Iijima et al., 2003; Teramoto et al., 2013). Many of these unrepaired experimental IAN transection injuries may regenerate. However, the mechanisms underlying the spontaneous regeneration and functional recovery of transected IANs without repair are still largely unknown.

Axonal injury in the peripheral nervous system can have a strong impact on neurons and surrounding non-neuronal cells. Successful regeneration in the mammalian peripheral nervous system requires a variety of changes in the expression of growth-associated proteins (Yuan et al., 2009; Benowitz and Routtenberg, 1997). This regeneration-associated protein expression is believed to enhance the growth potential of injured neurons. Although many molecules appear to correlate with a neuron's regenerative competence following transection, the most prominent molecule involved in regeneration is GAP-43 (a marker for regenerating nerve fibers) (Yuan et al., 2009). The increased synthesis of GAP-43 correlates with an increased propensity toward regeneration in neurons (Yuan et al., 2009; Benowitz and Routtenberg, 1997; Ikegami et al., 2005; Zhang et al., 2005). GAP-43 synthesis increases during neuron growth. GAP-43 protein moves to the periphery of regenerating axons via axonal transport and ultimately

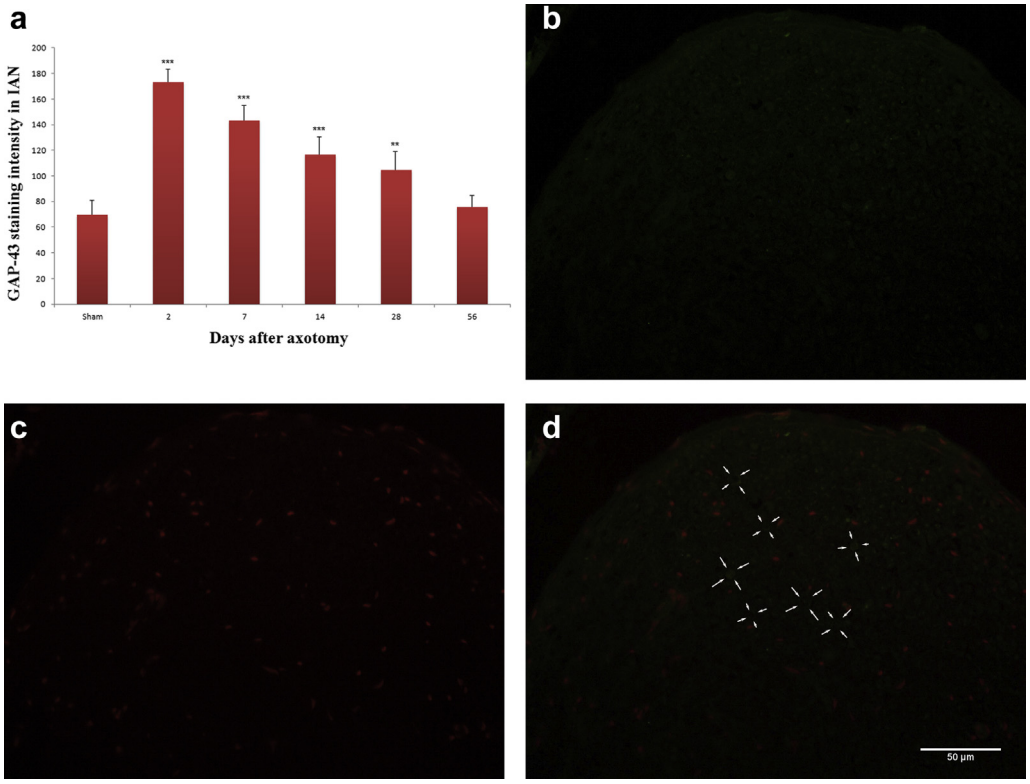

Fig. 2. (a) The fluorescence intensity of GAP-43 in the distal stumps of the axotomized IANs was significantly elevated at 2, 7, 14, 28, and 56 days following IAN axotomy compared to levels in sham-operated rats (***$p < 0.0005$, ***$p < 0.0005$, ***$p < 0.0005$, **$p = 0.007$, and *$p = 0.023$, respectively); (b) GAP-43 immunofluorescence staining of axotomized IAN; (c) S100 immunofluorescence staining of axotomized IAN; (d) double immunofluorescence staining shows that GAP-43 and S100 were colocalized in Schwann cells of the distal stump of the axotomized IAN; arrows indicate double staining. Scale bar = 50 µm.

concentrates in axonal growth cones (Benowitz and Routtenberg, 1997; Yin et al., 2003; Ikegami et al., 2005; Zhang et al., 2005). After axonal growth is complete, levels of GAP-43 decline in primary neurons. Previous developmental studies have reported that GAP-43 expression is downregulated after nerve fibers reach their targets, and GAP-43 is normally found at low levels in adults (Benowitz and Routtenberg, 1997; Oestreicher et al., 1997). After nerve injury in adult rodents (rabbits, mice, rats), GAP-43 is re-expressed and rapidly transported along the axons of successfully regenerating neurons (Skene and Willard, 1981; Starkey et al., 2009; Woolf et al., 1990). GAP-43 is usually labeled to identify growing axonal profiles (Ma et al., 2011). A previous study showed that GAP-43 immunoreactivity could be observed in adult rat avulsed spinal motoneurons as early as 1 day following cervical root avulsion, increases between 3 and 7 days post-avulsion, and peaks at 2 weeks post-injury (Yuan et al., 2009). This up-regulation of GAP-43 was coincident with axonal regeneration in reimplanted ventral roots and nerve in adult rats. GAP-43 immunoreactivity is not found in neonatal avulsed motor neurons at any examined post-injury time points. This failure of the up-regulation of GAP-43 was coincident with a lack of axonal regeneration in reimplanted nerves in neonatal rats (Yuan et al., 2009). However, root avulsion is different from nerve transection.

Recent studies have evaluated GAP-43 expression in primary neurons and in the proximal stumps of injured peripheral nerves after IAN ligation, crushing, and transection injury prior to regeneration (Elcock et al., 2001a, 2001b; Long et al., 1998; Berger and Byers, 1983; Fristad et al., 1995; Iijima et al., 2003; Teramoto et al., 2013). GAP-43 expression has also been evaluated in the proximal and distal stumps of transected sciatic nerves combined with a polyethylene nerve guide (Bryan et al., 2012). However, changes in the expression of GAP-43 in the distal stumps of unrepaired transected peripheral nerves have not been evaluated. The regenerating proximal stumps of injured nerves elongate if they find favorable terrain. In the absence of a guiding structure such as a distal nerve stump, regenerating axons take a tortuous course and form a neuroma. Previous studies have shown that axons regenerate from the proximal stumps of transected nerves in response to molecular secretions from the distal stumps of the injured nerves (Lundborg et al., 1982; Danielsen et al., 1983). Therefore, understanding whether changes in the expression of GAP-43 occur in the distal stumps of injured peripheral nerves may be important.

In the current study, GAP-43 was weakly expressed in samples from TGs and distal IAN stumps of sham-operated rats. GAP-43 expression in TGs and distal IAN stumps was significantly elevated between 2 and 28 days after transection. At 56 days after transection, GAP-43 expression in TGs and distal IAN stumps nearly returned to the corresponding levels in sham rats. GAP-43 expression in samples from the TGs and distal IAN stumps of transected rats peaked on day 2 after transection. Interestingly, the increased GAP-43 expression in the samples from TGs was also observed in the samples from the distal stumps of IANs at the same time points. The increased expression of GAP-43 in both TGs and distal IAN stumps suggests a role for GAP-43 in the regeneration of transected IANs. Changes in GAP-43 expression may reflect the induction of a nerve regenerative program by injured primary sensory neurons of the TG.

Our findings indicate that the changes in GAP-43 expression in TG neurons following IAN transection are similar to the changes observed in DRG neurons following transection. GAP-43 is

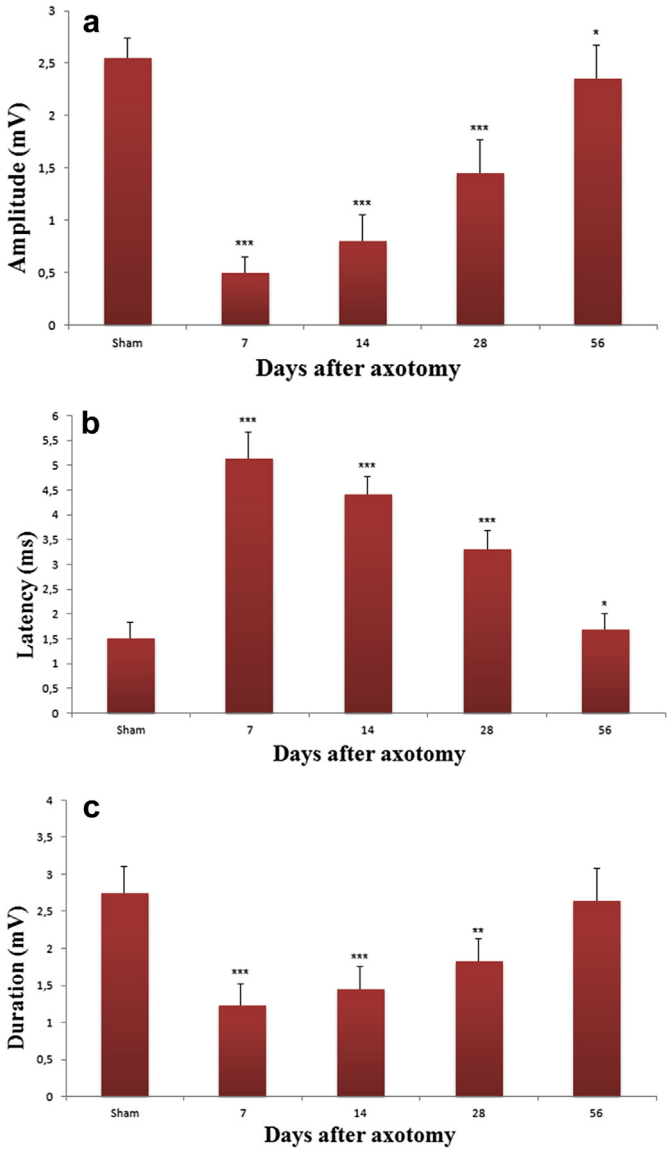

Fig. 3. Time-dependent changes in the (a) amplitude, (b) latency, and (c) duration of the CMAPs after IAN axotomy for stimulation of the distal stumps of the IAN adjacent to the transection site.

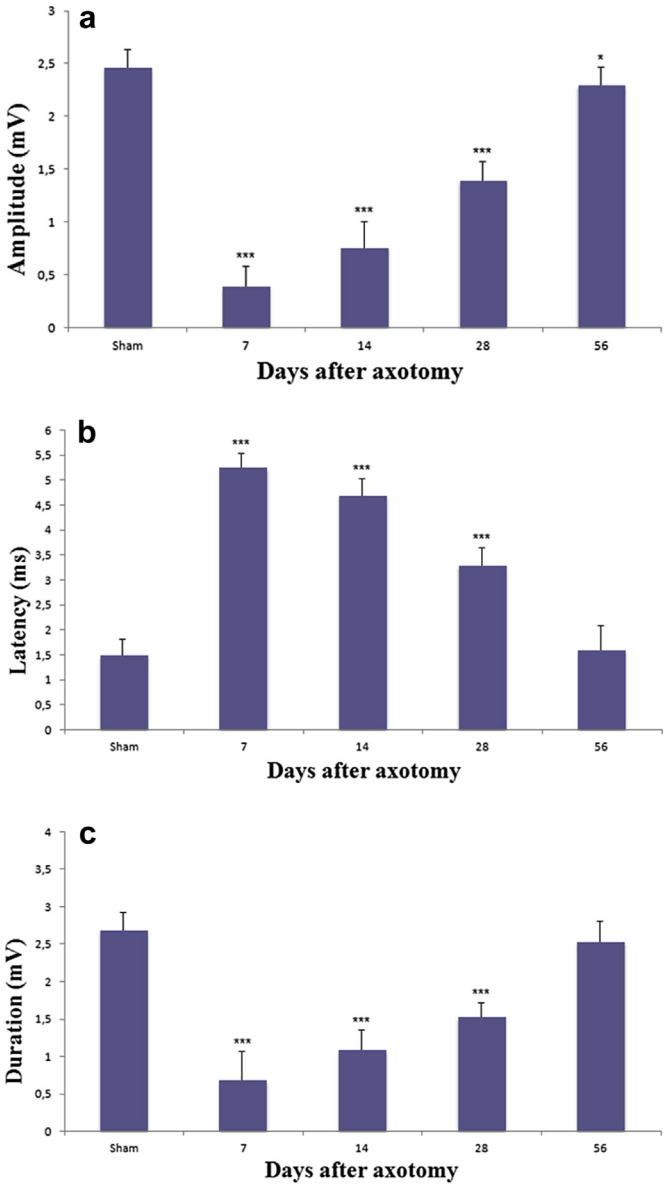

Fig. 4. Time-dependent changes in the (a) amplitude, (b) latency, and (c) duration of the CMAPs after IAN axotomy for stimulation of the proximal stumps of the IAN adjacent to the transection site.

expressed in DRG neurons 2 days after transection of the sciatic nerve and persists for 80 days (Liabotis and Schreyer, 1995). GAP-43 expression decreases after peripheral nerve regeneration is complete (Reynolds et al., 1991; Coggeshall et al., 1991). These findings suggest that the neural mechanisms underlying nerve regeneration following IAN transection or sciatic nerve injury are similar. Therefore, it is not clear why the microenvironmental conditions for the IAN as a cranial nerve are more favorable to regeneration than are spinal peripheral nerves. The IAN is unique among the cranial nerve branches because of its long intracanalicular course. The presence of guiding structures around injured nerves is very important for axonal sprouting and nerve regeneration (Teramoto et al., 2013). Conceivably, the mandibular canal might provide a natural conduit in IAN lesions, as in neurotmesis after experimental transection, and prevents the retraction of nerve ends, thereby maintaining their apposition. The mandibular canal thus may be suitable as a microenvironment for the diffusion of neurotrophic and neurotropic factors secreted by the injured nerve stump and may prevent the ingrowth of fibrous tissue into a nerve gap (Mohammadi et al., 2014).

IAN function was electrophysiologically evaluated based on the jaw-opening reflex (JOR) before and after axotomy. In contrast to its use in lower mammals, this electrophysiological method plays no role in clinical practice due to the different anatomic structures in humans. In humans, a disynaptic reflex through the supratrigeminal nucleus inhibits the jaw closing muscles completely, rather than directly exciting the jaw opening muscles to avoid the threat of damage. In lower mammals, the JOR is an actual trigeminal reflex that involves trigeminal afferents (IAN) and efferents (masticatory nerve) in its reflex arc. The primary trigeminal afferents synapse with excitatory sensory neurons of the spinal trigeminal complex. These neurons project to excite digastric motorneurons (Tsai et al., 1999). Even under general anesthesia, the JOR can easily be evoked by the electrical stimulation of the IAN afferents. Thus, the reflex seems to be an adequate model for

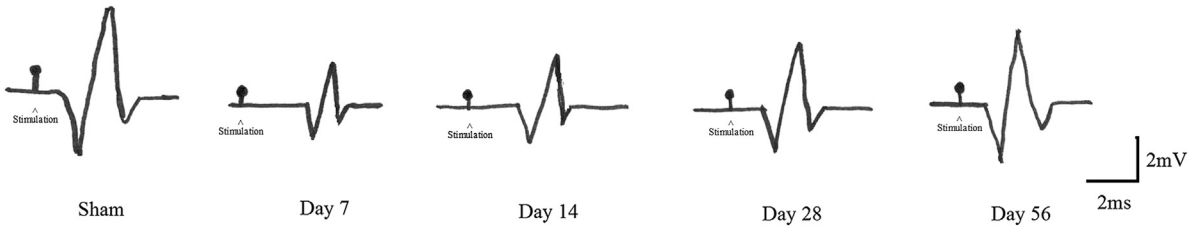

Fig. 5. Representative waveforms of digastric muscle action potentials following stimulation of the distal stumps of the IANs adjacent to the transection site inferior alveolar nerve.

investigating alterations to its excitability following transection that indicates the impairment of IAN function. In the present study, we used the parameters of the CMAP to objectively assess the changes in nerve regeneration over time in rats. The amplitude, latency, and duration of the CMAP nearly returned to their levels in sham rats on post-transection day 56.

The recovery of the parameters of the CMAP was favorable, and the results indicate restored myelination and an increased density of regenerated axons.

Our results show that there were almost identical values of amplitude, latency, and duration of CAPDM after proximal and distal stimulation through increasing postoperative intervals. Because the distance between stimulating and recording sites is distinct in distal and proximal stimulation, distance-dependent results, such as latency and duration of CAPDM, may cause distortion. Another important topic to discuss regarding evaluation of the functional recovery is the intensity of the stimulus. Amplitude is directly proportional to the intact or regenerated axonal fibers that the stimulus depolarizes. Therefore, below-normal amplitude may occur because of a low stimulus. Hence, it is necessary to establish a standard of stimulus intensity. In this sense, the supramaximal stimulus, which is the minimum intensity necessary to depolarize all the nerve fibers is ideal. Teramoto et al. used a single rectangular pulse of 0.01–1.0 mA, different from the supramaximal stimulus of 2 mA in our current study. Furthermore, stimulation pulses were delivered to the nerves for a total of 20 repetitions of each resulting signal in the current study. Taking into account the stimulation parameters used in our study, it is likely that amplitude of CAPDM may not increase when the stimulus intensity is more increased, and repetitive IAN stimulation may alter CAPDM values. These factors may cause inconsistency in CAPDM values in our study.

After IAN transection, reinnervation of denervated tissues can be classified into two principal areas: collateral reinnervation by adjacent non-injured nerves, and regeneration of the transected nerve. The first mandibular molar in rats is thought to be innervated via the IAN, whereas the second and third molars may exhibit collateral innervation mainly via the lingual nerve (Berger and Byers, 1983). However, subsequent to IAN transection, the first molar may become collaterally reinnervated by nerve branches from the lingual side perforating the alveolar bone. In the current study, we stimulated the distal stump of the IAN adjacent to the transection site and recorded the response in the digastric muscle. Thus, we were able to measure axonal regeneration across the lesion site. However, the possibility of collateral sprouting from adjacent, non-injured nerves cannot be excluded. Thus the recorded potentials in the digastric muscles were likely conducted through nerve fibers passing the transsection site. However, whether the IAN stimulation (either from a proximal or distal location) precludes the activation of adjacent nerve fibers is not finally known. The JOR might be elicited by stimulating nerve branches other than the IAN, a circumstance that is a principal methodological shortcoming.

The molecular mechanisms that control the dynamic regulation of GAP-43 signaling following IAN transection are not well understood. This study and previous studies have led to several hypotheses regarding the interactions of GAP-43 between TGs and IANs. The most appealing explanation for the concurrent expression of GAP-43 as early as 2 days post-transsection in both the TGs and the distal IAN stumps is a simultaneous synthesis in the two locations.

The concurrent expression of GAP-43 in the TGs and the distal stumps of transected IANs suggests that neuroendocrine-like reactions may be a mechanism for regulating GAP-43 signaling during IAN regeneration.

In the current study, the stumps of the transected IANs were left unrepaired. In the early follow up at 2 days post-transection, a spontaneous reconnection could not be confirmed, since CAPDM recordings at this time point were not included in the experimental series. At later time points, the CAPDM is indicative of axonal regeneration and reconnection, such that axonal transport mechanisms cannot be excluded as explanations for GAP 43 down-regulation. This hypothesis has not been formally tested.

There are limitations to our study design that must be considered. First, the sample size for each time point is small, and the results only show trends in changes to GAP-43 expression at multiple post-transection time points. The observed changes in the expression of GAP-43 should be verified using a larger sample size. Second, this is a preliminary study of spontaneous regeneration and functional recovery of transected IANs without repair. Therefore, additional groups with nerve coaptations and interpositional nerve grafts were not used. We will use these additional groups in future studies.

5. Conclusion

The recovery of the amplitude, latency, and duration of the CMAPs indicates increased myelination and an increased axon density in regenerated nerve fibers. Further studies are needed to clarify the role of GAP-43 in IAN regeneration.

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References

Antonarakis GS, Christou P: Quantitative evaluation of neurosensory disturbance after bilateral sagittal split osteotomy using Semmes-Weinstein monofilaments: a systematic review. J Oral Maxillofac Surg 70(12): 2752–2760, 2012

Benowitz LI, Routtenberg A: GAP-43: an intrinsic determinant of neuronal development and plasticity. Trends Neurosci 20: 84–91, 1997

Berger RL, Byers MR: Dental nerve regeneration in rats. II Autoradiographic studies of nerve regeneration to molar pulp and dentin. Pain 15: 359–375, 1983

Bloechlinger S, Karchewski LA, Woolf CJ: Dynamic changes in glypican-1 expression in dorsal root ganglion neurons after peripheral and central axonal injury. Eur J Neurosci 19: 1119–1132, 2004
Bryan DJ, Litchfield CR, Manchio JV, Logvinenko T, Holway AH, Austin J, et al: Spatiotemporal expression profiling of proteins in rat sciatic nerve regeneration using reverse phase protein arrays. Proteome Sci 10: 9, 2012
Ceber M, Mihmanli A, Kilic U, Sener U, Yuksek A, Durak MA, et al: Changes in expression of Slit1 and its receptor Robo2 in trigeminal ganglion and inferior alveolar nerve following inferior alveolar nerve axotomy in adult rats: a pilot study. Int J Oral Maxillofac Surg 44: 518–527, 2015
Chong MS, Reynolds ML, Irwin N, Coggeshall RE, Emson PC, Benowitz LI, et al: GAP-43 expression in primary sensory neurons following central axotomy. J Neurosci 14: 4375–4384, 1994
Coggeshall RE, Reynolds ML, Woolf CJ: Distribution of the growth associated protein GAP-43 in the central processes of axotomized primary afferents in the adult rat spinal cord; presence of growth cone-like structures. Neurosci Lett 131: 37–41, 1991
Curtis R, Stewart HJ, Hall SM, Wilkin GP, Mirsky R, Jessen KR: GAP-43 is expressed by nonmyelin-forming Schwann cells of the peripheral nervous system. J Cell Biol 116: 1455–1464, 1992
Danielsen N, Dahlin LB, Lee YF, Lundborg G: Axonal growth in mesothelial chambers. The role of the distal nerve segment. Scand J Plast Reconstr Surg 17: 119–125, 1983
Elcock C, Boissonade FM, Robinson PP: Neuropeptide expression in the ferret trigeminal ganglion following ligation of the inferior alveolar nerve. Arch Oral Biol 46: 729–743, 2001a
Elcock C, Boissonade FM, Robinson PP: Changes in neuropeptide expression in the trigeminal ganglion following inferior alveolar nerve section in the ferret. Neuroscience 102: 655–667, 2001b
Fristad I, Heyeras KJ, Jonsson R, Kvinnsland IH: Effects of IAN axotomy on immune cells and nerve fibres in young rat molars. Arch Oral Biol 40: 1053–1062, 1995
Hillerup S: Iatrogenic injury to the inferior alveolar nerve: etiology, signs and symptoms, and observations on recovery. Int J Oral Maxillofac Surg 37: 704–709, 2008
Iijima K, Harada F, Hanada K, Nozawa-Inoue K, Aita M, Atsumi Y, et al: Temporal expression of immunoreactivity for heat shock protein 25 (Hsp25) in the rat periodontal ligament following transection of the inferior alveolar nerve. Brain Res 979: 146–152, 2003
Ikegami T, Nakamura M, Yamane J, Katoh H, Okada S, Iwanami A, et al: Chondroitinase ABC combined with neural stem/progenitor cell transplantation enhances graft cell migration and outgrowth of growth associated protein–43-positive fibers after rat spinal cord injury. Eur J Neurosci 22: 3036–3046, 2005
Kassab A, Hage M, Jabbur SJ, Chidiac JJ: Modified technique for the exposure of the inferior alveolar nerve in rats. J Pharmacol Toxicol Methods 67: 182–186, 2013
Liabotis S, Schreyer DJ: Magnitude of GAP-43 induction following peripheral axotomy of adult rat dorsal root ganglion neurons is independent of lesion distance. Exp Neurol 135: 28–35, 1995
Long A, Bongenhielm U, Boissonade FMB, Fried K, Robinson PP: Neuropeptide immunoreactivity in ligature-induced neuromas of the inferior alveolar nerve in the ferret. Brain Res 791: 263–270, 1998
Lundborg G, Dahlin LB, Danielsen N, Gelberman RH, Longo FM, Powell HC, et al: Nerve regeneration in silicone chambers: influence of gap length and of distal stump components. Exp Neurol 76: 361–375, 1982
Ma CH, Omura T, Cobos EJ, Latrémolière A, Ghasemlou N, Brenner GJ, et al: Accelerating axonal growth promotes motor recovery after peripheral nerve injury in mice. J Clin Invest 121: 4332–4347, 2011
Marchena JM, Padwa BL, Kaban LB: Sensory abnormalities associated with mandibular fractures: incidence and natural history. J Oral Maxillofac Surg 56: 822–825, 1998
Mohammadi R, Yadegarazadi MJ, Amini K: Peripheral nerve regeneration following transection injury to rat sciatic nerve by local application ofadrenocorticotropic hormone. J Craniomaxillofac Surg 42: 784–789, 2014
Oestreicher AB, De Graan PN, Gispen WH, Verhaagen J, Schrama LH: B–50, the growth associated protein–43: modulation of cell morphology and communication in the nervous system. Prog Neurobiol 53: 627–686, 1997
Palacios G, Mengod G, Sarasa M, Baudier J, Palacios JM: De novo synthesis of GAP-43: in situ hybridization histochemistry and light and electron microscopy immunocytochemical studies in regenerating motor neurons of cranial nerve nuclei in the rat brain. Brain Res Mol Brain Res 24: 107–117, 1994
Qinyong S, Shanhui L, Pavan MP: Inferior alveolar and mental nerve injuries associated with open reduction and internal fixation of mandibular fractures: a seven year retrospective study. J Craniomaxillofac Surg 42: 1378–1381, 2014
Reynolds ML, Fitzgerald M, Benowitz LI: GAP-43 expression in developing cutaneous and muscle nerves in the rat hindlimb. Neuroscience 41: 201–211, 1991
Rustemeyer J, Gregersen J: Quality of life in orthognathic surgery patients: post-surgical improvements in aesthetics and self-confidence. J Craniomaxillofac Surg 40: 400–404, 2012
Schultze-Mosgau S, Erbe M, Rudolph D, Ott R, Neukam FW: Prospective study on post-traumatic and postoperative sensory disturbances of the inferior alveolar nerve and infraorbital nerve in mandibular and midfacial fractures. J Craniomaxillofac Surg 27: 86–93, 1999
Skene JH, Willard M: Axonally transported proteins associated with axon growth in rabbit central and peripheral nervous systems. J Cell Biol 89: 96–103, 1981
Starkey ML, Davies M, Yip PK, Carter LM, Wong DJ, McMahon SB, et al: Expression of the regeneration-associated protein SPRR1A in primary sensory neurons and spinal cord of the adult mouse following peripheral and central injury. J Comp Neurol 513: 51–68, 2009
Teramoto K, Tsuboi Y, Shinoda M, Hitomi S, Abe K, Kaji K, et al: Changes in expression of growth-associated protein–43 in trigeminal ganglion neurons and of the jaw opening reflex following inferior alveolar nerve transection in rats. Eur J Oral Sci 121: 86–91, 2013
Tetzlaff W, Alexander SW, Miller FD, Bisby MA: Response of facial and rubrospinal neurons to axotomy: changes in mRNA expression for cytoskeletal proteins and GAP-43. J Neurosci 11: 2528–2544, 1991
Tsai CM, Chiang CY, Yu XM, Sessle BJ: Involvement of trigeminal subnucleus caudalis (medullary dorsal horn) in craniofacial nociceptive reflex activity. Pain 81: 115–128, 1999
Woolf CJ, Reynolds M, Molander C, O'Brien C, Lindsay RM, Benowitz LI: GAP-43 appears in the rat dorsal horn following peripheral nerve injury. Neuroscience 34: 465–478, 1990
Yin Y, Cui QQ, Li Y, Irwin N, Fischer D, Harvey AR, et al: Macrophage derived factors stimulate optic nerve regeneration. J Neurosci 23: 2284–2293, 2003
Yuan Q, Hu B, Su H, So KF, Lin Z, Wu W: GAP-43 expression correlates with spinal motoneuron regeneration following root avulsion. J Brachial Plex Peripher Nerve Inj 4: 18, 2009
Zhang Y, Bo X, Schoepfer R, Holtmaat AJ, Verhaagen J, Emson PC, et al: Growth-associated protein GAP-43 and L1 act synergistically to promote regenerative growth of Purkinje cell axons in vivo. Proc Natl Acad Sci U S A 102: 14883–14888, 2005