Temporomandibular Joint Inflammation Potentiates the Excitability of Trigeminal Root Ganglion Neurons Innervating the Facial Skin in Rats

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INTRODUCTION
Since it has been reported that peripheral inflammation induces a release of neurotransmitters from the central terminal (Bereiter and Benetii 1996; Carleson et al. 1996; Garry and Hargreaves 1992) and increases the excitability of spinal dorsal horn neurons, the sensitization of the CNS is thought to contribute to inflammation-induced hyperalgesia (Coderre et al. 1993; Dubner and Ruda 1992; Ren et al. 1994). Moreover, there are many reports that inflammation activates silent nociceptors (i.e., receptive field-unresponsive) and potentiates the activity of dorsal root ganglion (DRG) nociceptive neurons (Schaible and Schmidt 1985, 1988; Xu and Zhao 2001), indicating that sensitization of nociceptive primary sensory neurons results in hyperalgesia (Noguchi et al. 1994).

Recent electrophysiological studies have shown that the activity of neighboring neurons elicits functional cross-excitation in the somata of affected sensory neurons in normal conditions (Amir and Devor 1996, 2000; Oh and Weinreich 2001), indicating that a nonsynaptically released diffusible chemical messenger may modify the somatic excitability of neurons within the sensory ganglia. In fact, there is a report that substance P (SP) is released from the somata of DRG neurons in vitro (Huang and Neher 1996). Recently, Matsuka et al. (2001) reported that SP could serve as a chemical messenger that mediates a nonsynaptic cross-excitation in trigeminal root ganglion (TRG) neurons. However, the relationships between the expression of SP receptors and the change in the electrophysiological properties of TRG neurons during the inflammatory pain state remain to be determined. In the trigeminal system, a complete Freund’s adjuvant (CFA) model of inflammation of the orofacial region has recently been developed in a persistent behavioral allodynia (Imbe et al. 2001; Iwata et al. 1999), and a persistent temporomandibular joint (TMJ) inflammation results in a hyperexcitability of the medullary dorsal horn neuronal activity (Iwata et al. 1999). TMJ inflammation is associated with spreading pain and hyperalgesia and/or allodynia (Sessle and Hu 1991). In fact, TMJ disorder patients complain of pain due to innocuous vibratory stimulation (Fillingim et al. 1998). Neubert et al. (2000) reported that SP release via a paracrine mechanism in the TRG was greatly increased after orofacial inflammation. Since application of SP to TRG neurons in a slice preparation can produce reversible depolarization (Spigelman and Puil 1990), it is possible that SP release within the TRG may modify the neighboring cells’ excitability after TMJ inflammation.

In general, peripheral inflammation-induced neuronal changes are observed in the DRG neurons innervating the inflamed area but not in the neurons innervating the neighboring noninflamed skin (Leslie et al. 1995). However, in inferior alveolar nerve transection rats, Iwata et al. (2001) found that the whisker pad area innervated by the second branches of
trigeminal nerves became hypersensitive to innoxious mechanical stimulation, suggesting that development of the mechanical allodynia occurs even in the area innervated by the uninjured nerves. A recent study has shown that changes in the excitability of A-fibers (medium and large TRG neurons) are mainly involved in inferior alveolar nerve injury–induced mechanical allodynia in the infraorbital nerve territory (Tsuboi et al. 2004).

Previous behavioral data indicated allodynia in perioral regions following TMJ inflammation (Sessle and Hu 1991). These results suggest that inflammation may lead to allodynia and changes in afferent properties in regions outside the area of inflammation. Thus the question arises as to whether a local release of SP within the TRG via a paracrine mechanism modifies the excitability of medium- and large-diameter TRG neurons, corresponding to the mechanical allodynia seen after the TMJ inflammation. To examine the sensory spreading phenomenon, the trigeminal system can provide an opportunity because the innervation territory of its three branches is well demarcated. To date, there have been no studies examining the effect of SP on the excitability of medium and large diameter TRG neurons innervating the facial cutaneous area. It is known that SP binds to a neurokinin receptor 1 (NK1-R) (Cao et al. 1998; Otsuka and Yoshioka 1993), but there are no reports showing the expression of mRNA and functional protein for NK1 receptor as well as their expression in TMJ inflammation.

Therefore the aim of this study was to investigate the basis of mechanical allodynia/neuronal changes in sensitivity at a site remote from the inflammation. We conducted experiments to clarify the following points: 1) to examine whether TMJ inflammation increases the SP and SP/NK1-R expression in TRG neurons innervating the TMJ and facial skin, respectively; 2) to investigate whether SP modifies the activity of the medium- to large-diameter TRG neurons innervating the facial cutaneous area after TMJ inflammation, by means of the perforated patch-clamp technique; and 3) to determine whether medium- and large-diameter TRG neurons express a message of mRNA and a functional protein of NK1 receptor by means of quantitative RT-PCR analysis and the immunohistochemical techniques.

**Methods**

The experiments were approved by the animal use and care committee of Nippon Dental University and were consistent with the ethical guidelines of the International Association for the study of Pain (Zimmermann 1983). For each experiment, the experimenters were blind to the experimental conditions. Efforts were made to minimize the number of animals used and their suffering.

**Induction of TMJ inflammation**

The experiments were performed on seventy-eight adult male Wistar rats (100–170 g body weight). Each animal was anesthetized with sodium pentobarbital (45 mg/kg, ip), and CFA (0.05 ml 1:1 oil/saline suspension) was injected into the left side of the TMJ capsule, as described in previous studies (Iwata et al. 1999; Ren 1999). In control rats, vehicle (0.9% NaCl) was injected into the TMJ capsule. In some experiments (n = 3), the CFA-induced inflammation was verified with Evan’s blue dye (50 mg/ml, 1 ml/kg, iv) extravasation. Postmortem examination of the injected TMJ region found accumulation of the blue dye in the TMJ and periaricular tissues, indicating plasma protein extravasation due to localized inflammation (Imbe et al. 2001). In preliminary experiments, we obtained evidence that there was no sign of inflammation of the overlying skin after CFA injection into the TMJ using the conventional histological method. Thus we confirmed that CFA induced only a local inflammatory reaction but did not affect the overlying skin. One day after CFA injection, the animals were checked for abnormal pain sensations by probing the injected sites and surrounding orofacial skin with von Frey filaments. The von Frey hair mechanical threshold (calibrated with a force transducer) was applied to the whisker pad (Takeda et al. 2000), and the mechanical threshold of escape behavior was measured in inflamed and naïve rats.

**Mechanical threshold for escape behavior**

In this study, mechanical threshold for escape behavior was conducted as described in a previous study (Ren et al. 1999). Two days after CFA or vehicle injection into the TMJ, the rat was habituated to lean against the hand of the experimenter who wore a regular leather work glove. The habituation required no more than normal petting of the rat, and it can be achieved within 20–30 min. The smell that developed on the glove through the handling of the animals seemed to facilitate the habituation. The ipsilateral and contralateral facial skin regions were tested, and the vibrisseae were carefully shaved. The von Frey hair mechanical threshold (calibrated with a force transducer) was applied to the whisker pad (Takeda et al. 2000), and the mechanical threshold of escape behavior was measured in naïve and inflamed rats. Each filament was tested three times at intervals of a few seconds. If head withdrawal was observed at least three times after probing with a filament, the rat was considered responsive to that filament. The response threshold was defined as the mean value for three trials.

**Retrograde-labeling of TRG neurons innervating TMJ and/or facial skin**

On the day of CFA/vehicle injection, to identify TRG neurons innervating the TMJ and/or facial skin, we used the Fluorogold (FG) labeling method (Takeda et al. 2004b, 2005). FG solution (2% in distilled water, 20 μl) was injected subcutaneously into the whisker pad and adjacent facial skin region in the ipsilateral region of CFA/vehicle injection as described in a previous study.

**Immunohistochemistry for TRG neurons innervating TMJ and/or facial skin**

The immunohistochemistry was conducted 2 days after CFA or vehicle injection and used the modified method described in previous studies (Takeda et al., 2004b, 2005). In the immunohistochemical study to identify both SP and NK1 receptors, we used three naïve and three inflamed rats. FG-injected rats (n = 9) were anesthetized with pentobarbital sodium (50 mg/kg, ip) and transcardially perfused with 50 ml heparinized 0.01 M PBS followed by 100 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.3). The left TRG was removed and incubated in 4, 10, and 20% sucrose solution (5 min × 3 times, 1 and 2 h, respectively) and 30% overnight. Frozen tissue was sectioned at 10 μm with a cryostat (Leica; Mannheim, Germany), and sections were mounted on silane-coated glass slides. Every third section was used for immunohistochemistry, and we analyzed 20 sections per ganglion. To show SP and/or NK1-R stainings, a fluorescent-immunohistochemical method was performed. Sections were incubated with rabbit anti-substance P (1:1,000; Chemicon) and/or rabbit anti-NK1-R serum (1: 500; Calbiochem) for 24 h at 4°C, followed by Alexa 568 goat anti-rabbit IgG (1:1,000; Molecular Probes). After washing (5 min × 2 times) in 0.01 M PBS, sections were incubated with mouse anti-NF200 monoclonal for a marker of the myelinated fiber (phosphorylated and nonphosphorylated neurofilament protein 200 clone N52, 1:1,000; Sigma) for 24 h at 4°C.
followed by Alexa 488 goat anti-mouse IgG (1:1,000; Molecular Probes). Labeled cryosections were rinsed consecutively in 0.01 M PBS for 5 min each. The samples were mounted with antifade-mounting medium (Molecular Probes). To determine whether the SP is increased in the TRG neurons innervating the TMJ region in inflamed rats, we compared the numbers of SP/NF200-positive (methylene blue fiber marker) and SP/NF200-negative neurons per section in naïve and inflamed rats. Also to determine whether SP and NK1-R are increased in the large TRG neurons in inflamed rats, we compared the numbers of SP- and NK1-R/NF200-positive neurons per section in naïve and inflamed rats. The control experiment was conducted without the first antibody. We have basically determined whether neurons express SP/NK1-R immunoreactivity compared with the background staining. The neurons with the least intense staining compared with those with primary antibody omitted was considered to be positive.

Digital images were collected and stored on a laboratory computer and later analyzed with Adobe Photoshop version 7.0 and a Leica Imaging Analysis Tool. Confocal images were generated in a Leica TCS NT laser scanning microscope (Leica).

Acute cell dissociation and whole cell patch-clamp recording

The patch-clamp recording was conducted 2 days after CFA or vehicle injection. Acute dissociation of TRG neurons was performed by a modified method of previous studies (Ikeda and Matsumoto 2003; Takeda et al. 2002a,b). For acute dissociation of trigeminal ganglion neurons, adult rats were decapitated. The left TRG was rapidly removed and incubated in modified Hank’s balanced salt solution (HBSS) containing (in mM) 130 NaCl, 5 KCl, 0.3 KH2PO4, 4 NaHCO3, 0.3 Na2HPO4, 5.6 glucose, and 10 HEPES, pH 7.3. They were incubated for 15–25 min at 37°C in HBSS containing collagenase-type XI (2 mg/ml; Sigma) and type II (2 mg/ml; Sigma). The cells were dissociated by trituration with a fire-polished Pasteur pipette and subsequently were plated onto poly-L-lysine–pretreated 35-mm dishes. The plating medium contained Leibovitz’s L-15 solution (Invitrogen) supplemented with (in mM) 10% newborn calf serum, 26 NaHCO3, and 30 glucose. The cells were maintained at 37°C in a humidified atmosphere of 95% air-5%CO2 and were used for recording between 2 and 8 h after plating. After incubation, poly-L-lysine–coated coverslips were moved into the recording chamber in a standard external solution containing (in mM) 155 NaCl, 3 KCl, 1 CaCl2, 1 MgCl2, 10 HEPES, and 20 glucose, pH 7.3. The recording chamber (volume, 0.5 ml) was mounted on an inverted microscope (Nikon) equipped with phase-contrast, a video camera, and two micromanipulators. The chamber was perfused under gravity with standard external solution at ~0.5 ml/min.

Whole cell recordings were conducted with the rapid perforated-patch technique (Takeda et al. 2002a,b, 2004a,b). Fire-polished patch-pipettes (2–5 MΩ) were filled with an internal solution containing (in mM) 120 potassium methanesulphonate, 20 KCl, 7.5 HEPES, and 2 EGTA, pH 7.3, with amphotericin B (100 μg/ml) and Lucifer yellow dipotassium salt (0.1%; Sigma). Current- and voltage-clamp recordings were conducted with an Axopatch 200B amplifier (Axon Instruments). Signals were low-pass filtered at 1 or 5 kHz and digitized at 10 kHz. To monitor changes in cell membrane resistance during recordings in the current-clamp mode, negative current pulses (50–600 pA, 250 ms, 0.2 Hz) were injected through the patch pipette. None of the cells displayed Lucifer yellow fluorescence, even when a low series resistance, resulting from patch perforation, was established. However, fluorescence could be observed when the membrane was ruptured by applying a negative pressure, and in that case, the resistance dropped to a lower value (usually <8 MΩ). All recordings were performed at room temperature. Data were stored in a computer disk for off-line analysis.

Application of drugs

All drugs were stored at -20°C, and stock solution was dissolved in distilled water (1 mM). On the day of the experiment, we dissolved the drugs in a standard external solution SP (ICN Biomedicals) and an NK1 receptor antagonist, L703,606 (Sigma), which were added to the perfusion for a period ranging from 30 to 60 s. In this study, the spontaneous fluctuations in membrane potential were <1 mV under experimental conditions. Thus we determined that the criterion for response to SP was 1-mV minimum depolarization. After confirmation of SP responsive cells, we further examined whether SP induced depolarization was blocked by the NK1 antagonist. In the current-clamp mode, the membrane potential in most cells was recovered within 10–15 min. In the voltage-clamp mode, repetitive 0.1-mM SP applications at 60-s intervals did not show any sign of desensitization. To determine the difference between naïve and inflamed rats in SP-evoked current density, some neurons were tested as described in a previous study (Takeda et al. 2002a) as follows: SP was pressure-ejected (Pneumatic Picopump, PV 800, WPI) through three-barreled pipettes (total tip diameter, 30 μm) positioned ~50 µm away from the TRG neurons (2–10 s injections, 7–11 psi; interval, 60 s). In a current-clamp mode, we examined whether SP rapid application (by using pressure injection) induces a rapid depolarization with repetitive discharges. To examine the effect of SP on the inward current, we tested whether the change in the inward current in response to SP application is blocked by pretreatment with an NK1 receptor blocker.

Data analysis

Data were stored on a computer disk for off-line analysis (pClamp 8.0). The values are expressed as means ± SE. Statistical analysis for behavioral test was made with Duncan’s new multiple range test. Data for immunohistochemistry and RT-PCR studies were analyzed by nonparametric statistical analysis (Mann-Whitney U test), whereas electrophysiological data were analyzed by parametric statistical analysis (a 2-tailed Student’s t-test). P < 0.05 was considered significant.

Quantitative RT-PCR with RNA extract from trigeminal ganglia and single cell RT-PCR

Total RNA was isolated from whole trigeminal ganglia of the rat (n = 3) with a Perfect RNA Eukaryotic kit (Eppendorf, Germany) as described in our previous study (Takeda et al. 2004b). Total RNA was treated with DNase (Invitrogen) at room temperature for 15 min to remove any contaminating genomic DNA. The enzyme was removed by a phenol-chloroform extraction. Optical density reading was performed to estimate the amount of total RNA before it was used in RT-PCR. The purity of the total RNA purification yielded an A260/A280 ratio of 1.9–2.0. The RT-PCR was conducted with the RT Applied Biosystems Kit (Applied Biosystems). A total 10-μl reaction volume containing 200 ng of total RNA, 1× buffer, 2.5 μM random hexamer, 5.5 mM MgCl2, 500 μM each of dNTP, 4 U RNase inhibitor, and 12.5 U RNase was incubated at 25°C for 10 min, transcribed at 48°C for 30 min, and terminated by heating at 95°C for 5 min. PCR was performed (Applied Biosystems TaqMan Universal PCR master mix, TaqMan rodent GAPDH control reagents, tachykinin 1 receptor gene expression assay kit; ID Rn00562004_m1) in a 50-μl reaction volume containing 5 μl RT product. Amplification was performed in an ABI 7700 sequence detection system (Applied Biosystems). The PCR products were separated on 1.5% agarose gel stained with SYBR Green I (Molecular Probes). Single-cell RT-PCR was performed by a modification of a technique used in previous studies (Takeda et al. 2002a,b). Cells were acutely dissociated and identified as FG-labeled large-diameter TRG neurons in both naïve and inflamed rats. Each dissociated neuron was aspirated into a pipette (tip diameter, 15–20 μm) containing RT buffer (7.7 μl). Care was taken to avoid aspiration of any cell debris. In this study, we used
careful inspection to avoid aspirating satellite cells along with neurons. The contents of the pipette were transferred into a 500-µl tube and freeze/thawed and vortexed to release RNA. Real-time RT-PCR was performed to quantify the relative expression levels (NK1-R/GAPDH) with the kits described above in an ABI 7700 sequence detection system (Applied Biosystems) (Haslett et al. 2002). Reaction mixtures lacking cDNA (no-template controls) were included during each session to assess contamination and nonspecific amplification. To examine NK1-R primer efficiencies, a standard curve was generated for each primer pair by regression analysis of PCR amplification on log10 serial dilutions of cDNA. Quantification for the ratio of NK1-R/GAPDH was performed by the comparative Ct method (Applied Biosystems). We confirmed that PCR amplification efficiency for GAPDH was the same as that for NK1-R, and we calculated the amount of mRNA expression in the difference between NK1-R and GAPDH as the ratio of the number of cycles in rising fluorescence intensity.

RESULTS

Induction of TMJ inflammation and allodynia

After CFA injection, the animals showed abnormal pain sensation on probing the injected site and/or the orofacial skin with von Frey filaments. The CFA-induced inflammation was verified with Evan’s blue dye extravasations. On postmortem examination, accumulation of the blue dye in the TMJ and periarticular tissue was found, indicating that plasma protein extravasation occurred after local inflammation. In the TMJ-inflamed rats, the ipsilateral threshold for escape from mechanical stimulation applied to the whisker pad area was significantly reduced from 53.5 ± 8.4 to 20.5 ± 3.2 mN at 48 h after CFA injection (Fig. 1; n = 10, P < 0.05). No significant changes in the contralateral threshold were observed in the whisker pad area (naïve vs. inflamed; 50.3 ± 7.1 vs. 47.2 ± 8.9 mN, n = 10, P < 0.05)

Inflammation resulted in an increase in the number of SP-positive neurons. The number of SP-/NF-negative neurons in inflamed rats was significantly increased compared with that in the naïve rats (naïve vs. inflamed; 1.1 ± 0.1 vs. 1.5 ± 0.1/section). Also, the number of SP/NF-positive neurons was significantly increased after TMJ inflammation (naïve vs. inflamed; 0.5 ± 0.1 vs. 0.7 ± 0.1/section). Figure 2, D and H, shows control sections without the first antibody.

Difference between naïve and inflamed rats in expression of the functional NK1 receptor in the TRG neurons innervating facial skin

The locations of FG-positive neuronal soma after FG injection into the TMJ corresponded to those in the second and third branches of the trigeminal nerve (Gregg and Dixon 1973; Ichikawa et al. 2004) in both naïve and inflamed rats. The area innervated by the second and third branches of the trigeminal nerve cell bodies were retrogradely labeled by FG in both the naïve (205/879, 23.3%) and inflamed (203/1,040, 19.5%) rats. There were no significant differences in the number of the FG-labeled neurons between naïve and inflamed rats. SP-immunoreactive cells were observed in all sections of the TRG in both naïve (84/205, 41.0%) and inflamed (123/203, 60.1%) rats. In this study, we classified TRG cell bodies by size as small (<30 µm), medium (30–39 µm), and large (>40 µm). As shown in Fig. 2, A–G and I, both in the naïve and inflamed rats, the most FG-labeled SP-positive neurons were observed in small-diameter TRG neurons. Medium-diameter neurons also expressed a marker for myelinated fiber, NF200 (Ma 2002), in both naïve and inflamed rats. As shown in Fig. 2I, there was a tendency for inflammation result in an increase in the number of SP-positive neurons. The number of SP-/NF-negative neurons in inflamed rats was significantly increased compared with that in the naïve rats (naïve vs. inflamed; 1.1 ± 0.1 vs. 1.5 ± 0.1/section). Also, the number of SP/NF-positive neurons was significantly increased after TMJ inflammation (naïve vs. inflamed; 0.5 ± 0.1 vs. 0.7 ± 0.1/section). Figure 2, D and H, shows control sections without the first antibody.
and 40–50 μm diameter TRG neurons) in the size of profiles, resulting from an increase in the number of NK1 receptor/NF 200-positive immunoreactivities (Fig. 3J). The numbers of NK1/NF 200-positive neurons in inflamed rats were significantly greater than those in naïve rats (naïve vs. inflamed; 4.3 ± 0.5 vs. 8.3 ± 0.5/section, n = 15, P < 0.05). Figure 3, D and H, shows control sections without the first antibody.

Increase in SP-immunoreactive TRG neurons innervating facial skin after TMJ inflammation

SP-immunoreactive cells were observed in all sections of the TRG innervating facial skin in both naïve (101/302, 33.4%) and inflamed (63/300, 54.3%) rats. The percentages of SP and NK1 receptor immunoreactive TRG neurons innervating the
NK1-R-immunoreactivity: TRG neurons innervating facial skin

Figure: Naïve and Inflamed Neurons

- A-D: FG-labeled, NK1-R-positive, NF200-positive, without anti-NK1-R (Naïve, Inflamed)
- E-H: Inflamed

Graphs:

- I: Naïve neuron distribution
  - NK1-R-positive
  - NK1-R/NF200-positive

- J: Inflamed neuron distribution
  - Number of neurons vs. Cell diameter (μm)
facial skin in the naïve and inflamed rats were 44.2 and 49.0%, respectively. As shown in Fig. 4, A–C and G, in the naïve rats, FG-labeled SP-positive neurons were observed in small-, medium- and large-diameter TRG neurons. Medium- to large-diameter neurons also expressed NF200 in rats. As shown in Fig. 4, I and J, there was a tendency for inflammation to result in an increase in the number of SP-positive neurons, and this is especially prominent in the medium- to large-diameter TRG neurons. The number of SP/NF-positive neurons in inflamed rats was significantly increased compared with that in the naïve rats ( naïve vs. inflamed; 2.2 ± 0.5 vs. 7.1 ± 0.7/section).

**General electrophysiological properties of TRG neurons innervating facial skin**

Rats used in these experiments were small (5–6 wk old), and it is reported that afferent fibers over the entire conduction velocity range conduct more slowly at 5–8 wk than they do in the adult rats, possibly at one-half to two-thirds of the adult conduction velocity (Harper and Lawson 1985). In our immunohistochemical experiments, the increase in SP in TRG neurons innervating facial skin was observed in the neurons with a cell body diameter between 30 and 40 μm (Fig. 3). Also, the increase in NK1 receptor was seen in TRG neurons with a cell body diameter >35 μm (2 peaks 35–40 and 40–50 μm; Fig. 4). Since the cell diameter size for SP/NK1-positive TRG neurons correspond to the A-fiber (>30 μm) type, we therefore decided to choose medium- and large-diameter TRG neurons (>30 μm) for the current analysis, as reported in a previous study (Tsubo et al. 2004). Medium- and large-diameter TRG neurons (>30 μm) were labeled at 48 h after FG injection into the orofacial skin. Acutely isolated TRG neurons were spherical in shape and had a bright appearance with a “halo” around the cell body when viewed under phase contrast (Fig. 5A). A typical example of a FG-labeled large-diameter TRG neuron (41 μm) is shown in Fig. 5B. In 93 acutely dissociated TRG neurons, after perforation of the cell membrane with amphotericin B, the series resistance dropped to <20 MΩ (17.6 ± 1.9 MΩ, n = 93) within 7–15 min and remained stable for >15 min. The value for cell capacitance in TRG neurons (>30 μm: 38.5 ± 6.7 μF) was 45.8 ± 2.6 pF (n = 93).

**Effects of SP on the resting membrane potential of TRG neurons innervating facial skin in naïve and inflamed rats**

To determine whether SP is related to the excitability of TRG neurons, we examined the effect of SP (0.1 μM) on the resting membrane potential of the FG-labeled TRG neurons in naïve and inflamed rats. As shown in Table 1, most neurons responding to the SP were depolarized, but the remaining neurons were hyperpolarized after SP application. In this study, we focused on the depolarizing response and obtained evidence that SP-induced depolarization was dose-dependent (0.01–10 μM) in both naïve and inflamed rats (Fig. 6, B and D). This study was concerned with the SP effect on the TRG neurons via the paracrine mechanism. To test whether SP application depolarizes the membrane potential of the TRG neurons (>1 mV), we used a 0.1 μM SP concentration. This was for the following reasons: 1) using nodose ganglion cells, Weinreich et al. (1997) suggested that 0.1 μM SP application may play an important paracrine/autocrine role in the regulation of sensory ganglion function and 2) reversible responses were easily obtained by SP application at 0.1 μM.

In naïve rats, the mean resting membrane potential recorded in the whole cell current-clamp mode was −61.5 ± 1.1 mV (n = 28). The values for capacitance in naïve rats were 44.2 ± 3.2 pF (n = 28). Relatively few neurons (medium: 5/17, 29.4% and large: 0/6, 0%) responded to 0.1 μM SP application (Fig. 5, C and D). A typical example of the large-diameter neuron depolarization induced by SP is shown in Fig. 6A. This depolarizing response was associated with a decrease in the cell input resistance (33 ± 2.1%, n = 5) and antagonized by the NK1 receptor blocker (1 μM, L703,606). The duration of depolarization ranged from 3 to 6 min (5.5 ± 0.7 min). As shown in Fig. 6E, the mean membrane potential evoked by 0.1 μM SP was significantly increased (control vs. SP: −61.2 ± 1.4 vs. −58.5 ± 0.8 mV, P < 0.05), and these changes were antagonized by the NK1 receptor blocker. In inflamed rats, the mean resting membrane potential recorded in the whole cell current-clamp mode was −58.5 ± 1.1 mV (n = 26). The value for capacitance in inflamed rats was 46.1 ± 4.1 pF (n = 26). As shown in Fig. 5, C and D, most of the TRG neurons (medium, 11/15, 73.3%; large, 6/7, 85.7%) responded to 0.1 μM SP application. In FG-labeled medium- and large-diameter TRG neurons in inflamed rats, the rate of occurrence of SP that induced the membrane depolarization was greater than that seen in naïve rats (Fig. 5D, naïve vs. inflamed; medium, 29.4% vs. 73.3%; large, 0% vs. 85.7%). As shown in Fig. 6C, in inflamed rats, 0.1 μM SP application depolarized the membrane potential (7.6 ± 1.1 mV, n = 5) and decreased the cell input resistance (42 ± 5.1%, n = 5) in medium- and large-diameter FG-labeled neurons (Table 1). No significant difference between naïve and inflamed rats was found on the input resistance ( naïve vs. inflamed; 24.5 ± 2.1 vs. 28.6 ± 3.1 MΩ, n = 5, not significant). The duration of depolarization ranged from 5 to 13 min, and the response was reversible. As shown in Fig. 6F, the SP-induced depolarization was blocked by co-administration of the NK1 receptor blocker (1 μM, L703,606; n = 5). The blockade by L703,606 of the SP effect was reversible and most cells took 5–10 min for response to SP to recover from the blockade (80–90% of control).

The summarized results for the difference between naïve and inflamed rats under current-clamp mode are as follows. There was a significant difference in the resting membrane potentials between naïve and inflamed rats (61.5 ± 11 vs. 58.4 ± 11 mV, n = 5, P < 0.05). The magnitude of depolarization induced by SP in the inflamed rats was significantly larger than...
SP-immunoreactivity: TRG neurons innervating facial skin

A
FG-labeled
Naive

B
SP-positive
Naive

C
NF200-positive
Naive

D
Inflamed

E
Inflamed

F
Inflamed

G

Naive

Number of neurons

SP-positive
SP/NF200-positive

H

Inflamed

Number of neurons

Cell diameter (μm)

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FIG. 4. Increased SP-immunoreactive TRG neurons innervating facial skin after TMJ inflammation. A–C: In naïve rats, fluorescent photomicrographs show FG-labeled TRG neurons innervating facial skin (A) and identified intact skin innervated by fluorescent FG in the same field (B). Arrowheads indicate the target cells after FG following FG injection into the whisker pad region C: size distribution of FG-labeled TRG neurons responsive to SP (0.1 μM) in both inflamed and naïve rats. Triangles show examples of FG-labeled SP/NF-positive TRG neurons.

FIG. 5. Occurrence of SP-induced membrane depolarization in the FG-labeled TRG neurons in both naïve and inflamed rats. A and B: dissociated large-diameter TRG neurons (41 μm diam) observed under phase contrast optics (A) and identified intact skin innervated by fluorescent FG in the same field (B). Arrowheads indicate the target cells after FG following FG injection into the whisker pad region C: size distribution of FG-labeled TRG neurons responsive to SP (0.1 μM) in both inflamed and naïve rats. D–F: different percentages of SP responsiveness in medium- (30–39 μm) and large- (>40 μm) diameter TRG neurons in naïve (n = 23) and inflamed (n = 22) rats.
that in naïve rats (3.1 ± 0.4 vs. 7.6 ± 1.1 mV, n = 5, P < 0.05). Also, the mean duration of depolarizing response in the TRG in inflamed rats was significantly longer than that in naïve rats (5.5 ± 0.7 vs. 12.5 ± 1.0 min, n = 5, P < 0.05). In this study, no spontaneous discharges in the TRG neurons were found in either naïve or inflamed rats.

### Difference between SP application–induced inward currents in the TRG neurons in naïve and inflamed rats

In the voltage-clamp mode (V_h = −60 mV), to determine whether TMJ inflammation changes in the functional NK1 receptor expression in medium- and large-diameter TRG neurons at the single cell level, we measured SP (0.1 µM)-evoked inward currents in the medium- and large-diameter TRG neurons in both naïve and inflamed rats. In naïve rats, 6 of 19 TRG neurons tested (31.5%) responded to SP, whereas in inflamed rats, 6 of 8 TRG neurons tested responded to SP (75.0%). Figure 7A shows typical examples of SP-induced inward currents in both naïve and inflamed rats. The repeated application of SP produced consistent responses applications at 60-s intervals (Fig. 7A). The mean SP-induced current densities in the inflamed rats were significantly greater than those in the naïve rats (naïve vs. inflamed; 4.1 ± 0.3 vs. 7.7 ± 0.5 pA/pF, n = 6, P < 0.05; Fig. 7B). In both naïve and inflamed rats, an increase in the current density induced by SP application (0.1 µM) was significantly attenuated in the presence of an NK1 receptor antagonist, L703,606 (Fig. 7, A and B). The blockade by

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<th>Membrane responsiveness</th>
<th>naïve (Responsiveness Cells/Tested Cells, %)</th>
<th>inflamed (Responsiveness Cells/Tested Cells, %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Depolarization</td>
<td>5/6 (83%)</td>
<td>17/19 (89%)</td>
</tr>
<tr>
<td>Hyperpolarization</td>
<td>1/6 (17%)</td>
<td>2/19 (11%)</td>
</tr>
<tr>
<td>Input resistance, % decrease from control</td>
<td>5/5 (33 ± 2.1%)</td>
<td>5/5 (42 ± 5.1%)*</td>
</tr>
</tbody>
</table>

Values are mean ± SE. *Statistical significance between naïve and inflamed rats (P < 0.05).
falling phase, whereas four of five TRG neurons in inflamed rats did not have an inflection (hump) in the falling phase (Fig. 8B). In both naïve and inflamed rats, the numbers of spike discharges during the current injection were significantly increased after SP application (control vs. SP, \( n = 5, P < 0.05 \); Fig. 8C). The relative number of spikes during the current injection (200 pA, 200 ms) in inflamed rats was significantly higher than that in naïve rats ( naïve vs. inflamed; 208 ± 23 vs. 310 ± 58%, \( n = 5, P < 0.05 \); Fig. 8D). Although in naïve rats, a small population of TRG neuron (1/5) showed signs of depolarization followed by spike discharges after 0.1 μM SP application (data not shown), in TRG neurons (4/5) in the inflamed rats, pressure application of SP (0.1 μM) induced strong depolarization associated with spike discharges (Fig. 8E).

**Difference in expression of mRNA for NK1 receptor in single TRG neurons innervating facial skin from naïve and inflamed rats**

The expressions of mRNA for NK1 receptors in the trigeminal ganglia were examined in some animals ( naïve, \( n = 3 \); inflamed, \( n = 3 \)). Total RNA was extracted from the trigeminal ganglia. RT-PCR revealed the expression of mRNAs for NK1 receptor and GAPDH (Fig. 9A). Controls that did not contain the RT enzyme were negative, indicating that genomic DNA was not amplified (Fig. 9A). The mRNA ratio of NK1 receptor/GAPDH in TRG tissue samples from both naïve and inflamed rats did not show any significant difference between them ( naïve vs. inflamed; 1.4 ± 0.1 vs. 1.5 ± 0.3, not significant).

Single cell RT-PCR analysis was performed in FG-labeled and medium- and large-diameter TRG neurons in naïve (38.3 ± 5.2 μm, \( n = 12 \)) and inflamed rats (39.6 ± 6.2 μm, \( n = 12 \)). In naïve rats, 4 of 12 TRG neurons tested (33.3%) expressed mRNA for NK1 receptor, whereas 9 of 12 TRG neurons tested (75.5%) expressed mRNA for NK1 receptor in the inflamed rats. As shown in Fig. 9B, the mRNA ratio in NK1 receptor/GAPDH of large diameter TRG neurons in inflamed rats was significantly larger than that in naïve rats ( naïve vs. inflamed; 1.4 ± 0.4 vs. 5.3 ± 1.8, \( P < 0.05 \)).

**DISCUSSION**

This study provided evidence that TMJ inflammation can enhance the excitability of medium- and large-diameter TRG neurons innervating the facial cutaneous area and that expression of up-regulated SP/NK1 receptors in their soma may contribute to trigeminal inflammatory allodynia in TMJ disorder.

**Methodological considerations**

The purpose of this study was to investigate whether TMJ inflammation alters the excitability of medium- and large-diameter TRG neurons innervating the facial cutaneous area using the techniques of perforated patch-clamp, quantitative RT-PCR, and immunohistochemistry. In this study, we used CFA as an inflammatory agent to produce both inflammation and allodynia in the TMJ region (Imbe et al. 2001; Iwata et al. 1999; Ren 1999), because the CFA-induced inflammation model is known to mimic a persistent orofacial pain represented by temporomandibular disorder (TMD) (Zhou et al.
There is evidence that inflammation due to mustard oil injected into the TMJ region appears within <1 h (Yu et al. 1995). Administration of CFA into the TMJ results in behavioral hyperalgesia that peaks within 4–6 h, and this hyperalgesic action lasts for 2 wk (Imbe et al. 2001). In this study, we obtained the following results: 1) 2 days after CFA injection into the TMJ, the threshold for escape from mechanical stimulation applied to the ipsilateral whisker pad in inflamed rats was significantly lower than that in naïve animals; and 2) the CFA-induced inflammation was verified with Evan’s blue dye extravasation, indicating plasma protein extravasation due to a localized TMJ inflammation. These results suggest that CFA-induced inflammatory model rats are available for studying the mechanism of orofacial pain including a persistent pain associated with TMD as described in previous studies (Imbe et al. 2001; Iwata et al. 1999; Ren 1999).

We tested medium- and large-diameter TRG neurons (30 ± 6.7 μm) responsiveness to SP, according to the results of immunohistochemical studies. Although there is good correlation between cell body size and axon conduction velocity at extreme ends of distribution (i.e., very small neurons tend to give rise to slowly conducting axons and very big neurons tend to give rise to rapidly conducting axons (Harper and Lawson 1985), the mean diameters of medium and large TRG neurons recorded (38.6 ± 6.7 μm) may belong to the category of slow or fast conducting myelinated afferent fiber (Aδ- or Aβ-) types. Irrespective of SP responsiveness, most neurons tested show the electrophysiological characteristics of a low-threshold mechanoreceptor, having a brief action potential that lacks the inflection (Ritter and Mendell 1992). In this study, we could not estimate the nerve conduction velocity in dissociated TRG neurons, but it is more likely that FG-labeled medium- and large-diameter TRG neurons are non-nociceptive A-type TRG neurons. Further studies are needed to elucidate the SP responsiveness of Aβ-TRG.
Increased SP immunoreactivity in TRG neurons innervating inflamed TMJ

Peripheral inflammation increases the content and transportation of SP in the sensory nerves innervating the inflamed tissues (Donnerer et al. 1992) and the SP release from spinal dorsal horn slice (Garry and Hagegreaves 1992). In this study, to determine whether TMJ inflammation increases in SP contents in the TRG neurons innervating the TMJ inflamed region, we examined SP immunohistochemistry for FG-labeled TRG neurons innervating the inflamed TMJ. We found that the areas innervated by second and third branches of TRG neuronal cell bodies were labeled by FG injection into the TMJ. This finding was consistent with the observation reported in a previous study (Ichikawa et al. 2004). We also obtained evidence that the number of SP-immunoreactive TRG neurons innervating the TMJ in the inflamed rats was significantly increased compared with that in naïve rats. Furthermore, TMJ inflammation resulted in a significant increase in the numbers of SP/NF-200-negative and -positive TRG neurons, suggesting that both nonmyelinated (C-neurons) and myelinated neurons (A-neurons) may contribute to the release of SP from TRG cell bodies. This suggestion is consistent with the results of previous studies demonstrating that the number of Aβ fibers expressing SP is increased after inflammation (Neumann et al. 1996; Xu and Zhao 2001). In fact, Fig. 2 shows a marginal shift to the right in the expression of SP with very few medium-diameter afferents expressing SP. Therefore it is possible to speculate that the increase in the SP expression seen after TMJ inflammation is mainly due to activation of nociceptive (Aδ- and C-type) TRG neurons.

Change in the excitability of TRG neurons innervating facial skin after TMJ inflammation

It has been reported that SP is an important neuromodulator in primary sensory neurons, and its effect is to bind primarily to the NK1 receptor (Cao et al. 1998; Otsuka and Yoshioka 1993). In DRG neurons, immunoreactive NK1 receptors are found predominantly in the smaller neurons represented by the Aδ- and C-types, and SP application depolarizes the membrane potential in these neurons (Szucs et al. 1999). In a preliminary experiment, we found that most FG-labeled small-diameter TRG neurons (<30 μm) in both naïve and inflamed rats had a depolarizing response to SP application ( naïve, 4/5, 80%; inflamed, 3/4, 75%). The results obtained in naïve rats were confirmed by evidence indicating that, in guinea pig TRG slice preparations, SP application (1–5 μM) produces reversible depolarization in the majority of TRG neurons (71%) (Spigelman and Puil 1990).

We found that the number of FG-labeled medium- to large-diameter (>30 μm) TRG neurons responding to SP application in inflamed rats was significantly greater than that in naïve rats ( naïve vs. inflamed: medium, 29.4 vs. 73.3%; large, 0 vs. 85.7%). In these neurons, the magnitude of SP-induced depolarization was concentration-dependent (0.01–1 μM), and the responses were associated with a decreasing effect of the cell input resistance. Application of SP to cultured spinal cord and brain stem neurons increases the membrane excitability due to the decreasing effect of K+ conductance (Norwak and MacDonald 1982; Stanfield et al. 1985). Concerning the depolarizing effect of SP in in vitro experiments with guinea pig TRG slice preparations, Spigelman and Puil (1990) have shown that SP could open nonselective ionic channels, resulting in a net inward current and this effect led to a blockade of K+ current, depending on the external [Mg2+]. Although in this study we did not test whether SP application induces the decrease in K+...
currents, we found that SP-induced depolarization and SP-activated inward current were blocked by co-application of a specific NK1 receptor blocker, L-703,606. Thus it can be assumed that concerning medium- and large-diameter TRG neurons innervating the facial skin, a combination of the receptor-activated inward current and deactivated outward current produces the membrane depolarization, and as a result, facilitates repetitive discharges of TRG neurons, as reported in a previous study using TRG slice preparations (Spigelman and Puil 1990). Furthermore, we obtained the following results in medium- to large-diameter TRG neurons innervating facial skin in this study: 1) the membrane potential of TRG neurons in inflamed rats was significantly smaller than that of TRG neurons in naïve rats, 2) the magnitude of depolarization induced by SP in TRG neurons in inflamed rats was significantly larger than that in TRG neurons in naïve rats, 3) duration of the depolarizing effect after SP application to TRG neurons in inflamed rats was significantly longer than that in TRG neurons in naïve rats, and 4) the increase in relative firings evoked by depolarizing pulses in the presence of SP in TRG neurons in inflamed rats was significantly larger than that in TRG neurons in naïve rats. When considering these results, taken together, it is reasonable to speculate that TMJ inflammation may potentiate the excitability of the medium- and large-diameter TRG neurons innervating intact skin area via the NK1 receptor.

Peripheral inflammation increases the SP expression of DRG Aβ-neurons (Neumann et al. 1996), which are released directly from the cell body, activates the SP receptor in the DRG soma, depolarizes the resting membrane potentials, and increases the spontaneous activity (Xu and Zhao 2001). In this study, the expression of mRNA for NK1 receptors on the trigeminal ganglia was found in both naïve and inflamed rats. We also observed that small-, medium-, and large-diameter TRG neurons expressed NK1 receptor immunoreactivity in two different types of animal groups, but that the number of NK1-positive medium- and large-diameter (>30 μm) neurons in inflamed rats was significantly greater than that in naïve rats. Most of these neurons in inflamed rats revealed the co-expression of NF-200 protein immunoreactivity as a myelinated A-fiber marker (Ma 2002). Since TMJ inflammation results in a significant increase of NF-200 protein immunoreactivity in the DRG neurons innervating the facial skin, a combination of the excitatory autoreceptor function and provide a pathway for paracrine signaling between nodose ganglia. In fact, we found that locations of FG-positive neuronal soma innervating the facial skin were adjacent to the TRG neurons innervating the TMJ in this study (2nd and 3rd branches). Therefore it is likely that SP enables release from the TRG through paracrine/autocrine mechanisms, which activate neighboring medium- and large-diameter TRG neurons. This was further supported by the fact that, in the medium- to large-diameter FG-labeled TRG neurons isolated from the inflamed rats, application of 100 nM SP caused depolarization with repetitive spike discharges. Moreover, we found that the increase in both SP and NK1 receptor expression in the cell bodies of TRG neurons innervating the facial skin was obtained in rats after TMJ inflammation, and SP application (0.01–10 μM) concentration-dependently depolarized the membrane potential of TRG neurons. In this study, neither estimated the extracellular concentration of SP inside the ganglion in both naïve and inflamed rats nor conducted the experiments in vitro conditions. Therefore
we cannot directly compare our results with in vivo experiments reported by Neubert et al. (2000). Since orofacial inflammation may induce a greater increase in the ganglionic SP concentration (Neubert et al. 2000), it is possible to speculate that the higher concentration of SP during TMJ inflammation may promote strong depolarization of TRG neurons.

Furthermore, other substances, including ATP and neurotrophic factors, would modify intraganglionic transmission. It has been reported that ATP is released concurrently with SP from intact TRG neurons (Matsuka et al. 2001). Peripheral inflammation is known to increase P2X receptors and to sensitize the P2X receptor–mediated response in DRG neurons (Xu and Huang 2002), but our study did not examine whether other substances modify the excitability of neighboring TRG neurons. Thus the possibility that P2X receptors contribute to changes in the excitability of TRG neurons after TMJ inflammation cannot be ruled out.

In this study, we obtained evidence that the numbers of SP/NF-200-positive immunoreactive TRG neurons innervating the facial skin in inflamed rats were higher than those seen in naïve rats. It can be assumed that the increased excitability of medium- and large-diameter TRG neurons innervating the facial skin may promote SP release from their soma, and this would further activate the neighboring small-diameter nociceptive TRG neurons innervating the facial skin. Accordingly, our data suggest that modification of NK1 receptors within the TRG assists in understanding the potential mechanism of the trigeminal inflammatory allodynia associated with a TMJ disorder. In other words, the NK1 receptor antagonist may be useful for the treatment of trigeminal inflammatory allodynia. Further studies are needed to investigate such an effect.

**REFERENCES**


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