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Chemokine ligand 2/chemokine receptor 2 signaling in the trigeminal ganglia contributes to inflammatory hyperalgesia in rats

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ABSTRACT

This study investigated the functional significance of hyperalgesia in the CCL2/CCR2 signaling system in trigeminal ganglion (TG) neurons following inflammation. Inflammation was induced by injection of complete Freund's adjuvant (CFA) into the whisker pad of rats. The escape threshold from mechanical stimulation applied to the whisker pad 2 days later was significantly lower in CFA-treated rats than in naïve rats. Fluorogold (FG) labeling was used to identify the TG neurons innervating the whisker pad. FG-labeled TG neurons were immunoreactive for CCL2/CCR2. The mean number of CCL2/CCR2-immunoreactive small/medium-diameter TG neurons was significantly higher in inflamed rats than in naïve rats. Using whole-cell patch-clamp experiments in small-diameter TG neurons, the threshold current of FG-labeled TG neurons in inflamed rats was significantly decreased compared to naïve rats. The number of spike discharges during current injections by FG-labeled TG neurons in inflamed rats was significantly increased compared to naïve rats. These characteristic effects were abolished by co-application of a CCL2 receptor antagonist. The present study provides evidence that CCL2 enhances the excitability of small-diameter TG neurons following facial skin inflammation via the upregulation of CCR2. These findings suggest that ganglionic CCL2/CCR2 signaling is a therapeutic target for the treatment of trigeminal inflammatory hyperalgesia.

1. Introduction

Chemokine C-C motif ligand 2 (CCL2) is a small chemotactic cytokine, also known as monocyte chemoattractant protein 1. It is a member of the chemokine family and secreted in damaged tissue, for example from neurons, glial cells and leukocytes (Subang and Richardson, 2001; Taskinen and Röyttä, 2000; White et al., 2005, 2007; Abbadie et al., 2009). The biological effects of CCL2 are mediated via interaction with its G protein-coupled receptor, chemokine C-C motif receptor 2 (CCR2) (Ransohoff et al., 2007; White et al., 2005, 2007; Abbadie et al., 2009). Several recent studies have revealed that the CCL2/CCR2 signaling system plays an important role in inflammatory and neuropathic pain (White et al., 2007; Abbadie et al., 2009). Actually it has been reported that mutant mice lacking CCR2 (CCR2-/-) do not develop mechanical allodynia after sciatic nerve injury (Abbadie et al., 2003), and animals with inactivated CCR2 do not show hyperalgesia (Thacker et al., 2009). Sun et al. (2006) reported that CCL2 enhances the excitability of nociceptive dorsal root ganglion (DRG) neurons via a decrease in the current threshold due to lowered potassium conductance associated with upregulation of the CCL2/CCR2 signaling system in chronically-compressed DRG models. Taken together, this evidence suggests that the CCL2/CCR2 signaling system contributes to the development of mechanical allodynia/hyperalgesia.

Since previous studies have demonstrated that non-synaptically-released diffusible chemical messengers, such as adenosine triphosphate, substance P (SP) and glial-derived neurotrophic factor, act via local paracrine mechanisms in the sensory ganglia to contribute to the development of inflammation-induced sensory abnormalities (Amir and Devor 1996; Matsuka et al., 2001; Takeda et al., 2005a, 2005b, 2007), it can be assumed that

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transganglionic communication is one mechanism by which central sensitization can be triggered, and that suppression of ganglionic sensitization can effectively reduce the central sensitization of secondary neurons (Takeda et al., 2019, 2012). In a rat model of pain induced by experimental tooth movement, expression of CCL2/CCR2 in small- and medium-diameter TG neurons is increased, as determined by Western blotting, and the time spent on directed face-grooming behavior also increases (Yang et al., 2014). Recently, Dauvergne et al. (2014) demonstrated that the CCL2/CCR2 system is involved principally in early events accompanying neuropathic pain, rather than in the long-term alteration and maintenance of trigeminal mechanical hypersensitivity. Additionally, the CCL2/CCR2 signaling system may contribute to the maintenance of orofacial pain via a trigeminal subnucleus caudalis astroglial-neuronal interaction, and targeting the CCL2/CCR2 signaling system may be a potentially important new treatment strategy for trigeminal orofacial pain (Zhang et al., 2012). Cao et al. (2016) demonstrated that the CCL2/CCR2 signaling system may regulate the maintenance of inflammatory pain via an autocrine/paracrine mechanism in DRG neurons. These findings suggest that local release of CCL2 from TG neuronal somata and/or nerve terminals may regulate the excitability of TG neurons following orofacial inflammation and may contribute to the development of hyperalgesia. Therefore, the present study investigated the functional significance of the CCL2/CCR2 signaling system in TG neurons on trigeminal hyperalgesia following orofacial inflammation, using behavioral, retrograde-labeling/immunohistochemical and electrophysiological patch-clamp techniques.

2. Materials and methods

All 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 of the Study of Pain (Zimmermann, 1983). Each experiment was performed such that the experimenter was blind to experimental conditions. Every effort was made to minimize the number of animals used and their suffering.

2.1. Induction of facial skin inflammation

The experiments were performed on 21 adult male Wistar rats (120–160 g; naïve, $n = 9$; inflamed, $n = 12$). Each animal was anesthetized with sodium pentobarbital (45 mg/kg, i.p.), and complete Freund's adjuvant (CFA; 0.05 ml of 1:1 oil/saline suspension; inflamed rats) or vehicle (0.05 ml of 0.9% NaCl, pH 7.2; naïve rats) was injected into the whisker pad on the left side of the face, as described previously (Takeda et al., 2013). In some experiments ($n = 2$), the CFA-induced inflammation was verified by the extravasation of Evan's blue dye (50 mg/ml, 1 ml/kg, i.v.). Postmortem examination of the injected facial skin showed the accumulation of blue dye in the facial skin, indicating that the plasma protein extravasation was due to localized inflammation (Takeda et al., 2013).

2.2. Mechanical threshold for escape behavior

The mechanical threshold for escape behavior was determined, as described in previous studies (Takeda et al., 2013). Briefly, two days after the injection of CFA or vehicle into the facial skin, hyperalgesia was assessed with calibrated von Frey filaments (Semmes-Weinstein Monofilaments, North Coast Medical, CA). To evaluate the escape threshold, a set of von Frey mechanical stimuli was applied to the skin in an ascending series of trials. Each von Frey stimulation was applied three times in each series of trials. The escape threshold intensity was determined when the rat moved its head away from at least one of the three stimuli.

2.3. Retrograde-labeling of TG neurons innervating the facial skin

For electrophysiological and immunohistochemical studies, fluorogold (FG; Fluorochrome, Englewood, CO) labeling methods were used (Takeda et al., 2005a, 2006). Male Wistar rats were anesthetized with pentobarbital sodium (45 mg/kg, i.p.) before FG solution (0.5% in distilled water, 10 μl) was injected into the whisker pad on the left side of the face using a Hamilton syringe with a 31-gauge needle.

2.4. Immunohistochemistry for CCL2 and CCR2

Immunohistochemistry was conducted using the modified method described in our previous studies (Takeda et al., 2005a, 2007). Rats (naïve, $n = 3$; inflamed, $n = 3$) were deeply anesthetized with pentobarbital sodium (50 mg/kg, i.p.), and transcardially perfused with 50 ml heparinized saline in 0.01 M phosphate buffered saline, followed by 100 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.3). The TGs were removed and incubated in 4% sucrose (3×5 min), 10% sucrose (1 h) and 20% sucrose (2 h), and then in 30% sucrose overnight. Frozen sections were cut at 10 μm with a cryostat (Leica, Nussloch, Germany) and mounted on silane-coated glass slides. Sections were incubated with rabbit anti-CCL2 primary antibody (1:100; Santa Cruz Biotechnology, Dallas, TX) and goat anti-CCR2 primary antibody (1:1000; Santa Cruz Biotechnology) for 24 h at 4 °C, washed, and then incubated in Alexa® 488 goat anti-rabbit IgG secondary antibody (1:1000; Molecular Probes, Eugene, OR) and Alexa® 568 rat anti-goat IgG secondary antibody (1:1000; Molecular Probes), respectively. Labeled cryosections were rinsed consecutively in 0.01 M phosphate buffered saline for 5 min each and then mounted with antifade mounting medium (Molecular Probes). A control experiment was conducted with primary antibody absorption. The fluorescence intensity difference between the staining with primary antibody omitted and the least intense staining was scored as positive. The cell body areas were calculated by assuming that TG neurons were elliptical and their major and minor axes were measured. In this study, we classified TG neurons by measuring the cell diameter as small (<30 μm), medium (30–39 μm), or large (>40 μm) (Tanimoto et al., 2005). Every third section was used for immunohistochemistry and twenty sections were analyzed per TG. At least 100 neurons were measured in each TG. The data are presented as percentages of the total neuron population, i.e., labeled/total population. Confocal images of the stained sections were generated on a Leica TCS NT laser scanning microscope system (Leica, Germany). Digital images were analyzed using Adobe Photoshop version 7.0 and the Leica Imaging Analysis Tool.

2.5. Acute cell dissociation and whole-cell patch-clamp recording

Twenty-one rats were used for the electrophysiological studies. Patch-clamp recordings were conducted two days after CFA (inflamed; $n = 36$ recordings) or vehicle injection (naïve; $n = 26$ recordings). Acute dissociation of TG neurons was performed as described previously (Takeda et al., 2005a, 2006, 2007a, 2008). Adult rats were anesthetized with pentobarbital sodium (45 mg/kg, i.p.) and decapitated. The left TG was rapidly removed and incubated for 15–25 min at 37 °C in modified Hank's balanced salt solution (130 mM NaCl, 5 mM KCl, 0.3 mM KH_2PO_4, 4 mM $NaHCO_3$, 0.3 mM Na_2HPO_4, 5.6 mM glucose, 10 mM N-2-hydroxyethylpiperazine-N'-2- ethanesulfonic acid [HEPES]; pH 7.3) containing collagenase type II and type XI (each at 2 mg/ml; Sigma-Aldrich, St. Louis, MO). The cells were dissociated by trituration with a fire-polished Pasteur pipette and then plated

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