Effect of a local application of an antibody to BDNF on neuroma formation after transection of the inferior alveolar nerve in the rat

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Abstract

Peripheral nerve injury sometimes induces neuroma formation at the injury site. The inferior alveolar nerve (IAN) is often damaged by dental treatment, resulting in painful symptoms. Antagonizing neurotrophins has been reported to reduce neuroma formation and neuropathic pain without damaging the transecting neurons following peripheral nerve injury, indicating the possibility that the inhibition of BDNF at the injury site can repress neuroma formation. The present study examined the effects of a local application of an antibody to BDNF at the injury site immediately after complete IAN transection, using immunohistochemistry and in situ hybridization histochemistry for trkB and BDNF. Histological analysis revealed a regular increase in the number of cellular elements and collagen fibers in the nerve cutting sites at both postoperative (PO) Weeks 1 and 2. At PO Week 2, a local administration of an antibody to BDNF clearly inhibited any proliferation of connective tissue at the injury site. Immunohistochemistry for PGP9.5 confirmed nerve fiber integrity in the anti BDNF-treated group. However, the control vehicle group showed no continuity of positive PGP 9.5 nerve fibers with neuroma formation at the proximal site. A fluorogold labeling technique revealed a significant difference ($p<0.05$) with a higher number of labeled trigeminal ganglion neurons in the anti BDNF- treated group than in the vehicle control groups. In situ hybridization histochemistry found intense signals for both BDNF and trkB mRNAs at the injury site of the vehicle control group. However, the signal for mRNAs for trkB and BDNF was localized in the area of the perineurium on the intact nerve fibers, indicating that fibroblasts encountered within the connective tissue were the cells expressing these signals. These findings suggest that a local inhibition of BDNF at a low dose clearly inhibited the proliferation of connective tissue at the injury site and neuroma formation.

Keywords: nerve injury, neuroma, IAN, BDNF, trkB

Introduction

Peripheral nerve injury sometimes induces neuroma formation, which represents a thwarted repair attempt at the nerve repair site [15]. Neuroma formation is associated with persistent painful symptoms such as dysesthesia or neuropathic pain which induces functional impairment [1, 10]. The mechanisms of neuroma formation are not yet fully understood though it has been considered a process in response to nerve tissue inflammation [32]. Cases such as that above can be identified as disorganized tissue with erosion of the epineurium regularly accompanied by the loss of funicular architecture [27].

Neurotrophins and inflammatory cells -- including mast cells -- play an important role in neuroma formation [12]. For instance, nerve growth factor (NGF) has been considered to have great influence on this process [13]. Mice lacking trkB, a high affinity neurotrophin receptor, have shown an increasing number of mast cells at the injury site during the chronic phase of regeneration. This accumulation may be down-regulated by some factors--including brain-derived neurotrophin factor (BDNF) and TGF-β1--that participate in nerve regeneration [12, 30]. The antagonization of neurotrophins has been reported to reduce neuroma formation without damaging the transecting neurons following peripheral nerve injury. A few trials have recently been carried out for clarifying the involvement of BDNF and trkB in neuroma formation and neurophatic pain. The exogenous application of BDNF to the proximal nerve stump has proven to cause an increase in neuroma development in rats in addition to enhancing behavioral autotomy [11, 12]. In fact, an experimental study has revealed that an administration of BDNF at a low dose (2μg/day) did not affect regeneration when administered following immediate repair [4]. This evidence has led us to consider the possibility that the inhibition of BDNF at the injury site can repress neuroma formation.

The inferior alveolar nerve (IAN) enters the mandibular foramen, passing through the inferior alveolar canal, to distribute widely in the mandible including the tooth and periodontal ligament. It is important for clinical dentistry to understand the processes of degeneration and regeneration of the IAN on nerve injury because clinical experience has shown a high frequency of injury of the IAN by trauma, the extraction of third molars, and orthognathic surgery, resulting in neuroma and

abnormal sensation in the injured receptive fields [9, 10, 22, 25, 28]. In terms of regeneration, some studies have revealed attenuation of the sciatic nerve regeneration and myelination in rats that were completely deprived of BDNF, a crucial factor for axonal regeneration by binding specific receptors called p75 and trkB [21]. However, it has also been involved in neurophatic pain models [29, 31]. Thus, this present study was undertaken to examine the effects of an immediate local administration of an antibody to BDNF following inferior alveolar nerve injury.

Material and Methods

All animal experiments were approved by the Niigata University Institutional Animal Use and Care Committee (approval number #42). The animals were housed in a temperature-controlled room under normal 12:12-hour light: dark laboratory conditions with free access to chow and water.

Animals and experimental procedure

Male Sprague-Dawley rats weighing 160-280g were used for the present study. All surgical procedures were performed under 8% chloral hydrate anesthesia (400mg/kg i.p.). Rats were placed in a right lateral recumbent position on a warm mat. A small incision was made on the surface of the left side of the facial skin over the masseter muscle, and the surface of the alveolar bone was exposed by separation of the muscle fibers with a separator. The left IAN was transected by using micro scissors at 7mm proximal to the angle of the alveolar canal and then immediately replaced back. Animals for this experiment were randomly assigned to three groups; sham (n=6), anti-BDNF treated (n=18), and vehicle-control groups (n=13). Furthermore, an additional 6 rats without any surgery were also treated as a naive group. For observation of histopathological changes and nerve regeneration processes, the animals in the naive group were sacrificed depending on time after transection of the IAN at either postoperative (PO) 1 week (n=2) or PO 2 weeks (n=4). In the anti BDNF-treated group, 1 µl of the antibody to BDNF (1mg/ml; ABCAM, Cambridge, UK) was injected into the transected IAN trunk using a Hamilton syringe. The vehicle control animals received a physiological saline injection in the transected nerve trunk. After an injection of either the antibody to BDNF or physiological saline, spongel (ASTELLAS Pharma Inc., Tokyo, Japan) and bone wax (Angiotech Pharmaceuticals, Inc. Vancouver, Canada) were applied. Then the skin was sutured and the rats were allowed to recover. The time course of this experiment is shown in Figure 1.

Fluorogold labeling

Five rats from each group were randomly selected for use in the Fluorogold (FG) tracing study. Under anesthesia with Sevofrane (Mylan Pharmaceutical, Co., Ltd, Osaka, Japan), they received a subcutaneous injection of 4% FG solution (10µl; Hydroxystilbamidine, Biotium, Hayward, CA, USA) into the mental skin at a depth of 5mm from the lower lip surface. One day after the FG injection, the animals were deeply anesthetized in the same way as described above and then perfused transcardially with 4% paraformaldehyde in 0.1M phosphate buffer (PB; pH 7.4). The removed trigeminal ganglia were post-fixed overnight in the same fixative and then stored for the next two days in 30% sucrose for cryoprotection. The frozen sections at a 20µm thickness were prepared in a cryostat, and every fifth section was mounted onto silane-coated glass slides and stained with propidium iodide. The examined area was selected as the root of the third branch of the trigeminal ganglion. To determine the proportion of the regenerated trigeminal ganglion neurons among the surviving neurons, the totals of both PI and FG labeled neurons were counted under a fluorescence microscope (n=3 per each animal; N=15). The results were processed for analysis of variance (ANOVA) with the aid of statistical analysis.

Histopathological evaluation

Two weeks following surgery, the animals were perfused transcardially with 4% paraformaldehyde in 0.1M PB under deep anesthesia as described above. The mandibles, including the IAN nerve of 17 animals from anti BDNF-treated (n=11) and vehicle control groups (n= 6), were decalcified with 5% formic acid and finally embedded in paraffin. Seven µm thick sections were subjected to azocarmin and aniline blue (AZAN) staining.

Immunohistochemistry for protein gene product (PGP 9.5), a general neuronal marker, was employed for the demonstration of neural elements according to our protocol [8]. The sections were primarily incubated with a rabbit polyclonal antiserum against human PGP 9.5 (1:2,000, Ultraclone Ltd., Wellow Isle of Wright, UK). They were then reacted with a biotinylated goat anti-rabbit IgG (1:1,000, Vector Laboratories, Buringame, CA) and subsequently with a peroxidase conjugated avidin

(ABC Kit, Vector Laboratories) at room temperature. The immunoreaction sites were visualized by incubation in 0.05 M Tris buffer (pH 7.6) containing 0.04% 3-3'-diaminobenzidine (DAB) and 0.03% hydrogen peroxide. The immunostained sections were counter-stained with 0.03% methylene blue. Results were visualized and documented using a digital camera (AxioCam HRc, Carl Zeiss, Oberkochen, Germany) mounted on an Axio Imager M1 (Carl Zeiss).

In situ hybridization histochemistry

The paraffin sections were processed for in situ hybridization histochemistry using a commercially available mRNA ISH kit (RNAscope 2.0 FFPE Assay Red; ACD, Hayward, CA, USA) in accordance with the manufacturer's instructions. Rat BDNF (accession number RNA scope probe MM-BDNF NP_0366) and trkB (accession number MM-NTRK2 NP_036) were designed and synthetized by ACD. The RNA transcript was visualized by Fast red.

Results

All animals survived the surgery and the two week post-operative period, demonstrating complete skin and muscle fascia closure. There was no clinical evidence of wound infection, and the rats recovered well after the experiment.

Neuroma formation after transection of the IAN

The sham group with the exposure of the IAN but without transection showed a slight proliferation of the connective tissue of collagen fibers and cellular elements in part of the IAN, suggesting that the opening of the inferior alveolar canal damaged the IAN trunk (Fig. 2a, d). The nerve transection induced the formation of scar tissue and neuroma at the injury site at PO Weeks 1 (b, e) and 2 (c, f). Observation of histologic sections confirmed that the IAN trunk was completely cut (Fig. 2b, c). In this injured area, connective tissues with rich collagen fibers proliferated to invade between the proximal and the distal stumps (Fig. 2b, c). The cellular elements at the neuroma appeared to increase in number more at PO Week 2 (Fig. 2f) than at PO Week 1 (Fig. 2e). Furthermore, the neuroma observed at PO Week 2 contained numerous vacuoles without stainability (Fig. 2f).

Effects of an antibody to BDNF on neuroma formation

The IAN passed through the inferior alveolar canal with integrity in the naive group (Fig. 3a) as confirmed by immunostaining with a PGP9.5 antibody (Fig. 3d). After two weeks post-operation, a local administration of the antibody to BDNF drastically inhibited the proliferation of connective tissue at the injury site (Fig. 3b) while the animals in the vehicle control group had a significant amount of connective tissue at the injury site, indicating the presence of neuroma (Fig. 3c). Immunohistochemistry for PGP9.5 indicated nerve fiber integrity in the anti BDNF-treated group (Fig. 3e). However, the vehicle control group showed no continuity of PGP 9.5 positive nerve fibers; the PGP9.5 positive nerve fibers ran a short distance in various directions to form the neuroma at the proximal site (Fig. 3f).

Labeling of trigeminal ganglion neurons with Fluorogold (FG)

Propidium iodide-staining could demonstrate all neurons in the trigeminal ganglion of all groups (Fig. 4a-c). Employment of FG, which was applied at the mental region, helped to visualize and enumerate the numbers of trigeminal ganglion neurons that regenerated their axons (Fig. 4). Many FG-labeled neurons were regularly localized in the root of the third branch of the trigeminal nerve of the naive group (Fig. 4d). No labeled neurons were observed in the root of the second branch. At PO week 2, the FG labeled neurons in the anti-BDNF treated group (Fig. 4e) surpassed those in the vehicle control group (Fig. 4f).

Many FG-labeled neurons were regularly localized in the root of the third branch of the trigeminal nerve of the naive group (Fig. 4a). No labeled neurons were observed in the root of the second branch. At PO week 2, the FG labeled neurons in the anti-BDNF treated group (Fig. 4b) surpassed those in the vehicle control group (Fig. 4c).

Figure 5, indicates the ratio of FG labeled neurons to all neurons in the trigeminal ganglion. The ratio amounted to 96.2±2.7% in the naive group (a total of 532 neurons), 74.9±4.9% in the vehicle control group (a total of 417 neurons), and 86.7±6.5% in the anti-BDNF treated group (a total of 416 neurons). Statistical analysis showed a significant difference among all experimental groups ($p<0.05$).

In situ hybridization histochemistry

A few isolated signals for BDNF and trkB mRNA, close to the border of the detection limit, were found in the nerve trunks of the naive group (data not shown). They were mainly localized in the area corresponding to the perineurium. In the anti BDNF-treated group, the signal observed at the injury site was very low and more confined to the area of the regenerating fibers (Fig. 6a). The nerve trunks of the vehicle control group had mRNA signals for BDNF at the area of the injury site containing fibrous and granular tissue (Fig. 6b).

For trkB mRNA, in the anti BDNF-treated group, fewer trkB mRNA signals were detectable in the region corresponding to the injury site (Fig. 6c). The signal was abundant in the saline group, being very specific to the injury site where fibrous tissue

predominated. Moreover, a prominent number of precipitates were also encountered in the region proximal to the injury site (Fig. 6d).

Discussion

In our study model, the local blocking of BDNF decreased the amount of scar tissue formation in the anti BDNF-treated group. A local application of an antibody to BDNF clearly inhibited neuroma formation after nerve transection while the vehicle control group showed a rich production of fibrous tissue and neuroma formation.

Histologic assessment demonstrated a great difference between anti BDNF-treated and vehicle control groups. The accumulation of scar tissue formation was coincident with an increasing number of various cellular elements that were encountered at the site of the injury. As shown in immunohistochemistry for PGP9.5, the vehicle control group had disorganized nerves in many directions, whereas the regenerated nerve fibers were arranged in a more organized fashion in the anti BDNF-treated group which had been administrated an antibody to BDNF. It is easy to consider that this contrasting phenomenon might correlate to an accumulation of connective tissue at the injury site. Previous studies have pointed out that a neuroma formed at the proximal nerve stump consisting of disorganized and splaying nerve fascicles with hemorrhagic fibrosis [10, 32]. Although the pathogenesis of neuroma development after nerve injury is not clear, we can easily suppose that the scar tissue at the repair site serves as a mechanical barrier to axonal regeneration.

During the nerve regeneration process, several factors -- including neurotrophins and tissue growth factor- β1 (TGF-β1) -- have been identified as promoters of neuronal survival, but it is apparent that they also play a chemotactic role for mast cells, macrophages, and inflammatory cells [12]. Experimental studies have suggested the involvement of mast cells in neurogenic inflammation [30]. Furthermore, it is well known that macrophages occur at the area of degenerating axons before and during the period of maximal proliferation of the Schwann cells as a part of the cellular response to peripheral nerve injury [14, 18, 20]. Previous studies have shown an infiltration of inflammatory cells and granular tissue accumulation around the injury site in significant numbers during the first three to five days in the animal model of peripheral nerve transection [20, 26]. In this experimental study, an application of an antibody to BDNF reduced the volume of connective tissue at the injury site, resulting in an inhibition of neuroma formation, as compared with the vehicle control group.

There have been different trials investigating neuroma prevention which include the local and endogenous antagonization of neurotrophins as well as cytokines. However, the antagonization of these neurotrophins has revealed results opposite to the treatment with these after peripheral nerve injury [2, 13]. It has been reported that exogenous treatment with BDNF itself not only induces neuroma formation [5, 11, 26]; BDNF application in low doses influenced regeneration after chronic axotomy, but could inhibit it when administrated in high doses [4]. The present administration of the antibody to BDNF induced a drastic decrease in connective tissue formation and inhibited neuroma formation at the injury site, as shown by AZAN staining and immunostaining with PGP9.5. Furthermore, fluorogold labeling did show that a percentage of labeled neurons in the anti BDNF-treated group totaled higher than in the vehicle control with a statistically significant difference, indicating that the application of the antibody to BDNF induced a regeneration of the IAN after nerve transection. These findings indicate that an antagonization of BDNF in low doses can repress neuroma formation via the inhibition of the proliferation of connective tissue at the injury site. This idea may be supported by previous experimental data showing that the antagonization of trkB, a specific neurotrophin receptor for BDNF, led to an accumulation of BDNF as well as to a remarkable increase in the levels of inflammatory cells and connective tissue [12].

In situ hybridization histochemistry succeeded in demonstrating a high number of precipitates that indicated the localization for BDNF mRNA and trkB mRNA at the injury site in the vehicle control group. Although it was difficult to identify the cell types expressing mRNAs for trkB and BDNF in this study, those cells were situated in the area of the perineurium on intact nerve fibers. This indicated that this signal corresponded to the area of fibroblasts encountered within the connective tissue. This finding suggested that fibroblasts were the great majority of the cells expressing these signals within the scar tissue area. These findings suggest the possibility that the fibroblasts synthesis, secrete, and bind BDNF in an autocrine/paracrine manner. Probably, an antibody to BDNF antagonized BDNF produced by the fibroblasts in fibrous tissue such as the perineurium via trkB, thereby inhibiting the proliferation of connective tissue. This notion may be explained by the involvement of fibroblast growth factor-2 (FGF-2) and fibroblast growth factor receptor

(FGFR), both of which are constitutively expressed in peripheral nerves. These molecules are up-regulated in the proximal and distal nerve stumps after peripheral nerve lesion. Their main sources are sensory neurons, fibroblasts, Schwann cells, and macrophages that invade the injury site [7].

In conclusion, an antagonization by a local application of an antibody to BDNF prevented neuroma formation. Through this experimental study, we found that IAN transection induced a neuroma formation containing an accumulation of collagen fibers at the injury site. The local administration of an antibody to BDNF clearly inhibited the proliferation of the connective tissue at the injury site. Therefore, we presume that the local inhibition of BDNF at low doses may be an alternative for therapeutic treatment on neuroma formation and its subsequent induction to neuropathic pain.

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Figure legends

Figure 1.

The time course of the experimental procedure used in the present study.

Figure 2.

Photomicrographs showing histological changes of the inferior alveolar nerve in the sham group (a, d) and the nerve-injury group postoperative Weeks 1 (b, e) and 2 (c, f). Figures d-f are magnified views of the boxed areas in a-c. AZAN staining. The opening of the inferior alveolar canal damaged the IAN trunk to allow the proliferation of a small amount of connective tissue (a) consisting of collagen fibers and cellular elements (d). IAN transection induces an increase in volume of the connective tissue at PO Weeks 1 (b) and 2 (c). Compared with PO Week 1 (e), the cellular elements come to increase at the injury sites at PO Week 2 (f). AB: alveolar bone, DP: dental pulp, NB: nerve bundle. Scale bars = 200μm (a-c), 50μm (d-f).

Figure 3.

Effects of a local application of an antibody to BDNF immediately after nerve transection. AZAN staining (a-c) and immunoreaction for PGP9.5 (d-f). The samples were obtained at 2 weeks after injection of either the antibody to BDNF (b, e) or physiological saline (c, f). The IAN bundle shows no damage, with integrity in the naive group (a) as confirmed by PGP9.5 immunostaining (d). Neither neuroma formation nor the proliferation of connective tissue is recognizable in the anti BDNF-treated group (b) while neuroma formation with connective tissue (asterisk) is found in the vehicle control group (c). PGP9.5 immunostaining demonstrates the disorganization of nerve fibers (arrows) in the vehicle control group (f) in contrast to the nerve fiber integrity in anti-BDNF treated group (e). AB: alveolar bone, BM: bone marrow, DP: dental pulp, NB: nerve bundle. Scale bars = 200 μm.

Figure 4.

Photomicrographs of images of propidium iodide (red, a-c) and Fluorogold (FG: yellow, d-f) staining in the trigeminal ganglion in the naive group (a, d), and anti BDNF-treated (b, e) and vehicle control groups (c, f). Almost all neurons are

labeled with FG in the trigeminal ganglion of the naive group (d). Note the difference in number of FG labeled trigeminal neurons between the antibody to BDNF- group (e) and saline-injected group (f). Scale bars = 100 μm.

Figure 5.

Histogram showing the ratio of FG labeled to all neurons in the trigeminal ganglion. A statistical difference exists among each group ($p<0.05$).

Figure 6.

Photographs showing expressions of mRNAs for BDNF (a, b) and trkB (c, d) at PO Week 2 as demonstrated by in situ hybridization histochemistry. Anti BDNF-treated (a, c) and vehicle control (b, d) groups. A few isolated signals for BDNF (a) or trkB (c) mRNAs are observed in the regenerating nerve fibers in the anti BDNF-treated group. The areas with neuroma formation contain BDNF (b) and trkB (d) mRNA signals in the fibrous and granular tissue. Scale bars = 20 μm

Experimental design

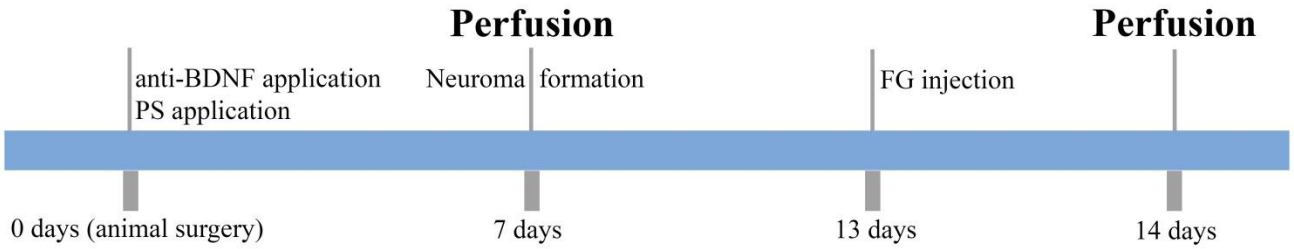

Figure 1

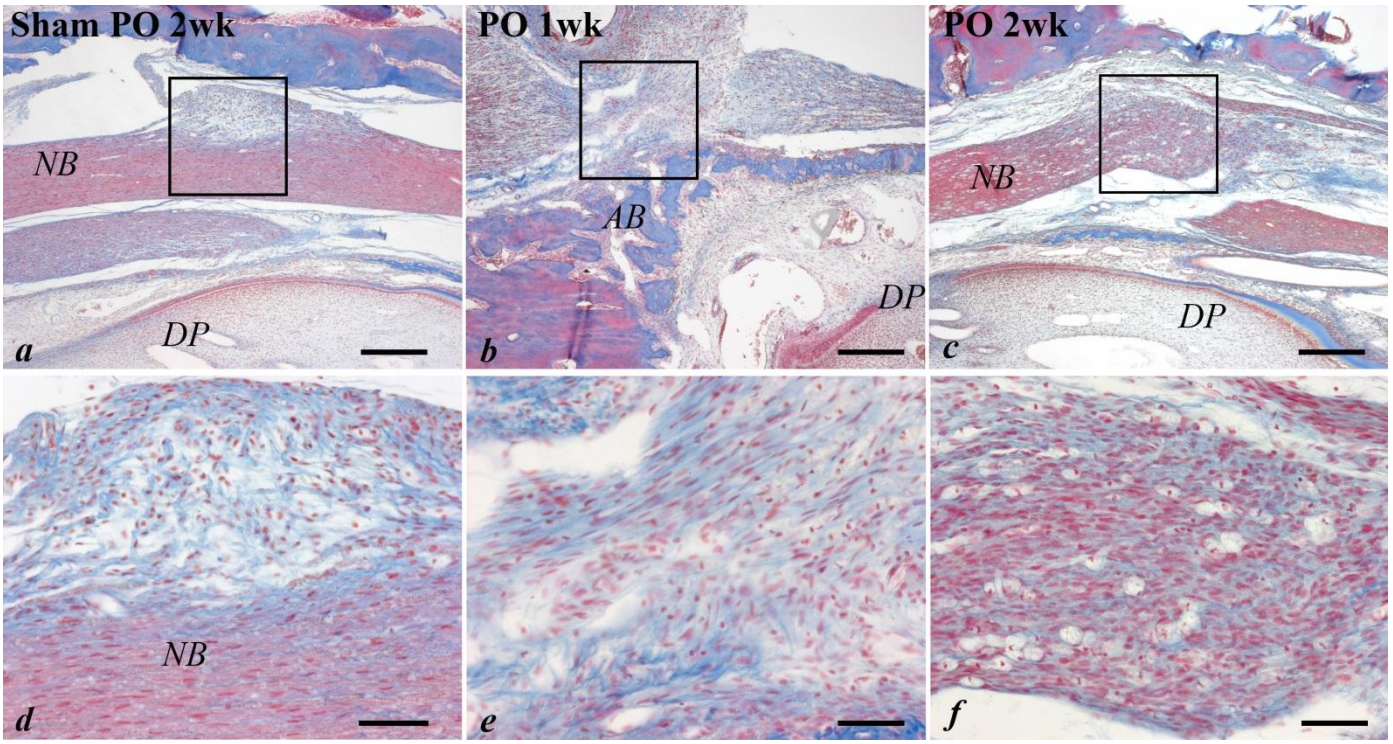

Figure 2

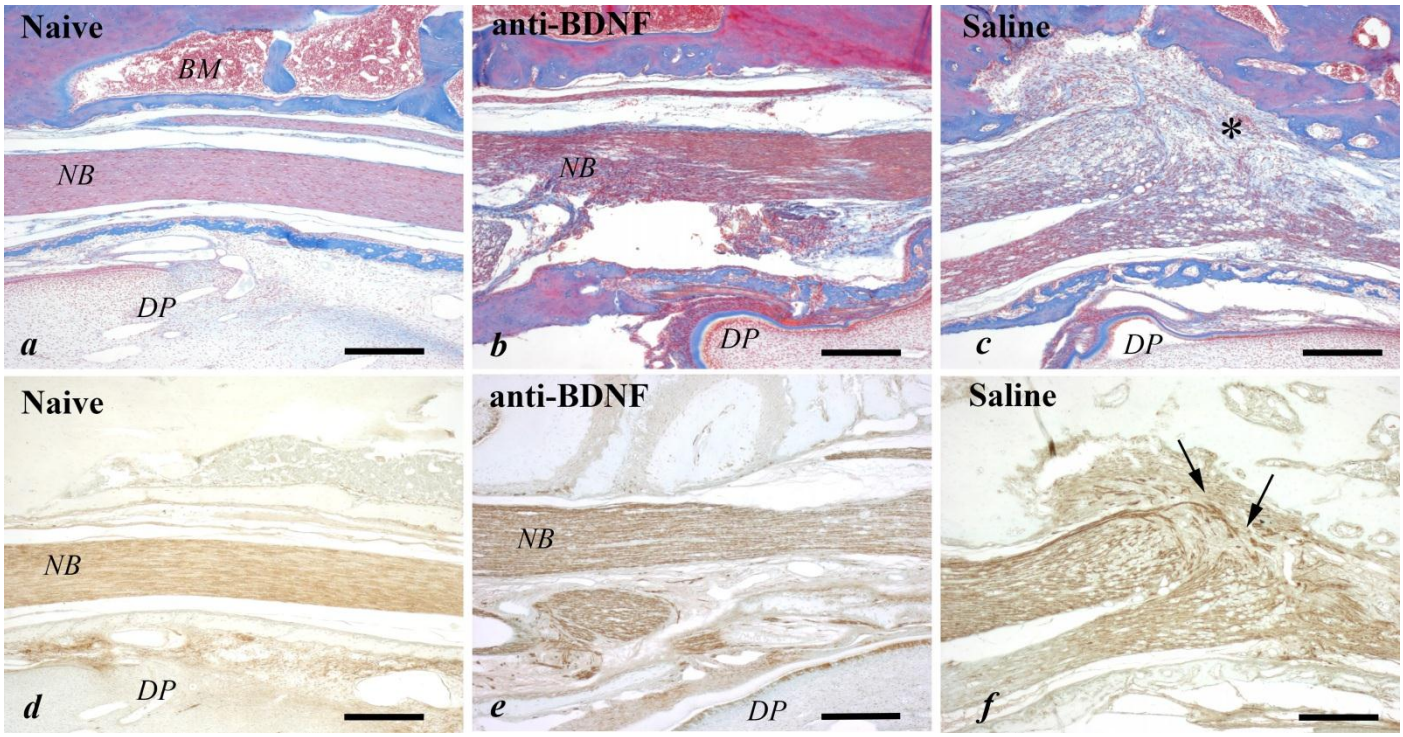
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Figure 3

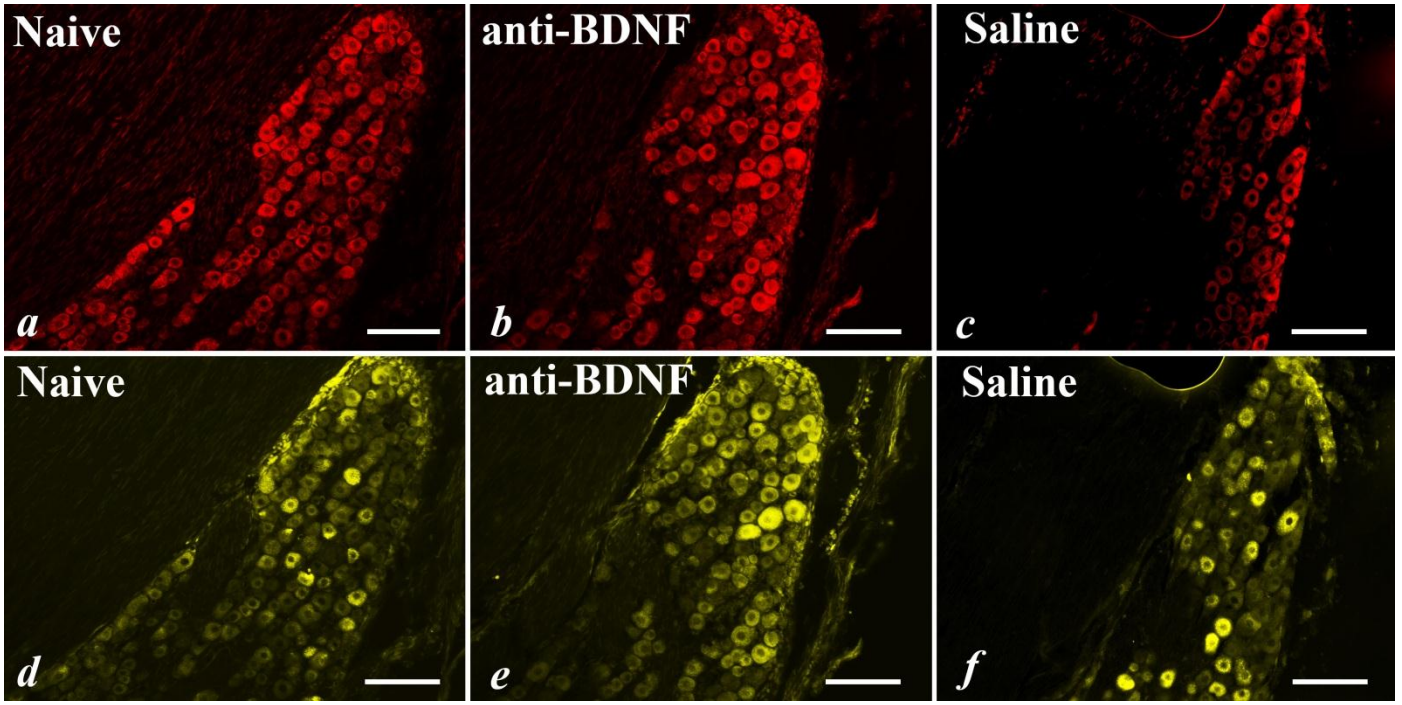

Figure 4

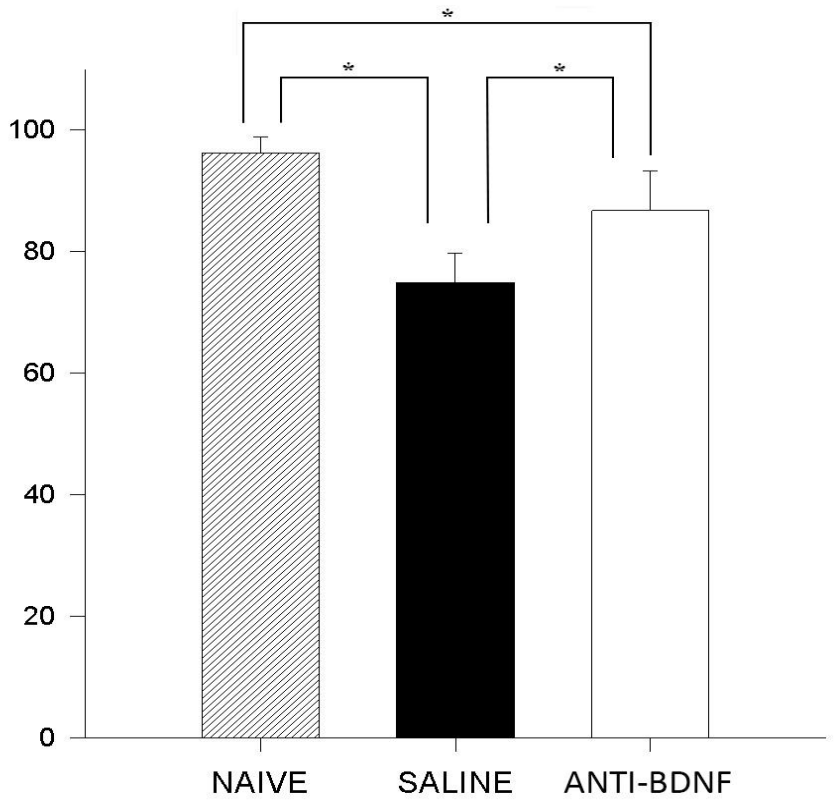

Figure 5

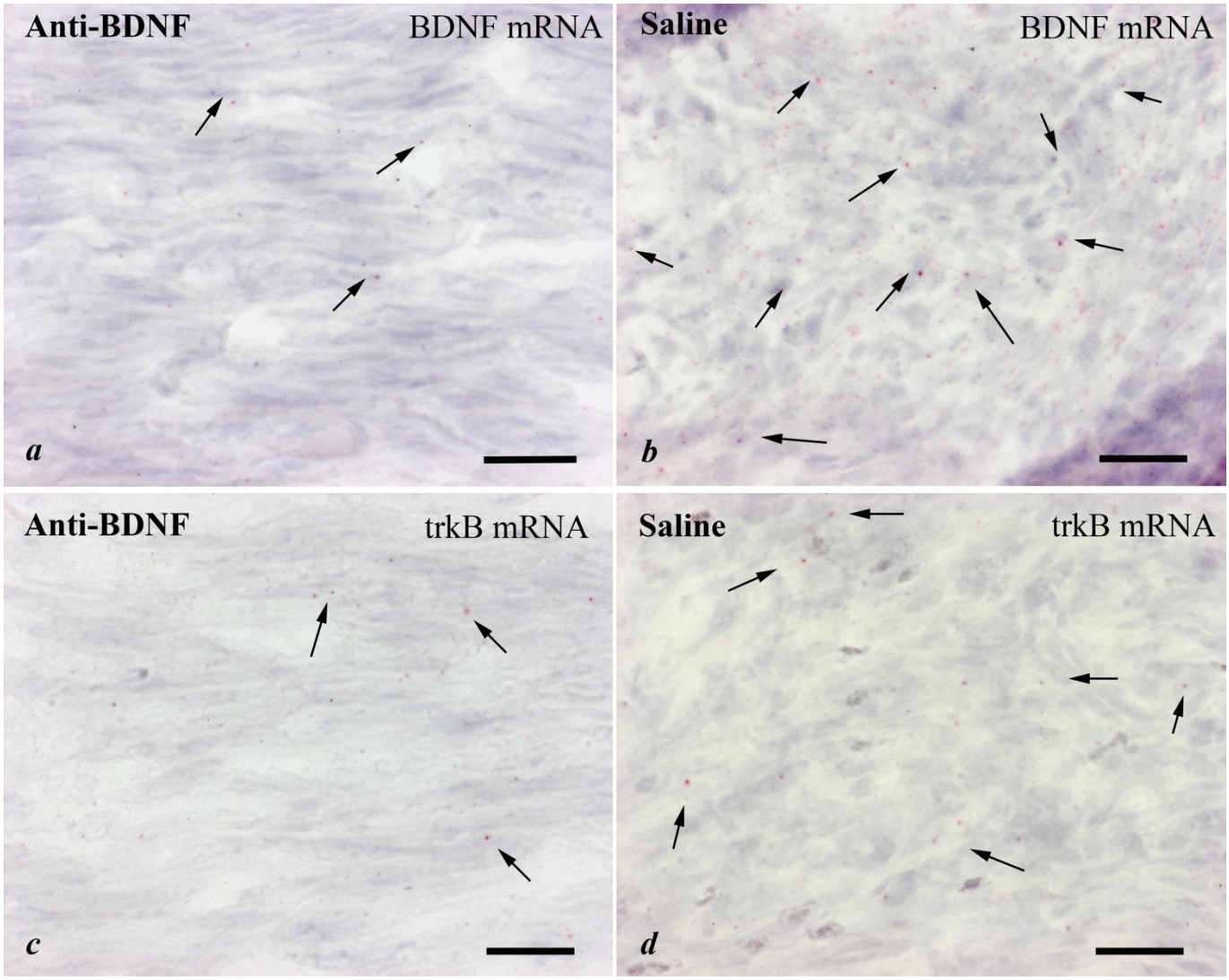
Anti-BDNF
BDNF mRNA
a
Saline
BDNF mRNA
b
Anti-BDNF
trkB mRNA
c
Saline
trkB mRNA
d

Figure 6