The involvement of brain-derived neurotrophic factor (BDNF) in the regeneration of periodontal Ruffini endings following transection of the inferior alveolar nerve*

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Summary. The present study employed immunohistochemistry for protein gene product 9.5 (PGP 9.5) to examine the regeneration process of Ruffini endings, the primary mechanoreceptor in the periodontal ligament, in heterozygous mice with targeted disruption of the brain-derived neurotrophic factor (BDNF) gene and their littermates, following transection of the inferior alveolar nerve. When immunostained for PGP 9.5, periodontal Ruffini endings appeared densely distributed in the periodontal ligament of the heterozygous mice, but the density of the positively stained nerve fibers in the ligament was 20% lower than that in the control littermates. At 3 days after surgery, the PGP 9.5-positive neural elements had disappeared; they began to appear in the periodontal ligament of both animals at 7 days. However, the recovery pattern of the PGP 9.5-positive nerves differed between heterozygous and wild type mice, typical periodontal Ruffini endings morphologically identical to those in the control group appeared in the wild-type mice at 7 days, whereas such Ruffini endings were detectable in the heterozygous mice at 28 days, though much smaller in number. On day 28, when PGP 9.5-positive nerves were largely regenerated in wild type mice, their distribution was much less dense in the ligament of the heterozygous mice than in the non-treated heterozygous mice. The density of PGP 9.5-positive nerve fibers was significantly lower in the heterozygous mice than in wild type mice at any stage examined. These data showing that a reduced expression of BDNF causes delayed regeneration of the periodontal Ruffini endings suggest the involvement of BDNF in the regeneration process of these mechanoreceptors.

Introduction

Neurotrophins play crucial roles in neural development, survival, maintenance, and regeneration both in central and peripheral nervous systems (for reviews, Barde, 1990; Richardson, 1991; Bothwell, 1991, 1995; Chao, 1992, Meakin, 1992). Brain-derived neurotrophic factor (BDNF) has been regarded as an essential neurotrophin for sensory neurons, but not motor neurons (Jones et al., 1994; Ernfors et al., 1994a, 1995a,b). A null mutation for BDNF induces a neuronal loss or the reduction/absence of sensory neurons, including large-sized neurons in trigeminal ganglion (Jones et al., 1994; Ernfors et al., 1994a, b, 1995a, b; Liu et al., 1995). Furthermore, BDNF-depletion has also been reported to cause the absence of cutaneous mechanoreceptors (Ernfors et al., 1994b; Fundin et al., 1997; Liebl et al., 1997), and a greater increase in mechanonotreshold than in littermate wild type mice (Carroll et al., 1998). These findings suggest the involvement of BDNF in the development, maintenance, and survival of mechanoreceptors.
The periodontal ligament functions as a tooth-anchoring, supporting, and sensory apparatus because of its rich sensory innervation (Schroeder, 1986; Maeda et al., 1999). Mechanical stimuli to the tooth evoke various oral reflexes via periodontal mechanoreceptors, which facilitate smooth mastication (Hannam, 1982; Matthews, 1975; Taylor, 1990). Although numerous morphological studies have reported various types of periodontal mechanoreceptors (Schroeder, 1986), immunocytochemical and electron microscopic investigations have revealed that the Ruffini ending is the primary mechanoreceptor in the periodontal ligament (Byers, 1985; Sato et al., 1988, 1989; Nakakura-Ohshima et al., 1993; Maeda et al., 1999). Experimental and clinical studies have suggested a high potential for neuronal plasticity in the periodontal Ruffini endings (for reviews, Maeda et al., 1999; Wakisaka et al., 2000). According to our previous studies, the periodontal Ruffini endings can regenerate around postoperative 28 days (PO 28d) following transection of the inferior alveolar nerve (IAN); quantitative analysis showed the neural density of the periodontal nerves decreased to a minimum around PO 3d and recovered to normal levels by PO 28d (Youn et al., 1997; Hiroshima et al., 1998; Wakisaka et al., 2000). Furthermore, the periodontal Ruffini endings express immunoreactivities for p75-neuropathy target protein (p75-NT) growth factor receptor (p75-NGFR) (Byers, 1990; Saito et al., 1993), and growth associated protein-43 (GAP-43) (Kobayashi et al., 1998) in adulthood, all of which are usually up-regulated during neural development/regeneration but down-regulated after the completion of neural development/regeneration. All this evidence supports the hypothesis that the periodontal Ruffini endings have a high potential for neuronal plasticity. 

BDNF mediates its effects by binding two kinds of receptors: trkB and p75-NGFR (Bothwell, 1991, 1995; Klein et al., 1991, Barbacid, 1994). The former is a high affinity neurotrophin receptor while the latter is a low affinity neurotrophin receptor which binds all neurotrophins. Our previous studies have reported that the periodontal Ruffini endings exhibit heterogenous immunoreactivities for trkB, which is capable of binding BDNF (Ochi et al., 1997; Atsumi et al., 1999). Recently, we further showed an approximately 20% reduction in neural density in the periodontal ligament of BDNF-deficient adult mice, compared with littermate wild type mice (Alkahramah et al., 2003). These findings suggest the involvement of BDNF in the regeneration of the periodontal Ruffini endings. The present study was therefore undertaken to investigate the regeneration process of the periodontal Ruffini endings in the lower incisor of BDNF heterozygous and wild type mice by an immunocytochemical technique using an antiserum against protein gene product 9.5 (PGP 9.5), a general neuronal marker (Doran et al., 1983; Thompson et al., 1983), following transection of the IAN. In addition, a quantitative analysis of neural density was performed by a computer-assisted image analyzer for a comparison between wild type and heterozygous mice. Since homozygous BDNF mice failed to survive over postnatal 3 weeks and since the terminal formation of mature periodontal Ruffini endings is completed over the first postnatal month (Nakakura-Ohshima et al., 1993, 1995), this immunocytochemical study compared wild littermate with BDNF heterozygous mice. BDNF heterozygous mice have been reported to exhibit BDNF protein levels approximately 50% lower than those in wild mice (Bianchi et al., 1996).

Materials and Methods

All animal experiments were done under the Guidelines for Intramural Animal Use and Care Committee of the Niigata University Faculty of Dentistry.

Animals

Breeding pairs of heterozygous mice with targeted disruption of the BDNF gene (strain-targeted mutation) were purchased from Jackson Laboratory (Bar Harbor, ME, USA). They were kept under normal 12 h light/12 h dark laboratory conditions, with free access to chow and water. The mating heterozygous parents obtained three types of newborn mice—a homozygous, heterozygous, and wild type—according to their genotype by polymerase chain reaction (PCR), as reported previously (Alkahrahmah et al., 2003). Briefly, genomic DNA was extracted from about 2–3 mm of tail tissues according to the method by Laird et al. (1991). PCR was done by the manufacturer's protocol using three kinds of primers (The Jackson Laboratory, ME: http://www.jax.org/html/informsearch/search DB_index.html). Amplified reaction products were run on 1.0% agarose gels in a TAE buffer (20mM Tris-acetate; 0.5mM EDTA; pH 8.0) at 100V and visualized using ethidium bromide under an ultraviolet transilluminator.

Animal preparation

Ninety-four 90-day-old mice, weighing 22–28 g at the time of surgery (47 wild type and 47 heterozygous), were used in the present study. The transection of the inferior alveolar nerve (IAN) was performed on 84 mice (42 animals each) according to the procedure by Atsumi et al. (1999, 2000). Briefly, under anesthesia with chloral hydrate (400 mg/kg, i.p., supplemented as necessary), the masseter muscle at each side was torn to expose the buccal surface of the
mandibular bone, and a small amount of the bone covering the mandibular canal was removed with the aid of a dental drill to expose the IAN. After transection of the IAN by fine scissors, the cut ends of IAN were returned into the mandibular canal, and the wound was sutured (experimental group). Those animals without any transection of the IAN (n=14; 7 wild type and 7 heterozygous) were used as a sham group. Ten mice (5 wild type and 5 heterozygous) without any surgical treatment served as a control group. No post-operative treatment such as the administration of antibiotics was given to the operated mice. The animals were allowed to survive for 3, 7, 10, 14, 21 and 28 days (n=7 each for wild type and heterozygous at each stage).

**Tissue preparation**

At appropriate survival periods, animals were deeply anesthetized with an intraperitoneal injection of chloral hydrate (400 mg/kg), and perfused transcardially with a fixative containing 4% paraformaldehyde and 0.125% glutaraldehyde in a 0.1 M phosphate buffer (pH 7.4). The mandibles, including the incisors, were removed en bloc and immersed in the same fixative at 4°C for 12 h. After decalcification with a 10% ethylene diamine tetraacetic acid (EDTA)-2Na solution for 3-4 weeks at 4°C, tissue blocks were equilibrated in a 30% sucrose solution overnight for cryoprotection. Frozen sections of mandibles including the incisors were serially cut at a thickness of 35 μm with a freezing microtome, collected in phosphate-buffered saline (PBS), and treated as free-floating sections.

**Immunohistochemistry**

Free-floating sections were processed for immunostaining using the avidin-biotin complex (ABC) method, as previously reported (Hsu et al. 1981; Nakakura-Ohashima et al., 1993, 1995). After the inhibition of endogenous peroxidase with 0.3% H₂O₂ in absolute methanol, frozen sections were treated with PBS containing 2.5% normal goat serum (Vector Lab., Burlingame, CA, USA). The sections were primarily incubated with a rabbit polyclonal antiseraum against human PGP 9.5 (1:10,000, Ultraclone Co. Ltd., Cambridge, U.K.) for 12 h at 4°C. They were then incubated with biotinylated goat anti-rabbit IgG (1:1000, Vector Lab), and subsequently with an avidin-peroxidase complex (ABC kit, Vector Lab.) for 90 min each at room temperature. The immunoreaction sites were visualized by incubation in a 0.05M Tris buffer (pH 7.6) containing 0.04% 3,3'-diaminobenzidine (DAB) and 0.003% H₂O₂ with nickel ammonium sulfate (0.1%) intensification. The immunostained sections were mounted on poly-L-lysine-coated glass slides, and counterstained with 0.03% methylene blue.

The origin, characterization, and specificity of the PGP 9.5 antiserum used in this study have been frequently reported elsewhere (Glubrecht et al., 1987, Nakakura-Ohashima et al., 1993, 1995).

**Quantitative analysis**

For quantitative analysis, the percentages of PGP 9.5-immunopositive areas to the surface areas of the restricted part of the periodontal ligament were measured by computer-assisted image analyzer (KS300; Carl Zeiss, Germany). The lingual periodontal ligament between the mesial and distal roots of the lower first molar was selected as the area to be statistically analyzed, as reported by Yoon et al. (1997). Images at a magnification ×10 (n=5 each) were directly transferred onto a computer. The periodontal ligament without blood vessels was extracted on a monitor and its surface area was measured (defined as the total area). Then the PGP 9.5-immunopositive area in the total area was determined by two-value threshold treatment, and its surface area was measured automatically by software equipped with an image analyzer. The percentage ratios of the PGP 9.5 immunopositive area to the total area were calculated (mean ± S.E.). Statistical comparisons were assessed between wild type and heterozygous mice at the same postoperative periods as well as between control and sham animals (unpaired Student's t-test), and between each postoperative period of the wild type or heterozygous mice (one-way ANOVA and Scheffe's post-hoc test). A p value of less than 0.05 was considered a significance difference.

**Results**

**Genotyping**

The PCR data were readily distinguishable between heterozygous and wild-type mice siblings (Fig. 1). Amplified PCR products were of uniform quality and clearly visible on agarose gel under ultraviolet trans-illumination. The C allele wild type mice were identified by single bands around 250bp. The amplified products of heterozygous siblings appeared double banded (250bp and 340bp), comparable with our previous report (Alkhambah et al., 2003) and the manufacturer's instructions (The Jackson Laboratory, ME: http://www.jax.org/html/search/search_DB_index.html).

**Innervation in the periodontal ligament of the control and sham groups**

A dense innervation of PGP 9.5-immunoreactive nerve fibers was found in the lingual periodontal ligament of the
wild type mice of the control group (Fig. 2a). As soon as thick nerve bundles showing PGP 9.5-immunoreactivity entered the periodontal ligament, they began ramifying to extend throughout the periodontal ligament. The nerve fibers were restricted to the alveolar half of the periodontal ligament (alveolus-related part) as described by Beets and co-workers (1974); no neural elements were observed in the tooth half (tooth-related part) or shear zone, a border between the alveolus- and tooth-related part (Fig. 2c). In the alveolus-related part, thick nerve fibers extensively ramified to form the Ruffini endings (Fig. 2c) as previously reported (Maeda et al., 1999). They possessed expanded axon terminals with tiny microprojections. However, a few thinner and less branched PGP 9.5-positive nerve endings were also recognized in the alveolus-related part. In addition, thin nerve fibers, beaded in appearance, were observed to terminate as free nerve endings among the periodontal collagen fibers (Fig. 2c).

The periodontal ligament of the heterozygous mice showed the same distribution pattern and terminal morphology of nerve fibers (Fig. 2b) as that of the wild type (Fig. 2c). Although the periodontal nerve fibers terminated as either a free or Ruffini ending, the Ruffini endings of the heterozygous mice were observed to be composed of thinner nerve fibers than those of the wild mice (Fig. 2d).

The distribution and terminal morphology of periodontal nerve fibers in the sham animals were identical to those of the control animals (data not shown).

**Postoperative Day 3 (PO 3d)**

Transection of the IAN almost induced the disappearance of the PGP 9.5-positive nerve fibers — including the periodontal Ruffini endings — from the lingual periodontal ligament of both wild (Fig. 3a) and heterozygous mice (Fig. 3b). However, a weak immunoreactivity persisted in the nerve fibers adjacent to the alveolar bone and blood vessels in the periodontal ligament.

**Postoperative Days 7–10 (PO 7–10d)**

In the wild type mice, PGP 9.5-immunopositive nerve fibers began to regenerate in the periodontal ligament on PO 7d (Fig. 3c). The regenerating nerve fibers appeared to increase in number and immuno-intensity gradually (Fig. 3c, f). The regenerating nerve bundles with intense PGP 9.5 immunoreaction entered the periodontal ligament. A major population of the regenerating nerve fibers with weak immunoreaction branched out to assume a tree-like ramification (Fig. 3d). A few nerve fibers, frequently beaded in appearance, were also observed in the alveolus-related part. On PO 10d, nerve terminals with dendritic ramifications
were recognized in the alveolus-related part (Fig. 3b).

The neural regeneration in the heterozygous mice appeared to lag behind that of the wild type mice on PO 7d (Fig. 3c). A few PGP 9.5-positive nerve fibers, thinner and with beaded profiles, were scattered in the periodontal ligament. Between PO 7d and PO 10d, the regenerating nerve fibers increased in number gradually. However, almost all nerve fibers appeared beaded and with fewer ramifications (Fig. 3i). No dendritic ramifications of PGP 9.5-immunopositive nerve fibers were recognizable in the periodontal ligament of the heterozygous mice at this stage.

Postoperative Day 14 (PO 14d)

In this stage, the neural regeneration had proceeded in the both the wild type and heterozygous mice (Fig. 4a, b). In the wild type mice, typical Ruffini endings as observed in the control group were found: thick PGP 9.5 positive nerve fibers ramified repeatedly to show tree-like ramifications with expanded portions. In addition to this type of nerve ending, dendritic terminals consisting of thin nerve fibers and free nerve endings with a beaded appearance were recognized in the periodontal ligament (Fig. 4c).
Fig. 3. Microscopic views of the distribution and terminal morphology of the PGP 9.5-immunoreactive nerve fibers in wild type (+/+; a, c, d, f, h) and heterozygous (+/-; b, e, g, i) mice following nerve injury. a and b: At PO 3d, the PGP 9.5-immunopositive nerve fibers have almost disappeared from both wild type (a) and heterozygous (b) mice. c-e: On PO 7d, regenerating nerve fibers with PGP 9.5-immunoreaction enter the periodontal ligament both of wild type (c) and heterozygous mice (e). d: A higher magnification of the boxed area in Figure 3c. The regenerating nerve fibers show a tree-like arborization. The periodontal ligament also contains thick nerve fibers showing a beaded appearance with a weak immunoreactivity. f-i: Neural distribution on PO 10d in wild type (f, h) and heterozygous (g, i) mice. The regeneration of nerve fibers has proceeded both in wild type (f) and heterozygous (g) mice, compared with the previous stages. h and i: Higher magnified views of the boxed area in (f) and (g), respectively. The nerve fibers have become thick and ramify to appear in a dendritic fashion (arrow) in the periodontal ligament of the wild type mouse. An arrowhead indicates a thin nerve fiber beaded in appearance. In contrast, although the thick nerve fibers appear in the heterozygous mouse (arrows), dendritic terminals are not found in the heterozygous mouse (i). BV: blood vessel. Scale bars = 50 µm (a–c, e–i), 25 µm (d)
Fig 4. Photomicrographs showing the distribution and terminal morphology in wild type (+/+; a, c, e, g) and heterozygous (+/-; b, d, f, h) mice on PO 14d (a–d), PO 21d (e, f) and PO 28d (g, h). a and b: The regeneration of nerve fibers has proceeded gradually in both the wild type (a) and the heterozygous (b) mice, compared with the previous stage, but is less innervated in the heterozygous mice. c: Higher magnification of the boxed area in Figure 4a. The distribution of PGP 9.5-immunoreactive fibers in the wild type mouse is almost identical to that in the control. The periodontal ligament contains Ruffini endings (arrow) and thin nerve fibers (arrowhead). d: High magnification of the boxed area in Figure 4b. The periodontal ligament in the heterozygous mouse contains thick (arrow) and thin (arrowhead) nerve fibers, but typical Ruffini endings do not occur at this stage. e–h: The maturation of the Ruffini endings (arrows) has proceeded further in the wild type mice (e, g), showing a distribution and terminal morphology identical to those seen in the control group. The periodontal Ruffini endings (arrows) appear in the heterozygous mouse on PO 21d (f) and PO 28d (h), though less developed than those in the wild type mice. Thin nerve fibers taking a straight course parallel to the tooth axis (arrowhead) remain in the periodontal ligament of the heterozygous mouse (f, h). AB: alveolar bone, T: tooth. Scale bars = 50 μm.
In contrast, the heterozygous mice contained fewer regenerated nerve fibers in the periodontal ligament, although the neural density was apparently higher than at the previous stage (Fig. 4b). The terminal formation, however, was delayed in the heterozygous mice; a major population of the PGP 9.5-positive nerve fibers was thin and appeared beaded. Although no typical Ruffini endings were recognized, a small number of nerve terminals with fewer ramifications of thick axons appeared in the periodontal ligament (Fig. 4d).

Postoperative Days 21–28 (PO 21–28d)

The periodontal ligament of wild type mice showed almost the same distribution pattern and terminal morphology at both PO 21d and PO 28d (Fig. 4c, g). Thick nerve fibers ramified repeatedly to form the Ruffini endings with irregular or smooth outlines in the alveolus-related part. Thinner nerve fibers were also distributed in the periodontal ligament.

On PO 21d, the PGP 9.5-immunopositive nerve fibers increased in number, and dendritic terminals consisting of thick nerve fibers were discernable in the periodontal ligament of heterozygous mice (Fig. 4f), though fewer than in the wild type. The periodontal ligament contained beaded and smooth nerve fibers. A part of these ran for a long distance parallel to the tooth axis in the periodontal ligament (Fig. 4f), though the occurrence of these nerve fibers was rare under normal conditions (Sato et al., 1988, 1989). No apparent differences in distribution and terminal morphology were recognized between PO 21d and PO 28d in the heterozygous mice (Fig. 4b).

Quantitative analysis

Figure 5 presents time-course changes in the quantitative analysis using a digital analyzer.

In the control animals, 17.0 ± 1.7% and 12.5 ± 1.6% were immunoreactive among wild type and heterozygous mice, respectively; there was a significant difference between

Fig. 5. Changes in density of PGP 9.5 nerve fibers in the periodontal ligament of heterozygous and wild-type mice after transection of the inferior alveolar nerve. Immunoreactive areas for PGP 9.5 compared with the defined total areas in the wild type (open column) and heterozygous (hatched column) mice. The percentage for wild type mice is significantly higher than that of heterozygous mice at the same postoperative period, except for PO 3d (*** p<0.001, * p<0.05). No significant difference exists in the percentages of immunoreactive areas to the defined area between control (cont.) and sham groups in either wild type or heterozygous mice (* p<0.05). In wild mice, the percentage of the immunoreactive area rapidly increases, and recovers to the level of sham animals on PO 28d. However, in heterozygous mice, it does not reach the level of the sham group even by PO 28d. ## p<0.001 and # p<0.05 when compared with values of the sham animals.
these two groups. The sham operation did not cause any alteration in the neural density (17.2 ± 1.1% in wild type mice and 13.6 ± 1.0% in heterozygous mice). Following nerve injury to the IAN, the neural density in the defined area drastically decreased, but thereafter increased gradually in both wild type and heterozygous mice, though the time course slightly differed between two groups. In the wild type, the percentage of the immunoreactive area decreased to 1.1 ± 0.3% on PO 3d, and then increased gradually. On PO 28d, the neural density almost returned to the normal levels (16.8 ± 2.0%). During regeneration, there were significant differences between each postoperative period.

In heterozygous animals, the neural density declined to a minimum level (0.8 ± 0.7%) on PO 3d, and increased slowly. Unlike the wild type, neural density was still significantly smaller even on PO 28d (8.2 ± 1.3%), compared with the sham-operated animals. Statistical differences were noted only between PO 10d and PO 14d.

During regeneration, there were significant differences between wild type and heterozygous mice at the same postoperative day except on PO 3d.

Discussion

This immunocytochemical study clearly demonstrated the regeneration process of the periodontal nerves as well as chronological changes in neural density in BDNF-heterozygous and littermate wild type mice following transection of the IAN.

Previous immunocytochemical studies have shown the expression of immunoreactivities for p75-NGFR (Byers, 1990; Saito et al., 1993) and trkB (Ochi et al., 1997; Atsumi et al., 1999) in the periodontal Ruffini endings even in adulthood, though their synthesis is usually down-regulated or diminished after the completion of neural development/regeneration. Both these neurotrophin receptors can bind BDNF (Klein et al., 1991; Bothwell, 1991, 1995; Barbacid, 1994), in spite of differences in binding affinity, indicating that BDNF is one of the key molecules for neural development, regeneration, maintenance, and survival in the periodontal ligament. In the present quantitative analysis of neural density in control and sham groups, BDNF heterozygous mice exhibited more than a 20% reduction in neural density, as compared with littermate wild type mice, a finding essentially comparable with our previous ones for the upper incisor ligament (Alkharnah et al., 2003). Enzyme-linked immunosorbant assay (ELISA) measurements of BDNF protein content from the dorsal root ganglion showed a dose-responsive curve for BDNF in wild, heterozygous, and homozygous mice, and indicated BDNF protein levels were reduced by approximately half in heterozygous mice compared with wild mice (Bianchi et al., 1996). Thus, the present observations of control and sham groups suggest the involvement of BDNF in the maintenance and survival of the periodontal nerves. However, since no complete depletion of the periodontal Ruffini endings was observed throughout this experimental period, other neurotrophins such as NT-4/5 and NT-3 are also considered to be involved in this process.

It is interesting that a BDNF-decrease induces a selective loss of Ruffini endings in the periodontal ligament. This ending in normal animals consisting of nerve fibers thicker than nociceptive free endings is characterized by extensive ramifications of expanded axon terminals (Byers, 1985; Sato et al., 1988, 1989; Kannari, 1990; Kannari et al., 1991; Maeda et al., 1999). Indeed, scanning electron microscopy combined with a chemical maceration technique was able to demonstrate a three-dimensional complex configuration of axon terminals (Takahashi-Iwanaga et al., 1997). On the other hand, BDNF-depletion has been reported to induce the absence of cutaneous receptors and large-sized neurons in the trigeminal ganglion (Ernfors et al., 1994b; Fundin et al., 1997; Liebl et al., 1997). Interestingly, BDNF has been regarded as an essential neurotrophin for the slowly adapting mechanoreceptors (Caroll et al., 1998) which includes the periodontal Ruffini endings (Chambers et al., 1972; Biemesderfer et al., 1978). Furthermore, observations of trkA- and trkB-knockout mice respectively showed defects in the thin nerve fibers terminating as free endings (Smeyne et al., 1994) and periodontal Ruffini endings (Matsuo et al., 2002). Even in BDNF-heterozygous mice, the cutaneous mechanoreceptors showed higher mechanosensitive thresholds than in littermate wild mice (Carroll et al., 1998). This evidence suggests that the reduction in neural density between littermate wild and heterozygous mice in control and sham groups may be caused by a decrease in the number of the Ruffini endings.

The present study also showed different recovery patterns between heterozygous and littermate wild mice, although no changes in the time course of neuronal density after IAN injury existed between them: the PGP 9.5-positive nerves reached minimum levels on PO 3d, and increased thereafter, consistent with the previous experimental data on rats (Youn et al., 1997; Hiroshima et al., 1998).

There are two findings of note on the time-course changes in the neural density of periodontal nerve fibers during regeneration. First, the neural density was less in the heterozygous than in the wild type mice at each stage throughout the observation period. Second, the neural density could not recover to sham levels in the heterozygous
while it could return to these in the wild mice: the neural density recovered to about 60% of the sham animals on PO 28d when wild type mice showed a recovery to normal levels. Previous experimental studies have confirmed the up-regulated expression of p75-NGFR, which is capable of binding all kinds of neurotrophins in the periodontal nerves during the neural regeneration in tooth replantation (Byers et al., 1992) and tooth movement models (Saito et al., 1993), indicating the involvement of certain neurotrophins in these regeneration processes. The periodontal Ruffini endings exhibit an immunoreactivity for trkB, but not for trkA nor trkC (Ochi et al., 1997; Atsumi et al., 1999), a high affinity neurotrophin receptor for BDNF as well as NT-4/5 and putative NT-3 (Klein et al., 1991, 1992; Bothwell, 1991; 1995). Thus, we speculate that the delayed and insufficient regeneration of the periodontal nerves was caused by the low concentration of BDNF in the target tissues, and that BDNF is involved in the regeneration of the periodontal nerve fibers, in particular of that of the Ruffini endings.

It is probable that heterozygous mice eventually recover to normal levels after PO 28d. This possibility, however, was excluded from the quantitative analysis; no significance difference was recognized between PO 21d and PO 28d, indicating that the neural regeneration was not active beyond PO 21d. Most likely, the regeneration of the periodontal Ruffini endings remains at 60% of the normal level. However, questions arise as to why the regeneration stops at this point, and further investigations will have to resolve this issue.

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