Nerve Growth Factor Involves Mutual Interaction between Neurons and Satellite Glial Cells in the Rat Trigeminal Ganglion

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Nerve growth factor (NGF) plays a critical role in the trigeminal ganglion (TG) following peripheral nerve damage in the oral region. Although neurons in the TG are surrounded by satellite glial cells (SGCs) that passively support neural function, little is known regarding NGF expression and its interactions with TG neurons and SGCs. This study was performed to examine the expression of NGF in TG neurons and SGCs with nerve damage by experimental tooth movement. An elastic band was inserted between the first and second upper molars of rats. The TG was removed at 0–7 days after tooth movement. Using in situ hybridization, NGF mRNA was expressed in both neurons and SGCs. Immunostaining for NGF demonstrated that during tooth movement the number of NGF-immunoreactive SGCs increased significantly as compared with baseline and reached maximum levels at day 3. Furthermore, the administration of the gap junction inhibitor carbenoxolone at the TG during tooth movement significantly decreased the number of NGF-immunoreactive SGCs. These results suggested that peripheral nerve damage may induce signal transduction from neurons to SGCs via gap junctions, inducing NGF expression in SGCs around neurons, and released NGF may be involved in the restoration of damaged neurons.

Key words: nerve growth factor, neurons, satellite glial cells, trigeminal ganglion, gap junction

I. Introduction

Nerve growth factor (NGF), a prototypical member of the neurotrophin family, is a candidate molecule that activates neurons following its secretion from satellite glial cells (SGCs) in the dorsal root ganglion (DRG) [22]. NGF plays essential roles in the survival and differentiation of sensory neurons. NGF plays a role in sensory neuron responses after peripheral nerve injury, including phenotype maintenance, sprouting, and regeneration [15, 20]. The actions of NGF on responsive cells are initiated by its binding to receptor molecules; NGF binds with low affinity to the p75 neurotrophin receptor (p75NTR) and with high affinity to the tyrosine kinase A (TrkA) receptor [16]. However, the expression of NGF in neurons or SGCs in sensory ganglia remains controversial. One study indicated that NGF mRNA is normally undetectable in sensory ganglia, and that NGF protein is synthesized in the target tissue and transported retrogradely to the responsive sensory neurons [12]. In contrast, Lee et al. [13] and Li et al. [14] found that NGF mRNA is expressed in neurons and SGCs of sensory ganglia. Zhou et al. [23] suggested that NGF plays an autocrine or paracrine role in DRG. Therefore, it is necessary to clarify whether NGF is expressed in the trigeminal ganglion (TG) neurons and/or SGCs.

The TG plays an important role in orofacial pain transmission. In the trigeminal somatosensory system, peri-
odontal tissues in the maxilla and mandible are mainly innervated by primary sensory neurons in the TG. During experimental tooth movement, the branches of the trigeminal nerve are particularly vulnerable to inflammatory or injury responses in periodontal tissues. In response to oral nociceptive stimulation, signals are transmitted to neurons in the TG from peripheral tissue. Each neuronal cell in the sensory ganglion is tightly wrapped by a layer of SGCs [9]. SGCs directly influence neuronal activity by controlling the microenvironment in the ganglion [17]. Capuano et al. [4] reported that SGCs, in addition to providing mechanical and metabolic support to neurons, directly modulate their functions. Recent research has illuminated the complex, mutual communication between SGCs and neurons [5, 11]. In addition, neuronal activity plays an important role in the activation of glial cells in the DRG [21]. Furthermore, a major component regulating the perineuronal ionic environment is the coupling between adjacent SGCs via gap junctions, which allows the rapid transcellular exchange of small molecules. Gap junctions are channels that are formed by members of the connexin family of proteins that allow the direct passage of ions and other small molecules (<1 kDa) from one cell to another [1]. This coupling is dependent on physiological and pathological conditions, and the numbers of gap junctions between SGCs increase markedly in response to nerve injury [8, 10]. The existence of gap junctions between neurons and SGCs in the TG was indicated by the distribution of connexins [6], whereas Suadicani et al. [18] reported that calcium signaling from SGCs to neurons and among SGCs was reduced in the presence of the broad-spectrum P2 receptor (ATP receptor) antagonist suramin or the gap junction blocker carbenoxolone. In contrast, suramin, but not carbenoxolone, reduced signaling from neurons to SGCs. Therefore, we postulated that the glial gap junctions between neurons and surrounding SGCs are involved in the signal transduction from neurons to SGCs.

The present study was performed to investigate the expression of NGF in TG neurons and SGCs with degeneration of the nerve fibers of the periodontal membrane on the pressure side and stretching and tearing of nerve fibers on the tension side associated with orthodontic tooth movement. First, we demonstrated that NGF mRNA is expressed in neurons and SGCs in the TG using in situ hybridization. Second, we investigated the temporal changes in NGF expression in neurons and SGCs after application of experimental tooth movement. Finally, we examined the changes in NGF expression in the presence of the gap junction inhibitor carbenoxolone, which blocked the gap junctions between neurons and SGC after tooth movement.

II. Materials and Methods

Animals

All experimental protocols involving rats were reviewed and approved by the Animal Care Committee of Kyushu Dental University (Permission number: 10-010). Fifty-eight male Sprague-Dawley rats weighing 200–250 g each were used. The animals were acclimated for at least 1 week prior to the start of the experiment.

Experimental tooth movement

The rats were anesthetized with an intramuscular injection of ketamine (70 mg/kg; Daiichi Pharmaceutical, Tokyo, Japan) and xylazine (13 mg/kg; Bayer, Tokyo, Japan). An elastic band was inserted between left maxillary first and second upper molars. The rats treated were fed powdered food. Untreated animals were used as controls.

In situ hybridization

To examine the localization of NGF mRNA, normal rats (n=6) were anesthetized with diethyl ether and perfused transcardially with 4% paraformaldehyde (PFA) in 0.2 M phosphate buffer. The TG and submandibular gland, as a positive control, were then removed and postfixed in the same fixative overnight. The tissues were rapidly frozen and then cut into sections 6 μm thick using a cryostat. The frozen sections were hybridized with digoxigenin-labeled antisense riboprobes corresponding to partial cDNA of NGF. Plasmid DNA was a gift from Dr. Michael W. Miller (State University of New York-Upstate Medical University) [3] (Table 1). Using T7 or SP6 RNA polymerase (Invitrogen, Carlsbad, CA, USA), antisense and sense riboprobes were transcribed in vitro in the presence of DIG-labeled nucleotides (Roche Applied Science, Indianapolis, IN, USA). Sections were fixed in 4% PFA for 5 min and washed in phosphate-buffered saline (PBS, pH 7.4). For partial proteolysis, sections were permeabilized with 1 μg/ml proteinase K in 0.1 M PBS at 37°C for 10 min, followed by a 5-min wash in PBS. The sections were fixed again in 4% PFA for 10 min. For neutralization, sections were washed in 0.2% glycine in PBS for 5 min. Endogenous alkaline phosphatase activity was quenched with 0.2 N HCl for 30 min and washed in PBS for 5 min. After prehybridization for 5 min, hybridization was carried out at high stringency (1.3× standard saline citrate (SSC), 50% formamide, 57°C). Sections were washed in decreasing concentrations of SSC as follows: 5× SSC at room temperature (RT) for 30 min, 5× SSC/50% formamide at 50°C for 1 hr, 2× SSC at 50°C for 1 hr, and 0.2× SSC at 50°C for 1 hr. The sections were incubated at RT in 1%
blocking buffer (Roche Applied Science) for 1 hr, followed
by reaction with anti-alkaline phosphatase antibody at 1:500
dilution in 1% blocking buffer at 4°C overnight. For detec-
tion, signals were developed using nitroblue tetrazolium
(NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP)
substrate (Roche Applied Science). Negative controls for in
situ hybridization were carried out using sense probes.

Real-time polymerase chain reaction
Control rats (n=5) and experimental rats at 2 and 4
days after tooth movement (n=11) were killed by sodium
tetobarbital injection. The TG of the experimental side
was removed, homogenized in a Nippi BioMasher II (Nippi,
Tokyo, Japan) with TRIzol Reagent (Invitrogen), and
allowed to stand for 5 min at room temperature. Total
RNA was dissolved in RNase-free water. The samples
were then treated with DNase. Total RNA was reverse
transcribed with random primers using a Superscript first-
strand kit (Invitrogen). Real-time PCR was performed using the TaqMan Gene Expression Assay (Applied Biosystems,
Foster City, CA, USA) and Eco Real Time PCR system
(Illumina, San Diego, CA, USA). The TaqMan primer ID
was NGF Rn01533872_m1. The level of target gene
expression was normalized relative to that of β-actin and
expressed relative to the control. The experiment was per-
formed five times in each group.

Cannula implant for injection in the maxillary division of
the trigeminal ganglion
The skull was exposed and a burr hole was drilled
above the maxillary division of the left TG 6.5 mm anterior
to interaural zero and 2.3 mm lateral to the midline. A guide
cannula was fixed to the skull over the burr hole using two
stainless steel screws and dental quick cure resin. The guide
cannula extended into the burr hole 1 mm below the
pedestal, but did not touch the surface of the cortex. At least
7 days were allowed for recovery from surgery before
injection into the TG. After 7 days, each animal was given
an injection of 10 μM carbenoxolone (LKT Laboratories,
St. Paul, MN, USA), a gap junction-specific blocker, or
normal saline. Before the injection, the rats were lightly
anesthetized with isoflurane. A 24-gauge beveled stainless
steel cannula was inserted through the guide cannula
(positioned over the maxillary division of the left TG as
described above) to 9.5 mm below the cortical surface. The
injection cannula was connected to a 200-μl osmotic pump
(ALZET mini-osmotic pump, model 2001; Alza, Palo Alto,
CA, USA) attached to a microinjection pump set to deliver
1.0 μl over a period of 1 hr. The rats (n=11) had an elastic
band inserted between their first and second upper molars.
Cannulated rats were sacrificed on the third day after
carbenoxolone injection. The experiment was performed
five times in each group.

Tissue preparation
One, 3, 5, and 7 days after tooth movement, the rats
(n=25, including the controls; the experiment was per-
formed five times in each group) were anesthetized with
diethyl ether and perfused transcardially with 4% PFA in
0.2 M phosphate buffer containing 0.2% picric acid. The
TG of the experimental side was then removed and post-
fixed in the same fixative overnight.

Immunohistochemistry
The TG was frozen rapidly and then cut into sections
6 μm thick using a cryostat (Leica Instruments, Wetzlar,
Germany). Immunohistochemical staining with double-
labeling for NGF and glutamine synthetase (GS), a satellite
glial cell marker, was performed as described below. First,
the sections were preincubated in 0.1 M PBS with 1%
normal goat serum (ICN Pharmaceuticals, Costa Mesa, CA,
USA) for 30 min at room temperature. The sections were
incubated with sheep polyclonal antibodies against NGF
(1:100; Abcam, Cambridge, MA, USA) for 2 hr at 37°C.
After rinsing with 0.1 M PBS, the sections were incubated with FITC-conjugated donkey anti-sheep IgG (1:400;
Molecular Probes, Eugene, OR, USA) for 2 hr at 37°C.
After rinsing with 0.1 M PBS, the sections were incubated with rabbit polyclonal antibodies against GS (1:200; Sigma,
St. Louis, MO, USA) washed in PBS, and incubated with TRITC-conjugated goat anti-rabbit IgG (1:400; Molecular
Probes) for 2 hr at 37°C. Negative control sections were
incubated with secondary antibody in buffer alone. The
sections were washed in 0.1 M PBS and covered with
coverslips.

Cell count
Images were acquired using a BIORÉVO BZ-9000
fluorescence microscope (Keyence, Osaka, Japan). Ten
randomly selected sections were obtained from each group.
Images were obtained from two non-overlapping areas of
each section. The size of one field was 221×166 μm, and
each field included approximately 40 cells. The images
were obtained at a standard exposure time across the
slides. The images were analyzed quantitatively using NIH
ImageJ software (NIH, Bethesda, MD, USA). Positive
SGCs were identified based on a pixel intensity value
over the threshold (background pixel intensity ±5 SD).
Individual neurons were selected with the freehand outline
tool in ImageJ on a pen tablet (XP-8060A; Active, Taipei,
Taiwan). The outlined neurons were saved, and measure-
ments were taken in the neuron area. The total number of
neurons enclosed by immunopositive neurons and neurons
surrounded by positive SGCs in the maxillary nerve region
was determined for each section. Only those neurons with
visible nuclei were counted.

Statistical analysis
One-way analysis of variance (ANOVA) was used to
determine the ratio of neurons with NGF-immunoreactive
(NGF-IR) neurons and neurons surrounded by NGF-IR
SGCs, followed by individual post hoc comparisons
(Scheffé’s test).
III. Results

Expression of NGF mRNA

To determine whether NGF mRNA is expressed in the neurons and SGCs of the TG, we performed in situ hybridization in specimens from untreated rats. The expression of NGF mRNA was confirmed in the submandibular gland as a positive control with the antisense probe (Fig. 1A). In TG, NGF mRNA was expressed in both neurons and the SGCs surrounding the neurons (Fig. 1C, D). No labeling was found with the NGF sense probe (Fig. 1B, E).

NGF mRNA expression in the trigeminal ganglion after tooth movement

The expression levels of NGF mRNA in the TG after tooth movement were analyzed by real-time PCR. Expression of NGF mRNA was significantly increased on day 2 compared with controls and then decreased on day 4 after tooth movement (Fig. 2).

NGF-IR neurons and SGCs after tooth movement

Temporal changes in the NGF-IR neurons and SGCs were examined in the maxillary nerve region of the TG. We used GS as a marker for SGCs. Three types of NGF-IR were observed: the neuron was immunopositive for NGF, but surrounding SGCs were negative [N(+)S(–), Fig. 3A]; both the neuron and surrounding SGCs were positive for NGF [N(+)+S(+), Fig. 3B]; and the SGCs were positive for NGF, but the neuron they surrounded was negative [N(–)S(+), Fig. 3C]. For NGF-IR neurons, we measured both N(+)+S(–) and N(+)+S(+) types. For NGF-IR SGCs, we measured the number of neurons surrounded by NGF-IR SGCs [i.e., N(–)+S(+) and N(–)+S(+) types] rather than counting the number of NGF-IR SGCs.

In the untreated controls, about 36.2% of the neurons were NGF-IR, and about 23.8% of the neurons were surrounded by NGF-IR SGCs (Fig. 4A–C). One day after tooth movement, the number of NGF-IR neurons [N(+)+S(–) and N(+)+S(+)] and the number of neurons surrounded by NGF-IR SGCs [(N(–)+S(+))] were increased slightly as compared with controls (NGF-IR neurons: 38.1% of the neurons in the maxillary nerve region; neurons surrounded by NGF-IR SGCs: 27.8% of the neurons in the maxillary nerve region) (Fig. 4D–F). Three days after tooth movement, the number of NGF-IR neurons was decreased, while the number of neurons surrounded by NGF-IR SGCs was increased significantly as compared with controls (NGF-IR neurons: 26.6% of the neurons in the maxillary nerve region, neurons surrounded by NGF-IR SGCs: 57.9% of the neurons in the maxillary nerve region) (Fig. 4G–I). Five days after tooth movement, the number of NGF-IR neurons was then increased, while neurons surrounded by NGF-IR SGCs were decreased as compared with 3 days after tooth movement (NGF-IR neurons: 50.9% of the neurons in the maxillary nerve region; neurons surrounded by NGF-IR SGCs: 37.1% of the neurons in the maxillary nerve region) (Fig. 4J–L). Seven days after tooth movement, the number of NGF-IR neurons had not changed as compared with the untreated controls.
Fig. 3. NGF-IR at satellite glial cells (SGCs) or neurons in the trigeminal ganglion (TG). Three types of NGF-IR were observed: (A) the neuron was immunopositive for NGF (arrow), but surrounding SGCs were negative [N(+)S(−)]; (B) both the neuron and surrounding SGCs (arrowhead) were positive [N(+)S(+)]; and (C) the SGCs were positive, but the neuron they surrounded was negative [N(−)S(+)]. Green: NGF-IR cells; red: GS-immunoreactive cells; and yellow: NGF and GS double positive cells. Bars=10 μm (A, B, and C).

Fig. 4. Distribution of NGF-IR cells and GS-immunoreactive (GS-IR) satellite cells in the maxillary nerve region of the trigeminal ganglion. NGF-IR cells (A, D, G, J, and M) and GS-IR satellite cells (B, E, H, K, and N) in control rats (A and B) and in rats at 1 (D and E), 3 (G and H), 5 (J and K), and 7 days (M and N) after tooth movement. (C, F, I, L, and O) are merged images of (A, D, G, J, and M) and (B, E, H, K, and N). Arrows in (A): NGF-IR neurons; arrowheads in (G): NGF-IR satellite cells. Bars=50 μm (A).
fifth day, while neurons surrounded by NGF-IR SGCs were increased slightly (NGF-IR neurons: 51.6% of the neurons in the maxillary nerve region; neurons surrounded by NGF-IR SGCs: 45.2% of the neurons in the maxillary nerve region) (Fig. 4M–O).

The number of NGF-IR neurons was decreased on day 3 and then increased on day 5, whereas the number of neurons surrounded by NGF-IR SGCs was increased on day 3 and then decreased on day 5 (Fig. 5A). Next, we examined the size-dependent changes in NGF-IR neurons. Both the NGF-IR neurons and the neurons surrounded by NGF-IR SGCs were mainly small (<500 μm²) or medium (500–1,200 μm²) in size (Fig. 5Ba, b). The ratio of small NGF-IR neurons decreased on day 3 and then increased on day 5, whereas the ratio of medium and large NGF-IR neurons remained constant (Fig. 5Ba). The ratio of small and medium-size neurons surrounded by NGF-IR SGCs was increased on day 3 and then decreased on day 5 (Fig. 5Bb).

NGF-IR neurons and SGCs after tooth movement when gap junctions were inhibited

We examined whether blocking of gap junctions with 10 μM carbenoxolone would alter the expression of NGF in neurons and SGCs after tooth movement. In the group injected with normal saline, the numbers of NGF-IR neurons and neurons surrounded by NGF-IR SGCs were similar to those in the no-administration group 3 days after tooth movement (Fig. 6A–C vs. Fig. 4G–I). However, 3
Fig. 6. The effects of carbenoxolone on the distribution of NGF-IR cells and GS-IR satellite cells. NGF-IR neurons and GS-IR SGCs in the maxillary nerve region of the TG 3 days after experimental tooth movement were stained using specific antibodies against NGF and GS, respectively. NGF-IR cells (A and D) and GS-IR satellite cells (B and E) in rats at 3 days. (C and F) are merged images of (A and D) and (B and E), respectively. (A–C) The rats were administered saline. (D–F) The rats were administered carbenoxolone. Arrowheads in (A): NGF-IR satellite cells; arrows in (D): NGF-IR neurons. Bars=30 μm (A).

Fig. 7. The effects of carbenoxolone on the ratio of NGF-IR neurons and neurons surrounded by NGF-IR satellite cells at 3 days after experimental tooth movement (ETM). (A) The ratio of NGF-IR neurons to all neurons in the maxillary nerve region. (B) The ratio of neurons surrounded by NGF-IR satellite cells to all neurons in the maxillary nerve region. The results are the means±SEM. *P<0.01 as compared with [Drug (+), ETM (+)].
IV. Discussion

In conclusion, this study demonstrated that experimental tooth movement induced signal transduction from the damaged neurons to the surrounding SGCs via gap junctions. The SGCs produce the NGF that induces activation of the damaged neurons. These findings suggest that there is a positive feedback loop between damaged neurons and surrounding satellite glial cells in the TG.

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VI. References


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