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Glial cell line-derived neurotrophic factor modulates the excitability of nociceptive trigeminal ganglion neurons via a paracrine mechanism following inflammation

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ABSTRACT

Previous our report indicated that acute application of glial cell line-derived neurotrophic factor (GDNF) enhances the neuronal excitability of adult rat small-diameter trigeminal ganglion (TRG) neurons, which innervate the facial skin in the absence of neuropathic and inflammatory conditions. This study investigated whether under *in vivo* conditions, GDNF modulates the excitability of nociceptive Aδ-TRG neurons innervating the facial skin via a paracrine mechanism following inflammation. We used extracellular electrophysiological recording with multibarrel-electrodes in this study. Spontaneous Aδ-TRG neuronal activity was induced in control rats after iontophoretic application of GDNF into the trigeminal ganglia (TRGs). Noxious and non-noxious mechanical stimuli evoked Aδ-TRG neuronal firing rate were significantly increased by iontophoretic application of GDNF. The mean mechanical threshold of nociceptive TRG neurons was significantly decreased by GDNF application. The increased discharge frequency and decreased mechanical threshold induced by GDNF were antagonized by application of the protein tyrosine kinase inhibitor, K252b. The number of Aδ-TRG neurons with spontaneous firings and their firing rates in rats with inflammation induced by Complete Freund's Adjuvant were significantly higher than control rats. The firing rates of Aδ-TRG spontaneous neuronal activity were significantly decreased by iontophoretic application of K252b in inflamed rats. K252b also inhibited Aδ-TRG neuron activity evoked by mechanical stimulation in inflamed rats. These results suggest that *in vivo* GDNF enhances the excitability of nociceptive Aδ-TRG neurons via a paracrine mechanism within TRGs following inflammation. GDNF paracrine mechanism could be important as a therapeutic target for trigeminal inflammatory hyperalgesia.

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1. Introduction

There is an increasing body of evidence for cross-excitation in the sensory ganglia. Recent studies have demonstrated that non-synaptically-released diffusible chemical messengers, such as ATP, substance P (SP) and calcitonin gene-related peptide (CGRP), modify the somatic excitability of neurons in the sensory ganglia, including the trigeminal ganglia (TRGs) (Amir and Devor, 1996, 2000; Takeda et al., 2005a,b; Zhang et al., 2007; Jing et al., 2008; Durham, 2008). An example of this phenomenon is the increased release of SP in trigeminal ganglion (TRG) neurons, which occurs predominantly after peripheral inflammation. This increase in SP release indicates that local paracrine mechanisms in the sensory ganglia contribute to the development of inflammation-induced sensory abnormalities (Matsuka et al., 2001; Takeda et al., 2005a, 2005b).

Glial cell line-derived neurotrophic factor (GDNF) plays an important role in adult sensory neuronal function. Ligands belonging to the GDNF family signal through a receptor complex consisting of the common transmembrane tyrosine kinase receptor Ret, and one of four ligand-binding receptor components, called GDNF family receptors (GFRα1-4). GFRα1 is the preferred receptor for GDNF (Airaksinen and Saarma, 2002). Recent studies demonstrated that small- and medium-diameter human TRG neurons express GDNF and the GFRα1 receptor (Quartu et al., 1999a,b, 2006). We recently reported that: (1) most of the small- and medium-diameter GDNF immunoreactive TRG neurons in rats, also express GFRα1; (2) acute application of GDNF enhances the neuronal excitability of adult rat small-diameter TRG neurons, which innervate the facial skin in the absence of neuropathic and inflammatory conditions; (3) potentiation of small-diameter TRG neuronal excitability is mediated by inhibition of voltage-gated outward K^+ channels via activation of the GDNF-induced intracellular signaling pathway (Takeda et al., 2010). Recent studies have indicated that increased amounts of GDNFs are released during

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inflammation (Mendell et al., 1999; Okragly et al., 1999; De et al., 2004; von Boyen et al., 2006). Also, Schmutzler et al. (2009) reported that GDNF ligands enhance capsaicin-stimulated release of CGRP from dorsal root ganglion (DRG), suggesting that this sensitization could contribute to the inflammatory hyperalgesia induced by GDNF. Therefore, we hypothesized that local release of GDNF from TRG neuronal somata and/or nerve terminals may regulate normal sensory function, including nociception. GDNF family ligands are a group of peptides that have been implicated as important factors in inflammation. The aim of the present study was to test the hypothesis *in vivo* that GDNF alters the excitability of nociceptive Aδ-TRG neurons innervating the facial skin via paracrine mechanism following inflammation.

2. Material and 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). Every effort was made to minimize the number of animals used and their suffering.

2.1. Induction of cutaneous inflammation

The experiments were performed on twelve adult male Wistar rats (250–350 g, body weight). Each animal was anesthetized with sodium pentobarbital (45 mg/kg, i.p.), and Complete Freund's Adjuvant (CFA) (0.05 mL 1:1 oil/saline suspension) was injected into the left side of the facial skin (vibrissae in the whisker pad area were carefully shaved to avoid follicle responding unit) ($n = 4$), as described previously (Takeda et al., 2005a). For control rats, vehicle (0.05 mL, 0.9% NaCl) was injected into the left side of the facial skin (vibrissae were carefully shaved) ($n = 8$). We previously reported that the CFA-induced inflammation was verified with Evan's blue dye (50 mg/mL, 1 mL/kg, i.v.) extravasation and the accumulation of blue dye in the skin by postmortem examination of the injected facial region, which indicated that the plasma protein extravasation was due to localized inflammation (Takeda et al., 2005a,b, 2008).

2.2. Mechanical threshold for escape behavior

Mechanical threshold for escape behavior was conducted as described in previous studies (Takeda et al., 2005a,b). In brief, two days after CFA or vehicle injection into the facial skin, mechanical hyperalgesia was assessed with a set of von Frey hairs (Semmes–Weinstein Monofilaments, North Coast Medical, CA). To evaluate the rat's escape threshold, the von Frey mechanical stimuli were applied to whisker pad in ascending series of trials. Each von Frey stimulation was applied three times in each series of trials. Escape threshold intensity was determined when rats moved their heads away from at least one of three stimuli.

2.3. Extracellular single unit recording of TRG neuronal activity with a multibarrel electrode

Electrophysiological recordings were conducted two days after the injection of CFA or vehicle. Each animal was first anesthetized with sodium pentobarbital (45 mg/kg, i.p.) and anesthesia was maintained as required with additional doses of 2–3 mg/kg/h through a cannula into the jugular vein. The level of anesthesia was confirmed by the absence of the corneal reflex and the lack of response to paw pinching. The trachea was cannulated and the rectal temperature was maintained at 37 ± 0.5 °C with a radiant heater. Arterial blood pressure was monitored by means of a pressure transducer through a cannula inserted into the femoral artery. All wound margins were continuously covered with a local anesthetic, lidocaine (Xylocaine®), throughout the experiments. The animal was then placed in a stereotaxic frame and a craniotomy and hemispherectomy were performed to expose the trigeminal ganglia (TRGs) at base of the skull. Single TRG neuronal activity was recorded from the left side of TRGs, by means of a four-barreled glass micropipette filled with 2% pontamine sky blue with 0.5 M sodium acetate, as described in our previous studies (Takeda et al., 2000, 2005b). The neural activity was amplified (WPI, DAM80), filtered (0.3–10 kHz) and monitored with an oscilloscope (Nihon Kohden, VC-11). Of the three lateral barrels of the micropipette, one barrel containing 160 mM NaCl was used for balancing currents to prevent the occurrence of tip polarization artifacts. The remaining barrels contained the following aqueous solutions respectively: GDNF (10 μg/mL, R&D Systems, Minneapolis, MN, USA) in 160 mM NaCl (pH 3.5); and the protein tyrosine kinase inhibitor, K252b (1 μg/mL, Sigma–Aldrich, St. Louis, MO, USA) in 160 mM NaCl. The currents for ejecting, retaining, and balancing were provided by a constant current unit (Dia Medical, DPI-25, Japan). The drugs were ejected with 10–90 nA cationic currents, and 10–25 nA retaining currents were used.

TRG neuronal activity in response to electrical stimulation (0.3 ms, 1–3 mA, 1 Hz) of the orofacial skin was analyzed. In this experiment, neurons with a conduction velocity of 2–13 m/s were classified as Aδ-fibers (Takeda et al., 2005b). The conduction velocity for each TRG neuron was calculated by dividing the distance between the site of electrical stimulation and the recording site by the latency between the stimulus artifact and the evoked spikes. The number of spike discharges was counted by means of a spike counter (bin width 100 ms). Iontophoretic application of GDNF (10–90 nA, 30–120 s) was considered to be effective if the firing spontaneous discharges changed by more than 20%. The neuronal activity was recorded on a polygraph (NEC-Sanei, 8M14) and stored on magnetic tape for off-line analysis.

2.4. Experimental protocol

Recordings of the extracellular TRG unit activity were performed using the following procedures. The single unit activity was measured in the Aδ-TRG neurons that responded to transcutaneous electrical stimulation through bipolar stainless steel electrodes inserted into facial skin (whisker pad area). We determined whether there was a spontaneous discharge and compared the discharge rates induced by non-noxious and noxious mechanical (pinch) stimuli in control and inflamed rats. Noxious pinch stimulation was applied to the orofacial area with forceps that evoked a pain sensation when applied to a human subject. The threshold for mechanical stimulation was determined as follows: (1) each mechanical stimulation point-of-discharge rate was plotted against the mechanical stimulation, after calibrated von Frey filaments were applied, (4, 8, 15, 26 and 60 g); (2) the mechanical threshold was then determined by extrapolating the mechanical stimulus-induced discharge rate response curve (Takeda et al., 2005b). Therefore, the mechanical response threshold for each TRG neuron was calculated by using a stimulus–response curve. The neuronal discharges in the TRG neurons induced by non-noxious and noxious mechanical stimuli were quantified by subtracting the background activity from the evoked activities. Spontaneous discharge frequencies were determined by means of a spike counter over 2–5 min and if no discharge neurons were found, the cell was deemed to be a silent neuron. After a stable spontaneous discharge was obtained, we tested whether the neuronal firing was related to the activation of GDNF receptors. To determine the ejection current-related effect of GDNF, the baseline unit activity was averaged over a 30–60 s period. In some

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