Heterogeneous Localizations of Trk B among Individual Periodontal Ruffini Endings in the Rat Incisor*

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Summary. The present immunocytochemical study examined the localization of Trk B, a high affinity neurotrophin receptor, in the neural elements of the periodontal ligament of the rat incisor. In light microscopy, the immunoreactivity was demonstrated in dendritic profiles in the alveolar half of the periodontal ligament. Their location and morphological features indicated that they were periodontal Ruffini endings. Occasional rounded cells associated with periodontal Ruffini endings, which had immunonegative kidney-shaped nuclei, were immunoreactive; these were judged to be terminal Schwann cells. Immunoelectron microscopy revealed the heterogeneous localization of Trk B among individual Ruffini endings. Some terminal Schwann cells contained immunoreactive products for Trk B in the cytoplasm, while others did not. Similarly, a part of the Schwann sheaths covering the axon terminals showed Trk B immunoreactivity. Most axon terminals associated with periodontal Ruffini endings were immunopositive for Trk B, though a few of them were immunonegative. The ordinary Schwann cells did not contain Trk B immunoreactive products. These findings imply that Trk B is required for the maintenance of periodontal Ruffini endings. The different expression pattern of Trk B suggests that neuronal and glial elements comprising individual periodontal Ruffini endings are subject to heterogeneous conditions with regard to the requirement of Trk B.

Neurotrophins are a family of diffusible molecules that play crucial roles during neural development and regeneration both in the peripheral and central nervous systems. They include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), NT-4/5 and NT-6. Their biological effects are initiated by binding to specific

transmembrane receptors, the neurotrophin receptors. They have been divided into two subtypes according to their binding affinity: low- and high-affinity neurotrophin receptors. The former so-called low-affinity neurotrophin receptor or p75 NGF receptor (p75-NGFR) is capable of binding all the neurotrophins with similar kinetics, but cannot mediate biological activity. The latter receptors are encoded by proto-oncogenes known as the *Trk* family: Trk A is the receptor for NGF, Trk B for BDNF and NT-4/5, and Trk C binds NT-3, although NT-3 also interacts with Trk A or Trk B (for reviews, BOTHWELL, 1991, 1995; MEAKIN and SHOOTER, 1992).

The periodontal ligament contains at least two types of nerve endings: nociceptive free nerve endings, and mechanoreceptive specialized nerve endings. Many studies have shown that the Ruffini ending, categorized as a slowly-adapting type II mechanoreceptor, is a primary mechanoreceptor in the ligament. Morphologically, the axonal elements of the periodontal Ruffini ending are characterized by extensive arborization, and the association of specialized Schwann cells called lamellar or terminal Schwann cells which possess kidney-shaped nuclei (for reviews, BYERS and MAEDA, 1997; MAEDA et al., 1999). The active remodeling of tissue elements including periodontal fibers and alveolar bone takes place in the ligament in response to the constant occlusal forces and tooth eruption, suggesting that the periodontal nerve endings also possess a high potential for neural plasticity. This hypothesis is supported by the recent immunocytochemical analysis showing that the periodontal Ruffini ending expresses growth-associated protein-43 (GAP-43), a key

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molecule for neural development and regeneration (MAEDA and BYERS, 1996; YOUN et al., 1998). In addition, p75-NGFR has also been detected in the periodontal Ruffini endings (BYERS, 1990; SAITO et al., 1993). It is unknown, however, whether high-affinity neurotrophin receptors are present in these endings.

Previous studies have showed that Trk B mRNA and protein are localized in the medium- to large-sized trigeminal ganglion neurons as well as the mesencephalic trigeminal nucleus neurons (MU et al., 1993; YAN et al., 1997), both sending peripheral processes to the periodontal mechanoreceptors. FOSTER et al. (1995) reported the presence of Trk B-immunoreactive nerve fibers in the dental pulp of the rat molar. Recently, OCHI et al. (1997) demonstrated the presence of Trk B-immunoreactive neural elements in the periodontal ligament of the rat molar, but did not show the detailed morphology of those neural structures. In the present study, therefore, we investigated the ultrastructural localization of Trk B-like immunoreactivity in the periodontal ligament of the rat incisor, particularly in the neural elements.

MATERIALS AND METHODS

Five male Sprague-Dawley rats, weighing 180–200 g, were deeply anesthetized with chloral hydrate (500 mg/kg, i.p.), and perfused transcardially with 0.02 M phosphate-buffered saline (PBS) followed by a mixture of 4% paraformaldehyde and 0.05% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). Upper jaws were dissected out *en bloc* and further fixed in 4% paraformaldehyde in 0.1 M phosphate buffer for an additional 1–2 days at 4°C, and decalcified with 7.5% ethylene diamine tetraacetic acid (EDTA) for 5–6 weeks under gentle agitation. The decalcifying solution was changed at least 3 times a week. Following the completion of decalcification, the specimens were soaked in PBS containing 25% sucrose overnight at 4°C, embedded in Tissue-Tek OCT compound (Sakura Finetechnical Co., Ltd., Tokyo), and rapidly cooled by liquid nitrogen. They were sectioned at a thickness of 40 µm with a cryostat, collected in PBS, and stained as free-floating sections. Some specimens were cut at a thickness of 20 µm, and thaw-mounted onto poly-L-lysine-subbed glass slides. All experiments were reviewed and approved by the intramural Animal Use and Care Committee of Osaka University Faculty of Dentistry prior to the experiments.

Following the blocking of endogenous peroxidase activity with methanol containing 0.3% H₂O₂ for 30 min, these sections were incubated with rabbit polyclonal anti-Trk B serum (1 : 400 : Santa Cruz Biotech-

nologies Inc., Santa Cruz, CA) for 16–18 h at room temperature. Following several rinses in PBS, the sections were incubated with biotinylated swine anti-rabbit IgG (1 : 500; Dako, Denmark) and subsequently with ABC complex (Vector, Burlingame, CA) for 90 min each at room temperature. The horseradish peroxidase (HRP) was visualized by incubation with 0.04% 3-3' diaminobenzidine and 0.003% H₂O₂ in 0.05 M Tris-HCl buffer with nickel ammonium sulfate intensification (0.08–0.1%). The immunostained sections were counterstained with methyl green, dehydrated through an ascending series of ethanol, and coverslipped with Permount (Fisher Scientific, NJ).

For immunoelectron microscopy, HRP was developed without nickel enhancement. The immunostained sections were further fixed with 1% OsO₄ reduced with 1.5% potassium ferrocyanide in 0.1 M cacodylate buffer for 30 min, dehydrated and embedded in Epon 812 resin. Ultrathin sections were prepared with a diamond knife and examined with a Hitachi H-7000 transmission electron microscope (Hitachi, Tokyo), following brief staining with uranyl acetate and lead citrate.

The specificity of the primary antibody against Trk B was tested by preabsorption. The primary antibody was preabsorbed with an excess amount of antigen (10 µg/ml of diluted antibody; Santa Cruz). Sections stained with the preabsorbed antibody showed no immunoreactions, leading us to consider the immunoreactions to be specific for Trk B.

RESULTS

Although Trk B-like immunoreactivity was recognized in both neural and non-neural elements of the periodontal ligament, we shall primarily describe its distribution in the neural elements.

Immunostaining with the antibody against Trk B was able to demonstrate structures with dendritic ramification located in the alveolar half (alveolus-related part) of the periodontal ligament. Occasionally, rounded cells were immunostained closely associated with these structures, but their nuclei were devoid of any immunoreactivity B (Fig. 1).

Under the transmission electron microscope, the localization of immunoreaction products for Trk B was variable between individual periodontal Ruffini endings; some terminal Schwann cells contained the immunoreaction products in their cytoplasm (Fig. 2), while others lacked them (Fig. 3). The number of Trk B-immunoreactive terminal Schwann cells appeared smaller than that of immunonegative ones. Not all Schwann sheaths extending from the terminal

Fig. 1. Immunoreaction for Trk B is observed in the cytoplasm of the rounded cells (*arrowheads*) at the alveolus-related part (*ARP*). Thick Trk B immunoreactive structures (*arrows*) arise from the Trk B immunoreactive cells, showing dendritic ramifications. $\times 500$

Fig. 2. The cytoplasm of a terminal Schwann cell with a kidney-shaped nucleus (*TSC*) contains immunoreaction products. A cell (*F*) which contains rich rough-endoplasmic reticulum also shows a weak immunoreaction. $\times 7,000$

Schwann cells displayed the immunoreactivity for Trk B (Fig. 3). Most of the axon terminals were filled with the immunoreactive products except for the cell organelles (Fig. 3). These axon terminals were usually surrounded by the immunopositive Schwann sheaths, but occasionally by immunonegative Schwann sheaths (Fig. 3). In some cases, both immunopositive and immunonegative Schwann sheaths surrounded an immunopositive axon terminal (Fig. 3, inset). The ordinary (non-terminal) Schwann cells lacked the immunoreactivity (Fig. 4). Near the Ruffini endings, cells which were probably dendritic cells, as evidenced by the presence of tubulovesicular structures in their cytoplasm, showed a strong immunoreaction for Trk B (data not shown). Throughout the entire periodontal ligament, some fibroblasts also exhibited the immunoreactivity for Trk B (Figs. 1, 2).

DISCUSSION

The present immunocytochemical study demonstrated the occurrence of Trk B in both neural and non-neural elements in the periodontal ligament of the rat incisor. The presence of Trk B in non-neural elements such as fibroblasts and dendritic cells was in agreement with a previous report on the rat molar periodontal ligament (OCHI et al., 1997). Interestingly, the present analysis clearly showed that the localiza-

tion of the immunoreactivity for Trk B differed between the individual periodontal Ruffini endings, as immunopositive and immunonegative terminal Schwann cells were intermingled. Moreover, a part of the axon terminals lacked the immunoreactivity. Although we do not have a precise explanation for such heterogeneous staining patterns, one possibility is that the requirement of Trk B differs among the individual Ruffini endings.

Previous immunohistochemical studies (BYERS, 1990; SAITO et al., 1993; MAEDA and BYERS, 1996; YOUNG et al., 1998) have revealed that the terminal Schwann cells associated with the periodontal Ruffini endings exhibit p75-NGFR and GAP-43 — both proteins are up-regulated during development/regeneration, but repressed or down-regulated in mature animals. Moreover, non-specific cholinesterase activity, a representative histochemical marker for specialized Schwann cells in mechanoreceptors, has been detected in immature Schwann cells, not in mature ones (MUNGER and IDE, 1988). This enzyme activity was also found in the terminal Schwann cells of the mature periodontal Ruffini ending (MAEDA et al., 1990). This line of evidence suggests that the terminal Schwann cells associated with periodontal Ruffini endings are immature Schwann cells. Active tissue remodeling usually takes place in the periodontal ligament of the rat incisor due to occlusal forces and continuous eruption. Such a peculiar environment as

Figs. 3 and 4. Immunoelectron microscopic views of Trk B in the terminal (**Fig. 3**) and ordinary Schwann cells (**Fig. 4**) in the lingual periodontal ligament of the incisor.

Fig. 3. A terminal Schwann cell (*TSC*) lacks the immunoreactivity. The Schwann sheaths (*SS*₁) and axon terminals (*AT*₁) are immunoreactive. Another Trk B immunoreactive axon terminal (*AT*₂) is surrounded with an immunonegative Schwann sheath (*SS*₂). **Inset:** Both immunopositive (*SS*₁) and immunonegative (*SS*₂) Schwann sheaths surround an immunopositive axon terminal (*AT*). $\times 7,000$, inset: $\times 7,000$

Fig. 4. Two ordinary Schwann cells (*SC*) are devoid of the immunoreaction. The myelin (*My*) and axon (*Ax*) lack the immunoreaction. $\times 6,000$

the periodontal ligament leads us to speculate that the periodontal nerve fibers have a high potential for neural plasticity. It is suggested that the heterogeneous localization of Trk B between individual periodontal Ruffini endings might reflect their different statuses with regard to neural plasticity.

The origin of the Trk B-immunoreactive axon terminals remains undetermined, though FOSTER et al. (1995) reported Trk B-immunoreactive nerve fibers in the dental pulp of the molar teeth. The pulpal primary afferents are known to be derived from the trigeminal ganglion neurons. Moreover, the injection of an anterograde radioactive tracer ($[^3\text{H}]$ proline) into the trigeminal ganglion labels the Ruffini endings of the rat incisor (BYERS and DONG, 1989). Thus, it seems reasonable to presume that the axon terminals exhibiting immunoreactivity for Trk B in the periodontal Ruffini endings of the rat incisor have their somata in the trigeminal ganglion. However, the possibility of a mesencephalic trigeminal nucleus origin is not fully excluded because both neurons in the trigeminal ganglion and mesencephalic trigeminal nucleus have been demonstrated to be immunoreactive for Trk B (MU et al., 1993; YAN et al., 1997).

The functional implications for Trk B in the periodontal Ruffini endings remain unclear. A denervation study of cutaneous mechanoreceptors suggests that trophic factors from the specialized Schwann cells are essential for the induction of the sensory receptor (DELLON and MUNGER, 1983). It has been revealed that the development of the Ruffini endings in the mystacial pad is severely affected in mice lacking genes for Trk B (FUNDIN et al., 1997). Moreover, BDNF null mutant mice also show deficits in cutaneous Ruffini endings (FUNDIN et al., 1997). Thus, it is easily speculated that Trk B and BDNF, the ligand for Trk B, are essential for the development and maturation of periodontal Ruffini endings. To our knowledge, there is no report on an immunocytochemical analysis of BDNF in the periodontal ligament. Further studies are required to examine the presence of Trk B and BDNF in the periodontal Ruffini endings during development and regeneration to clarify their functional roles in these endings.

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