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Original

Activation of trigeminal intranuclear pathway in rats with temporomandibular joint inflammation

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Abstract: We examined the anatomical connections of trigeminal neurons between the trigeminal subnuclei interpolaris/caudalis (Vi/Vc) transition and caudal subnucleus caudalis/upper cervical dorsal horn ($Vc/C_{1,2}$) zones in rats, using the fluorogold (FG) retrograde tracing method combined with Fos expression, a marker of neuronal activation, following temporomandibular joint (TMJ) inflammation. The head withdrawal threshold was also measured in rats 3 days after complete Freund's adjuvant (CFA)-induced TMJ inflammation. The head withdrawal threshold on the inflamed side was significantly decreased after CFA injection into the TMJ. FG was injected into the Vi/Vc transition zone and retrogradely labeled FG-positive cells were observed in the $Vc/C_{1,2}$ region. Numerous Fos protein-expressing cells were present both in the Vi/Vc transition zone and in the laminated $Vc/C_{1,2}$ zone. A population of cells was double-labeled with Fos and FG in the $Vc/C_{1,2}$ zone. Fos/FG cells were only observed in the deep laminae of the $Vc/C_{1,2}$ zone. These findings suggest that Vi/Vc transition zone activity is modulated by activation of the caudal laminated zone after orofacial tissue injury. (J. Oral Sci. 47, 65-69, 2005)

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Introduction

It has been reported that trigeminal nociceptive information is sent to the nucleus caudalis of the trigeminal spinal nucleus and upper cervical cord (1-3). Following peripheral inflammation, Fos studies have revealed that two peaks of Fos protein-like immunoreactive cells (Fos protein-LI cells) are expressed; one in the transition zone between the trigeminal spinal nucleus interpolaris and caudalis (Vi/Vc zone), and one in the caudal laminated region between the caudalis and upper cervical dorsal horn ($Vc/C_{1,2}$ zone) (4). Further studies have suggested that these two areas functionally differ in the processing of inflammation-induced nociception in the trigeminal region. However, it is unknown whether nociceptive neurons that are activated by peripheral inflammation, are anatomically connected within these two regions. In the present study, we examined the anatomical relationship between nociceptive neurons in the Vi/Vc and $Vc/C_{1,2}$ zones through fluorogold (FG) retrograde tracing combined with Fos expression following temporomandibular joint (TMJ) inflammation.

Materials and Methods

The present experiment was approved by the Animal Experimental Committee of Nihon University School of Dentistry and animals were treated according to the guidelines of the International Association for the Study of Pain (5). Adult male Sprague-Dawley rats (260-320 g) were used for all experiments.

Rats were anesthetized with pentobarbital Na (50 mg/kg, i.p.) and Complete Freund's adjuvant (CFA, 0.05 ml) was injected into the left side of the TMJ capsule through the facial skin. CFA was suspended in an oil/saline (1:1) emulsion. Three days after CFA injection, rats were tested for mechanical hyperalgesia. Ten rats that had limited access to a 10% sucrose solution (100 ml/kg/day for 1 week) were used for behavioral study. The animals were able to drink the sucrose solution through a hole in the front panel of the plastic cage. They were trained to continue drinking sucrose solution during noxious mechanical stimulation of the lateral face. The mechanical stimulation was applied with von-Frey monofilaments through a small hole (1 cm in diameter) in the lateral wall of the plastic cage. The mechanical stimulation was applied to the lateral face 2 mm below the posterior edge of the zygomatic arc. The rats showed normal weight gain during the training session. The training was considered to be successful when the rats were able to continue drinking sucrose solution for 10 min without escaping from 36g mechanical stimuli. At the end of the training period, injection was performed (CFA TMJ group; n = 5, naive group; n = 5). Three days after injection, all animals were tested with von-Frey hairs. Each hair was applied twice at intervals of 5 s and the two values were averaged. The bending force of the first hair to evoke an escape response was designated as the escape threshold intensity.

Fifteen rats were used for Fos and Fluorogold (FG) tracing experiments. Rats were anesthetized with pentobarbital Na (50 mg/kg, i.p) and CFA was injected into the left TMJ. Animals were then fixed on a stereotaxic flame, the Vi/Vc zone was exposed and FG was injected into the left Vi/Vc zone. Three days after CFA and FG injection, animals were anesthetized with pentobarbital (80 mg/kg, i.p.) and perfused through the aorta with 500 ml of 0.02 M phosphate buffer saline (PBS, pH 7.4) followed by 500 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). The medulla and the upper cervical cord were removed, post-fixed in the same fixative for 3 days at 4°C with agitation, and transferred to 30% sucrose (w/v) in phosphate buffer for several days for cryoprotection. Fifty-micron sections were cut on a freezing microtome and every fourth section was collected in PBS. The FG injection sites were examined under a fluorescent microscope before immunohistochemical treatment. If the injection site was identified in the Vi/Vc zone, sections were processed for FG and Fos immunocytochemistry.

Free-floating tissue sections were rinsed in PBS, 3% normal goat serum (NGS) in PBS for 1 hour, and then incubated for 72 hours with rabbit anti-c-fos (1:20000: c-fos ab-5, Oncogene, MA, USA) in 3% NGS at 4°C. After three washes with PBS containing 0.75% Triton X-100 and with PBS, sections were incubated with biotinylated secondary IgG (1:200; Vector Labs, Burlingame, CA, USA) for 24 hours at 4°C. Following three rinses in PBS containing Triton, sections were incubated in peroxidase-conjugated avidin-biotin complex (1:100; ABC, Vecter Labs) for 2 hours at room temperature. To develop the ABC reaction product, sections were incubated in 0.035% 33'-diaminobenzidine-tetra HCI (DAB; Sigma), 0.2% nickel ammonium sulfate, and 0.05% peroxide in 0.05 M Tris-buffer (TB, pH 7.4). After Fos immunohistochemistry, sections were rinsed in PBS followed by 3% normal goat serum (NGS) in PBS for 1 hour, and were then incubated for 24 hours with rabbit anti-FG (1:5000:Chemicon, USA) in 3% NGS at room temperature. After three washes in PBS containing 0.75% Triton X-100 and in PBS, sections were incubated with biotinylated secondary IgG (1:200; Vector Labs, Burlingame, CA, USA) for 24 hours at 4°C. Following three rinses in PBS containing Triton, sections were incubated in peroxidase-conjugated avidin-biotin complex (1:100; ABC, Vecter Labs) for 2 hours at room temperature. To develop the ABC reaction product, sections were incubated in 0.035% 33'-diaminobenzidine-tetra HCI (DAB; Sigma) and 0.05% peroxide in 0.05 M Tris-buffer (TB, pH 7.4). Finally, sections were rinsed in PBS, mounted on gelatin-coated slides, dehydrated in alcohol, cleared in xylene, and covered with Eukitt (O. Kindler, Germany).

Cells with black deposits in the nuclei were considered to be Fos protein-LI cells. Every fourth section was selected and camera lucida drawings of these sections were produced. The number of Fos protein-LI cells was counted per section per rat. The mean number of Fos protein-LI cells of all sections (number of Fos protein-LI cells per section) was calculated from five rats. Data are presented as means ± SEM. Statistical comparisons were made by one-way ANOVA. *P* values of less than 0.05 were considered significant.

CFA-induced hyperalgesia was quantified by measuring the head withdrawal threshold to mechanical stimulation applied to the lateral face of rats with CFA injection into the TMJ and untreated naive rats. It has been reported that a large number of Fos protein-LI cells are expressed at 3-7 days after CFA injection into the TMJ. We measured the

head withdrawal threshold and analyzed immunohistochemical data at 3 days after CFA injection into the TMJ.

Results

CFA injection into the TMJ significantly decreased the head withdrawal threshold on the injected side (Fig.1), thus suggesting the development of mechanical allodynia/hyperalgesia. Figure 2 illustrates the site of FG injection in the Vi/Vc transition zone (Fig. 2A). Retrogradely labeled FG-positive cells were observed in the Vc/$C_{1,2}$ region (Fig. 2C-F), indicating the intranuclear connection between the Vi/Vc and Vc/$C_{1,2}$ zones. Consistent with previous studies, numerous Fos protein-LI cells were present in both the Vi/Vc transition zone and the laminated Vc/$C_{1,2}$ zone (Fig. 3A). Many neurons in the deep and superficial laminae exhibited Fos protein immunoreactivity after TMJ inflammation. There were also cells that were double-labeled with Fos and FG (Fos/FG cells) in the Vc/$C_{1,2}$ zone (Fig. 2D). Most of the Fos/FG cells had spindle-shaped or multipolar soma (Fig. 2C-F). Although Fos-positive cells were observed in the superficial and deep laminae of the medullary dorsal horn, Fos/FG cells were only observed in the deep laminae of the Vc/$C_{1,2}$ zone (Fig. 3A). Double-labeled cells appeared to be present in all segments of the Vc/$C_{1,2}$ zone (Fig. 3B).

Discussion

It has been reported that Fos protein is induced in a large number of cells in the trigeminal spinal nucleus, including the Vi/Vc transition zone and laminated Vc/$C_{1,2}$ regions,

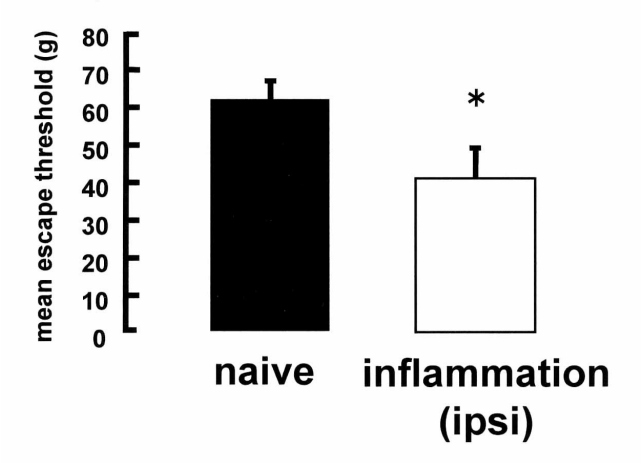

Fig. 1 Mechanical escape thresholds of naive and TMJ-inflamed rats. Mechanical stimuli were applied to the lateral face and escape threshold was measured using von-Frey filaments. *: $P < 0.05$

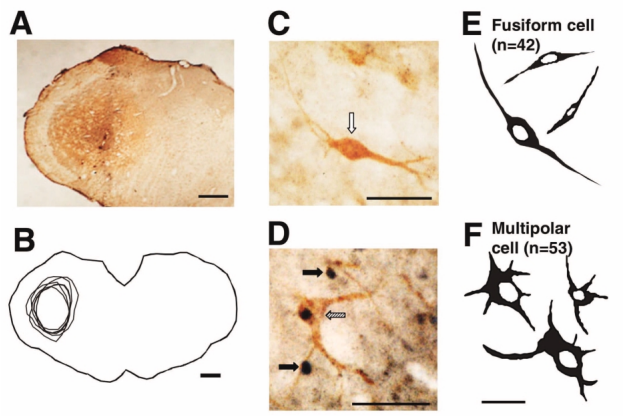

Fig. 2 Fluologold injection sites (A and B), retrogradely labeled FG-positive cells (C), Fos protein-LI cells and Fos/FG cells (D) and camera lucida drawings of Fos/FG cells (E and F). A: Photomicrograph of the FG injection site. B: camera lucida drawings of FG injection sites analyzed in this study. C: Photomicrograph of Vc/$C_{1,2}$neuron retrogradely labeled with FG. D: Fos protein LI-cells and Fos/FG cells in the Vc/$C_{1,2}$ zone. E: Camera lucida drawings of Fos/FG fusiform cells in Vc/$C_{1,2}$ zone. F: Camera lucida drawings of Fos/FG multipolar cells in Vc/$C_{1,2}$ zone.

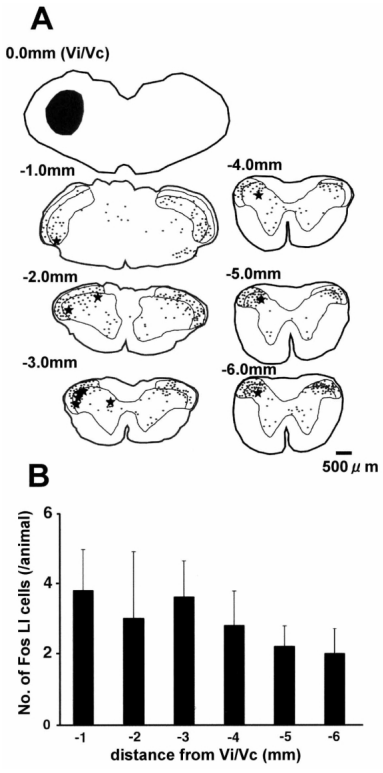

Fig. 3 Rostro-caudal distribution of Fos protein-LI cells and Fos/FG cells in the Vc/$C_{1,2}$ zone. A: Camera lucida drawings of Fos protein-LI cells and Fos/FG cells in the Vc/$C_{1,2}$ zone. Small dots indicate Fos protein-LI cells and large solid stars indicate Fos/FG cells. B: Rostro-caudal distribution of Fos/FG cells in the Vc/$C_{1,2}$ zone.

following CFA injection into the TMJ (4,6,7). Fos protein-LI cells are distributed into two populations; one in the Vi/Vc zone and one in the Vc/$C_{1,2}$ zone. The two populations of Fos protein-LI cells may play different roles in trigeminal nociceptive processing after orofacial tissue injury. Hirata et al. (8) reported that neurons in these two zones differentially respond to morphine. Neurons in the Vi/Vc transition zone may be activated after injury in order to participate in pain modulation. Previous studies have reported that there are neuronal networks within the trigeminal spinal nucleus or the cervical cord that contribute to control of trigeminal nociception (9,10-13). However, the functional connection within the trigeminal spinal nucleus is unknown.

The present study demonstrates that neurons in the Vc/$C_{1,2}$ zone are activated by TMJ inflammation and directly project to the Vi/Vc zone. Our results also indicate that Vc/$C_{1,2}$-Vi/Vc projection originates mainly from neurons located in the deep laminae of the Vc/$C_{1,2}$ zone. This is consistent with previous suggestions that the ascending pathway from Vc/$C_{1,2}$ exerts a modulatory effect on the activity of neurons in the more rostral subnuclei of the trigeminal brain stem nucleus (14). More recently, Meng et al. (15) showed that topical application of glutamate and morphine onto the Vc/C_1 region altered the evoked activity of ~30% of corneal units tested in the Vi/Vc. Both facilitatory and inhibitory effects were noted. These findings suggest that transition zone activity is modulated by activation of the caudal laminated zone. The Vi/Vc transition zone has been suggested to play a unique role in trigeminal responses to orofacial injury (16), but the region, particularly the ventral portion of the transition zone, generally does not receive direct primary afferent input from the orofacial region.

The present results indicate that the information from the injured TMJ is specifically relayed to the Vi/Vc transition zone via the caudal laminated Vc/$C_{1,2}$ zone. This pathway is activated by TMJ injury and may contribute to altered central processing after injury.

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