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RESEARCH ARTICLES

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Role of capsaicin-sensitive primary afferent inputs from the masseter muscle in the C_1 spinal neurons responding to tooth-pulp stimulation in rats

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Abstract The aim of the present study was to demonstrate the convergence of inputs from masseter muscle (MM) and tooth pulp (TP) onto C_1 spinal neurons and to determine whether the afferent fibers express the functional vanilloid receptor (VR1). Extracellular single-unit recordings were made from 61 C_1 units responding to TP electrical stimulation with a constant temporal relationship to a digastric electromyogram signal in pentobarbital anesthetized rats. Eighty-four percent of C_1 neurons responding to TP stimulation also responded to the ipsilateral MM stimulation. Of these neurons, 61% were considered to be afferent inputs from Aδ-fibers and the remaining units (39%) were C-fibers, based on calculation of the nerve conduction velocity. Intramuscular injection of capsaicin (0.05 and 0.1%) produced a reduction in a MM-induced C_1 neuronal activity in a dose-dependent manner and this effect was antagonized by pretreatment with an antagonist of VR1, capsazepine. Some of these units were also excited by noxious heat stimulation (> 43° C). The trigeminal root ganglion (TRG) neurons that innervated the MM were retrogradely labeled with Fluorogold (FG) and the small-diameter FG-labeled TRG neurons expressed the immunoreactivity for VR1. After intramuscular mustard oil injection (noxious chemical stimulation), the C_1 neuronal activity induced by both touch and pinch stimuli was enhanced and their receptive field sizes were significantly expanded. These changes were reversed within 15–20 min. These results suggest that there may be the convergence of noxious afferents inputs from the MM and TP afferents on the same C_1 neurons in rats, and that the afferent fibers expressing the functional VR1 may contribute to the hyperalgesia and/or referred pain associated with temporomandibular joint disorder.

Keywords Capsaicin · C_1 spinal neuron · Masseter muscle · Referred pain · Tooth pulp · VR1

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Introduction

In regard to the trigeminal referred pain, it has been reported that SpVc neurons receive noxious information from the temporomandibular joint (TMJ) and/or masseter muscle (MM), suggesting that SpVc neurons may also be important in the spread and referred pain accompanying dysfunction of the masticatory system (Kojima 1990). Muscle primary afferents from the masseter to the trigeminal sensory nuclei consist of smaller myelinated and unmyelinated fibers (Nishimori et al. 1986). Activation of these small-diameter nociceptive fibers by mustard oil (MO) injection into the MM can produce mechanical hyperalgesia as well as receptive field expansion in the SpVc neurons, suggesting that muscle primary afferents from the masseter nerve contribute to the development of temporomandibular joint disorders (TMJD) (Hu et al. 1992, 1993). Bereiter et al. (2002b)also pointed out the possibility that the nociceptive inputs from receptors in deep craniofacial tissues are relayed to the ventral trigeminal subnucleus interpolaris/caudalis transition region (SpVi/Vc-vl) through the trigeminal subnucleus caudalis/cervical dorsal horn C_2 (SpVc/C_2) junction region. C_1 spinal neurons are known to contribute to the pain referred to the neck and jaw regions because the neurons responded to electrical stimulation of the ipsilat-

eral and contralateral phrenic nerves above the heart and are also excited by noxious stimulation of somatic receptive fields involving the neck and jaw regions (Razook et al. 1995). In addition, Matsumoto et al. (1999) demonstrated that most of C_1 spinal neurons responding to tooth pulp (TP) stimulation receive afferent inputs from the ipsilateral phrenic nerves. From these observations, it is therefore possible that there may be a convergence of face, neck, jaw, TP and phrenic afferents on the same C_1 spinal neurons in rats. Based on the fact that complete or partial relief from TMD occurred after receiving endodontic treatment including tooth extraction, Wright and Gullickson (1996) suggested that pulpalgia acts as a significant factor forming TMD symptoms. Therefore, these results led us to suggest that convergence inputs from noxious information from the MM and TP afferent inputs onto C_1 spinal neurons may play an important role in masticatory pain.

Capsaicin-induced burning pain is mediated by activation of specific (vanilloid) receptors on sensory nerve endings (Szallasi and Blumberg 1999). The cloned vanilloid receptor 1 (VR1), (transient receptor potential V1, TRPV1), consists of a nociceptive neuron-specific capsaicin-gated ion channel responding to heat, proton, anandamide, and lipoxygenase (Caterina et al. 1997; Caterina and Julius 2001; Hwang et al. 2000; Tominaga et al. 1998; Zygmunt et al. 1999). The experiments on mice lacking TRPV1 showed that this specific channel was required for expression of both pain sensation and thermal hyperalgesia (Caterina et al. 2000). The VR1 antagonist capasazepine has the ability to reverse mechanical hyperalgesia in an inflammatory and neuropathic pain model (Walker et al. 2003). Therefore, it is possible to speculate that the VR1 may contribute to the development of neuropathic and inflammatory pain. In fact, immunohistochemical and in situ hybridization studies have revealed expression of the VR1 in small and medium diameter trigeminal root ganglion (TRG) neurons (Caterina et al. 1997; Helliwell et al. 1998; Ichikawa and Sugimoto 2001; Szallasi et al. 1995). These neurons project to the superficial layer dorsal horn of the spinal cord and trigeminal spinal nucleus and are considered to have unmyelinated axons (Guo et al. 1999), suggesting that capsaicin receptor referred to as the VR1 is a marker for small-sized neurons such as nociceptors. The VR1 is also expressed in Aδ-fiber neurons as well as C-fiber neurons in DRG neurons (Ma 2002). Thus, the question arises as to whether MM afferent fibers expressing the VR_1 may contribute to the referred pain associated with TMJD, but no studies have tested this possibility.

In the present study, we examined whether the noxious information from the MM convergences on C_1 spinal neurons responding to TP, and whether the muscle afferent fiber expresses the VR1 with electrophysiological and immunohistochemical techniques. In addition, we also determined whether noxious stimulation of the MM activates neuroplastic changes in the cutaneous mechanical receptive field properties of C_1 neurons.

Materials 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.

Animal preparation

The experiments were performed on 43 male Wistar rats (320–360 g). Each animal was initially anesthetized with pentobarbital (50 mg/kg, i.v.), and anesthesia was maintained with additional doses of 2–3 mg/kg/h through a cannula in the jugular vein, as required. The trachea was cannulated and rectal temperature was maintained at 37.0±0.5°C with a radiant heater. Arterial blood pressure was measured by means of a pressure transducer through a cannula inserted into the femoral artery. Adequacy of the anesthesia was determined by the lack of a response to pinching a paw.

TP stimulation

Bipolar stimulating electrodes made from stainless steel wire (diameter 150 μm, enamel insulated except for the tip) were inserted into the pulp of upper incisors and insulated from the surrounding tissue with dental cement to limit current spread, as described in previous studies (Takeda et al. 1998, 1999, 2000).

MM stimulation

To determine whether C_1 neurons responding to TP stimulation receive afferent inputs from the ipsilateral masseter muscle, electrical stimulation with constant current single pulses (0.1–3.5 mA, 0.1 ms, 1 Hz) was delivered by a pair of stainless steel wires (interpolar distance: 2 mm) inserted into the MM.

Receptive field

Somatic receptive fields of C_1 spinal neurons that responded to TP stimulation were examined by tactile stimulation with a small brush (<150 mN) and pinching the skin with forceps. Noxious pinch stimulation was applied to the orofacial area with calibrated forceps at an intensity (4.0 N) that evoked pain sensation when applied to human subjects. The mechanical receptive fields of neurons were mapped by probing the skin with von Frey filaments, and then outlined on a life-sized drawing of rats on the tracing paper. The size of the receptive field was quantified by means of a planimeter (Tamaya, Planix, Tokyo) as described in a previous study (Takeda et al. 2000).

Recording of single unit and dEMG activities

The animals were then placed in a stereotaxic apparatus, and a laminectomy was performed to exposure the C_1 spinal region of the spinal cord. Single neuronal activity was recorded extracellularly from the C_1 neuron by means of a glass micropipette filled with 2% pontamine sky blue and 0.5 M sodium acetate. The neuronal activity was amplified (WPI, DAM 80), filtered (0.3–10 KHz), and monitored with an oscilloscope (Nihon Kohden, VC-10). By means of stainless steel electrodes (interpolar distance 2 mm, insulated except for the tip) the digastric electromyogram (dEMG) was recorded from the ipsilateral anterior belly of the digastric muscle to record JOR.

Noxious heat stimulation of MM

After surgical exposure of the muscle, noxious heat was also used in the nociceptive stimulation (Dia Medical, DPS-705, Japan). Radiant heat, at preselected temperatures (43–55°C), was focused on the surface of the MM with a feedback-controlled projector lamp. A copper-constantan thermocouple was placed in the center of the field of heat stimulation. Heat stimulation was repeated at more than 3-min intervals to minimize tissue damage.

Intramuscular injection of capsaicin

Responses to small diameter fiber activation were tested for sensitivity to intramuscular injection of an agonist for the VR1, capsaicin. Capsaicin (0.05 and 0.1% equal to 1.5 and 3 mM, respectively) (8-methyl-N-vanillyl-6-noneamide; Sigma) was prepared in solvent intralipid (Pfrimmer Kabi and Co) and was freshly made on the day of each experiment, as reported in a previous study (Dallel et al. 1999).

Noxious chemical stimulation of MM

In order to stimulate the nociceptor in the MM, 10 µl of 20% mustard oil (MO) (allylisothiocyanate, Sigma) in paraffin oil was injected into the MM by mean of a blunt Hamilton syringe. Rats receiving 10 µl of pure paraffin oil into the MM were used as a control.

Experimental protocol

Recordings of C_1 unit and dEMG activities responding to TP stimulation and their data analysis were carried out in the following steps: the threshold for JOR was determined from the dEMG; pulse duration was set at 0.1 ms and pulse intensity (at a stimulation frequency of 1 Hz) was increased until three of five consecutive dEMG responses to TP stimulation were obtained. The peak-to-peak amplitudes in five stimulus trials were averaged. Poststimulus histograms of C_1 unit activity induced by TP stimulation (intensity of TP stimulation was set at 3.5 times the dEMG threshold) were constructed (16–32 sweeps, bin width=1 ms). After we confirmed that the neurons had TP afferent inputs, and we also examined whether these C_1 neurons receive afferent inputs from the ipsilateral MM by using electrical stimulation. After confirmation of convergence input from MM to C_1 neurons responding to TP, we also tested the responsiveness to capsaicin injection into the MM and noxious heat to the MM. Before capsaicin administration, vehicle (same volume as capsaicin) was administered to the MM, and no changes in the C_1 neuronal activity were found within 60 min. Concerning capsaicin (0.1 and 0.05%) sensitivity to the effect of the afferent fiber inputs from MM to C_1 neurons, we evaluated the capsaicin effect by the difference between the number of spikes before and after capsaicin administration (10-min intervals, up to 60 min). If the change from control activity was more than 20%, a given stimulus was considered effective. Inhibition was then expressed as a percentage decrease in the number of spikes with reference to the control. To determine whether inhibition of the MM-evoked C_1 neuronal activity induced by capsaicin is related to the activation of VR1, we tested the effect of a specific VR1 blocker, capsazepine (30 min) on the MM-evoked TRG neuronal activity, as described in a previous study (Kwak et al. 1998). In some experiments, we tested whether the noxious chemical stimulation (MO) of MM activates neuroplastic changes in the cutaneous receptive field properties. After noxious chemical stimulation of the MM, we evaluated the change in cutaneous mechanical receptive field properties (response to pinch, touch stimuli, and receptive field size), by examining at 5-min intervals for 40 min. Before MO injection, vehicle (same volume of MO) was administered to the MM, and no changes in the C_1 neuronal activity were found within 60 min. The neuronal discharges of C_1 neurons after MO application were quantified by subtracting background discharges from the evoked activity. The nerve conduction velocity for each C_1 neuron was calculated by dividing the distance between the stimulation site (TP and MM) and the C_1 region by the latency between the stimulus artifact and evoked spikes. The value for the latency was corrected by a 0.5-ms synaptic delay. Latency was determined from the peak of the poststimulus histogram. Neurons with a conduction velocity less than 2 m/s and within 2–15 m/s were defined as C-fibers and Aδ-fibers, respectively. Statistical significance was calculated by Duncan's new multiple range test. A probability less than 0.01 was considered significant. Values are expressed as the mean ± SEM.

Identification of recording sites

At the end of recording sessions, the rats were deeply anesthetized. Then cathodal DC currents (30 µA, 5 min) were passed through a recording micropipette. The animals were transcardially perfused with saline and 10% formalin. Frozen coronal sections were cut into 30-µm thicknesses and stained with hematoxylin-eosin. Recording sites were identified from the blue spots, and construction with micropipette tracks was done by means of a combination with micromanipulator readings.

Retrograde-labeling of TRG neurons and immunohistochemistry for VR1

Rats (n =3) were deeply anesthetized with pentobarbital sodium (45–50 mg/kg, i.p.), and the ipsilateral MM was surgically exposed. Fluorogold (FG) (1%, 20–30 µl, Fluorochrome, Englewood Co, USA) was intramuscularly injected into the MM by Hamilton syringe as described in a previous study (Takeda et al. 2004). After FG injection the skin incision was sutured and the rats were allowed to recover. After 2 days, FG-injected rats (n =3) were anesthetized with pentobarbital sodium (50 mg/kg, i.p.), and transcardially perfused with 50 ml heparinized saline in 0.01 M phosphate buffer (PBS), followed by 100 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.3). The TRG were removed and incubated in 4, 10 and 20% sucrose solution (5 min × 3 times, 1 and 2 h, respectively) and in 30% sucrose solution overnight. Frozen sections at 10 µm with a criostat (Leica, Germany) were mounted on silan-coated glass slides. To demonstrate the VR1 staining, a fluorescent immunohistochemical method was performed. Sections were incubated with rabbit anti-VR_1 serum (1:1000, TRANSGENIC, Japan) for 24 h at 4°C, followed by Alexa 568 goat anti-rabbit IgG (1:1000, Molecular Probes, USA). Labeled cryosections were rinsed consecutively in 0.01 M PBS for 5 min each. The samples were covered with antifade-mounting medium (Molecular Probes, USA). Digital images were collected and stored on a laboratory computer and later analyzed by means of Adobe Photoshop v. 7.0 and Leica Imaging Analysis Tool. Confocal images were generated on a Leica TCS NT laser scanning microscope system (Leica, Germany).

Results

General properties of C_1 neuronal and dEMG activities in response to TP stimulation

Extracellular single unit activity was recorded from 61 C_1 neurons responding to TP stimulation. As shown in Fig. 1a, recording sites (90.1%) were founded in the layers I–III, and four neurons were located in lamina IV of the dorsolateral area in the C_1 dorsal horn. The activity of all

61 C_1 neurons evoked by TP stimulation with the same time course as simultaneously recorded dEMG activity was tested for response to electrical stimulation of TP. TP stimulation-induced reflex responses of digastric muscle had a latency of 6–14 ms (n =43). The mean threshold intensity was 0.9±0.1 mA (n =61). Out of 61 units, fifteen showed a spontaneous discharge at the rate of 0.5–16 spikes/s. The latency of C_1 spinal neurons (n =61) during TP stimulation was 8–14 ms. The mean threshold was 0.7±0.3 mA. As the stimulus intensity was increased (Fig. 1b), the number of C_1 spikes and amplitude of dEMG increased proportionally during 1.0–3.5 times the threshold for dEMG (Fig. 1c). Since the distance from the site of TP stimulation to the ipsilateral C_1 segment was approximately 46 mm, the average conduction velocity was 4.1±0.2 m/s (n =61). As shown in Fig. 1d, these C_1 neurons responding to both TP and MM stimuli had a somatic receptive field in the orofacial area. All of these neurons also responded to mechanical stimulation (touch and pinch): every neuron recorded was wide dynamic range.

Effect of MM stimulation on the activity of spinal C_1 spinal neurons responding to TP stimulation and their afferent fiber properties

The effect of ipsilateral MM electrical stimulation on C_1 spinal neurons responding to TP stimulation was tested in 61 neurons. Fifty-one out of 61 units (84%) that responded to TP stimulation were also excited by the ipsilateral MM stimulation. Typical C_1 unit responses to both TP and MM stimuli are shown in Fig. 2a. The mean threshold for activation of MM was 1.1±0.2 mA. The latency of C_1 spinal neurons during MM stimulation was 11.6±0.8 ms. The discharge rates of these units increased proportionally

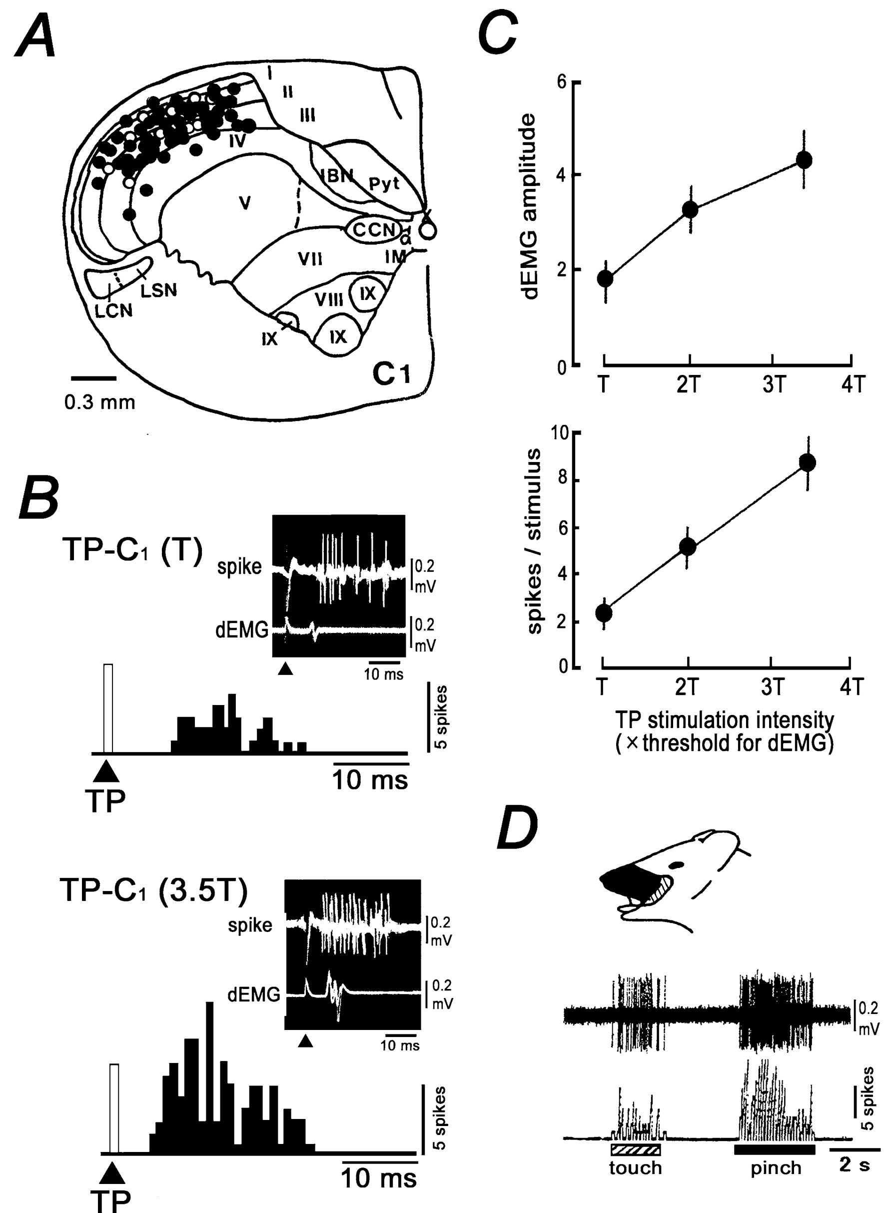

Fig. 1a–d Characterization of C_1 neurons responding to TP electrical stimulation. **a** Distribution of recorded neurons in the first cervical dorsal horn (n =61). *Open circle* indicates that the neuron responded to TP stimulation. *Closed circle* indicates that neurons responded to both TP and MM stimuli. **b** Typical example of C_1 unit response and dEMG activity. Corresponding poststimulus histogram (1-ms bin width) was obtained from 16 successive responses. Timing of TP stimulation is indicated by *triangles* (0.3 ms, 0.5–2.5 mA, 1 Hz). **c** Threshold-related responses of dEMG and C_1 neurons to TP stimulation. *Vertical bars* are the mean ± SE. **d** Examples of mechanoreceptive field properties. *Blacked area* indicates the location and size of receptive fields responding to pinch stimulation; *hatched area* indicates the location and size of receptive field responding to touch stimulation

as the stimulation intensity was increased (Fig. 2b). Thirty-one out of 51 units (61%) were considered to receive afferent input from activated Aδ-fibers and the remaining units (20/51, 39%) were due to C-fibers, based on the calculated conduction velocity (CV). Most C_1 neurons that had afferent inputs from Aδ-fibers (5/10) and C-fibers (7/10) showed a transient excitation induced by intramuscular 0.1% capsaicin injection. A typical example of excitation of C_1 neuronal response (CV=Aδ-fiber range) to capsaicin injection is shown in Fig. 2c. The mean firing rate was significantly increased after capsaicin injection (2.1±0.9 imp/s vs. 16.4±3.3 imp/s, n =5, p <0.05). In some of these neurons (Aδ-fiber, n =5; C-fiber, n =7), we tested whether C_1 neurons (CV=C-fiber range) responding to TP were also activated by noxious heat stimulation. Figure 2d shows on example of C_1 neuronal response to noxious heat stimulation. The activation threshold for noxious heat ranged from 43 to 47°C (45±3.2°C). The mean firing rate of C_1 neuronal response to noxious heat stimulation was significantly increased (2.5±0.9 imp/s vs. 17.9±4.3 imp/s, n =5, p <0.05).

Inhibition of the MM stimulation evoked C_1 neuronal activity induced by intramuscular capsaicin injection

The effect of intramuscular injection of capsaicin on the MM stimulation which evoked C_1 neuronal activity was evaluated in ten units with Aδ-fiber input. The mean conduction velocity was 4.9±0.5 m/s (n =10). As shown in Fig. 3a, Aδ-fiber evoked C_1 neuronal activity was time-dependently suppressed by intramuscular 0.1% capsaicin administration. Figure 3b shows the time course effect of capsaicin administration for 60 min on the Aδ-fiber evoked unit activities. The suppressive effect was gradually increased and this suppression was time- and dose-dependent. The response was reduced by 60% from control at 50 min after administration. The suppression did

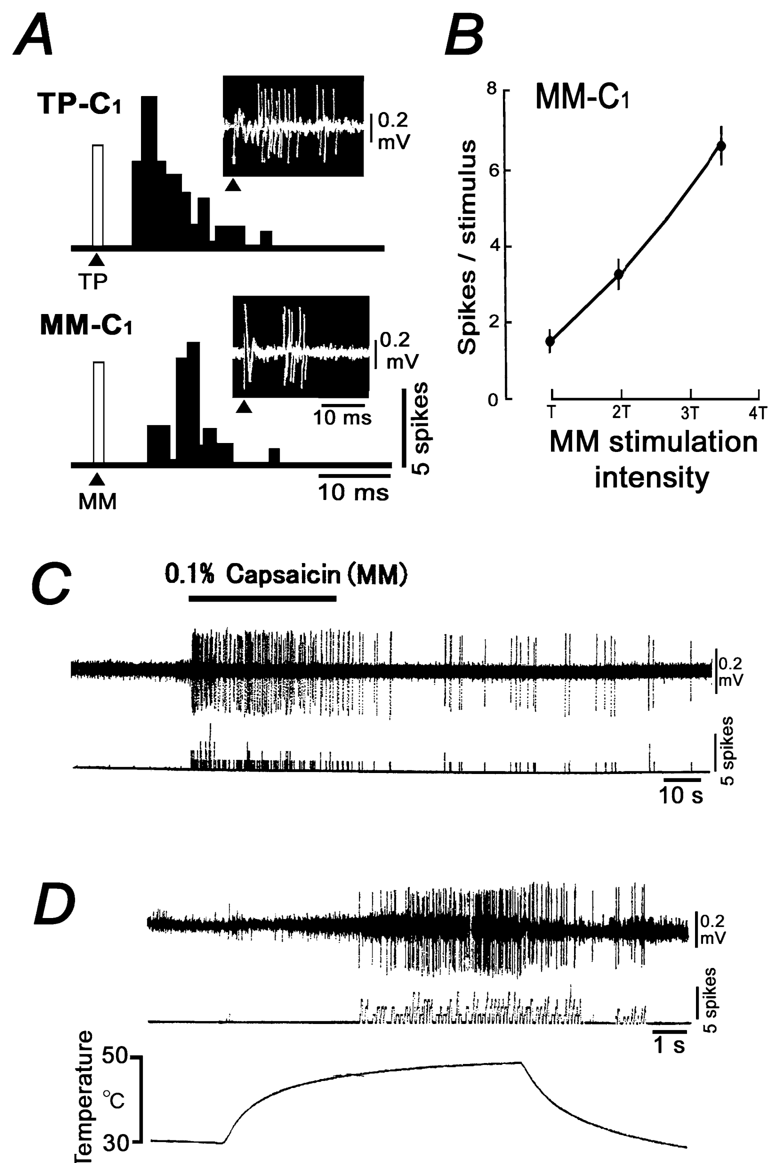

Fig. 2a–d General properties of the C_1 neurons responding to both TP and MM electrical stimulation. **a** Example of C_1 unit response to both TP and MM stimulation. **b** Threshold-related responses of C1 neurons to MM stimulation. **c** Excitatory response of C1 neuron (Aδ-fiber input) caused by intramuscular injection of 0.1% capsaicin (50 µl) **d** Example of C_1 neuronal firing (Aδ-fiber input) in response to noxious heat stimulation of MM

not recover throughout the observations. As shown in Fig. 3c, the suppressive effect of capsaicin on the Aδ-fiber evoked C_1 unit activity was antagonized by a VR1 antagonist, capsazepine (0.1%, n =4). But vehicle had no effect on the unit activity.

Similarly, the effect of capsaicin intramuscular injection on the MM stimulation evoked C_1 neuron activity was evaluated in ten units with C-fiber input. The mean conduction velocity was 1.8±0.2 m/s (n =10). Figure 4a shows a typical example of the time course effect of capsaicin administration for 60 min on the C-fiber evoked unit activities. C-fiber evoked C_1 unit activities were also inhibited after capsaicin administration, and the inhibition of C-fiber evoked activity was greater than that of Aδ-fiber evoked activity. Vehicle caused no significant change in these firings (Fig. 4c). The suppressive effect of C-fiber evoked activity after capsaicin treatment was dose-dependent (0.05 and 0.1%) and was gradually increased and the response was reduced by 80% from control at 30–40 min after administration (Fig. 4c). This effect did not recover throughout the observations. The suppressive effect of capsaicin on the C-fiber evoked C_1 unit activity was antagonized with pretreatment by a VR1 antagonist, capsazepine (0.1%, n =4) (Fig. 4c).

Expression of functional protein for VR1 in FG-labeled small diameter TRG neurons

As shown in Fig. 5a, 23.8% (106/446) of TRG neurons had the immunoreactivity for the VR1. Two days after FG injection into the ipsilateral MM, TRG neurons innervating ipsilateral MM were retrogradely labeled. Figure 5b shows a typical example of FG-labeled TRG neurons (24.4%:109/446) and one of these neurons also expressed immunoreactivity for VR1 (Fig. 5c). Figure 5d shows the distribution of size frequency in FG-labeled and FG-labeled/VR1 positive neurons. Most of the FG-labeled TRG neurons with a small diameter (<30 μm) (37.5%; 30/80) showed immunoreactivity for VR1.

Effect of noxious chemical stimulation of MM on the C_1 neuronal activity responding to TP

The effect of MO intramuscular injection on the C_1 neurons responding to both MM and TP stimuli was tested in 11 out of 61 C_1 neurons; ten of these units showed an increase in firing in response to the intramuscular application of MO. Figure 6a shows an example of responses induced by electrical stimulation of the MM. The typical response of these neurons to application of MO is shown in Fig. 6b. Before MO application there were no spontaneous discharges, but firing spikes appeared just after its application. After MO application, firing frequencies gradually increased and then returned to the control level within 15–20 min (Fig. 6b). After MO application, the mean maximum firing rates of the neuronal activity was significantly increased compared with those before the application (Fig. 6c).

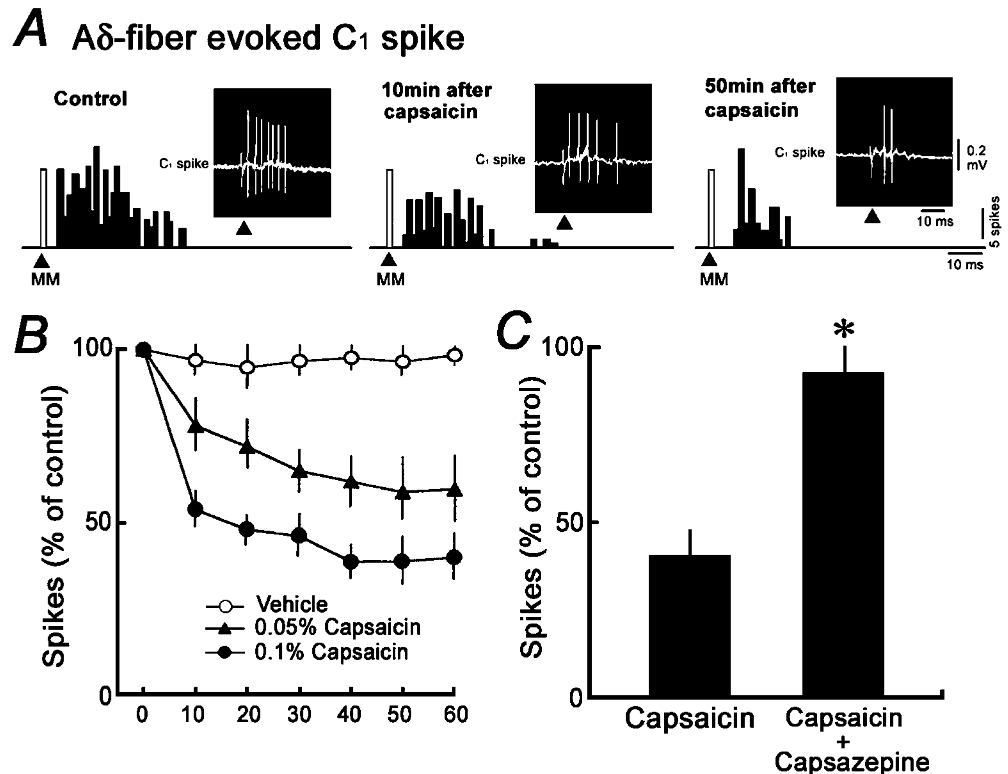

Fig. 3a–c Effect of intramuscular capsaicin injection on Aδ-fiber evoked responses of C_1 neurons responding to TP stimulation. **a** Aδ-fiber evoked response to poststimulus histogram (16 sweeps, 1-ms bin width) corresponding to each sampling time after capsaicin injection. *Insets:* C_1 unit response to MM stimulation. **b** The time course of effects of intracutaneous injection of vehicle (n =4), 0.05% capsaicin (n =5) and 0.1% capsaicin (n =5) on Aδ-fiber evoked response of C_1 neurons. *Vertical bars* are the mean ± SE. **c** Summary of effects of pretreatment with capsazepine (0.1%) on the capsaicin (0.1%) induced inhibition of Aδ-fiber evoked response of C_1 neurons. Each *column* represents the mean ± SE (n =5). * p <0.05

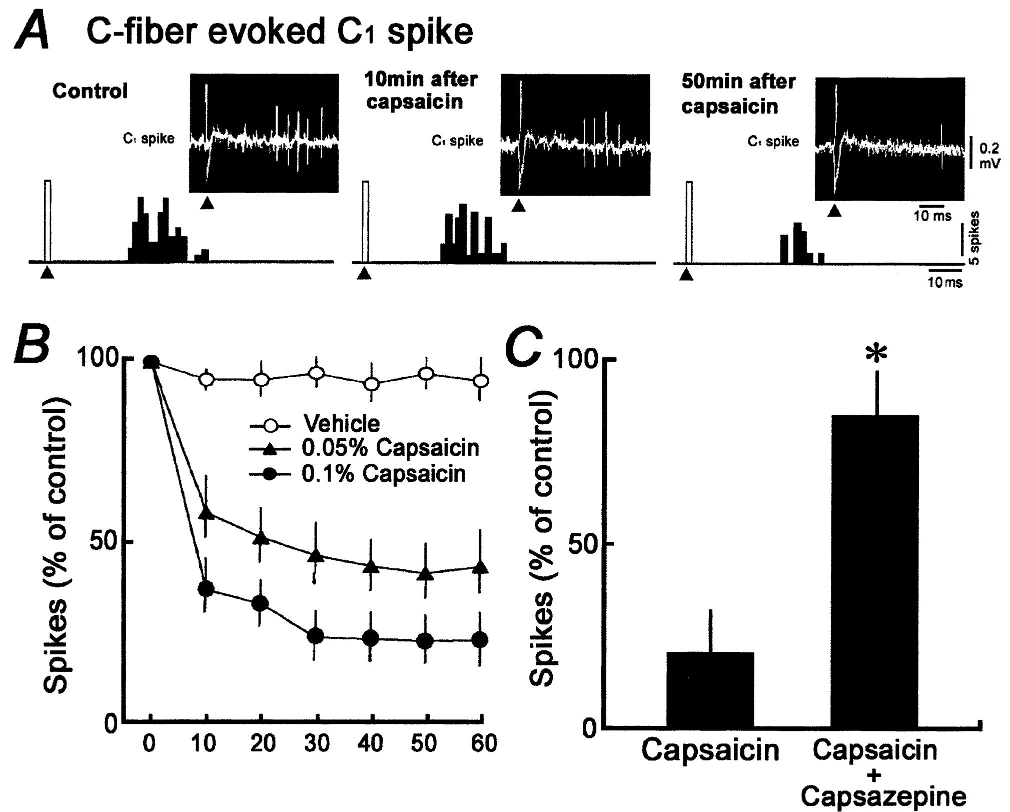

Fig. 4a–c Effect of intramuscular capsaicin injection on C-fiber evoked responses of C_1 neurons responding to TP stimulation. **a** C-fiber evoked response to poststimulus histogram (16 sweeps, 1-ms bin width) corresponding to each sampling time after capsaicin injection. *Insets:* C_1 unit response to MM stimulation. **b** The time course of effects of intracutaneous injection of vehicle (n =4), 0.05% capsaicin (n =5) and 0.1% capsaicin (n =5) on C-fiber evoked response of C_1 neurons. *Vertical bars* are the mean ± SE. **c** Summary of effects of pretreatment with capsazepine (0.1%) on the capsaicin (0.1%) induced inhibition of C-fiber evoked response of C_1 neurons. Each *column* represents the mean ± SE (n =5). * p <0.05

Change in the mechanical receptive field properties of the C_1 neurons responding to TP after noxious chemical stimulation of MM

The effect of noxious chemical stimulation of MM on mechanical receptive field properties of the C_1 neurons responding to TP stimulation was tested in 15 out of 61 C_1 neurons (ten neurons were considered to be afferent inputs from Aδ-fibers and the remaining five units derived from C-fibers inputs). Typical examples of the effect of intramuscular administration of MO on the C_1 neuronal response to receptive field mechanical stimulation are shown in Fig. 6d. Ten minutes after MO administration, increases in the excitability of C_1 neurons were observed by touch and pinch, and also the expansion of the receptive field evoked by touch and pinch stimuli occurred. These increases returned to control levels within 20–30 min. As shown in Fig. 6e, the mean firing rates of these neurons evoked by touch and pinch stimuli were significantly increased compared with those before the application (touch: 12±2.9 vs. 26.6±7.3 spike/s, p <0.05; pinch: 38.8±7.9 vs. 64.8±11.6 spike/s). The mean receptive field size also significantly expanded compared with that before the application (touch: 1.8±0.6 vs. 4.1 ±0.5 cm^2; pinch: 0.4±0.2 vs 0.9±0.1 cm^2, p <0.05). These changes were reversed within 15–20 min.

Discussion

In the present study, we obtained the following major findings: (1) most C_1 spinal neurons (84%) responding to TP stimulation also responded to ipsilateral MM stimulation, and the conduction velocity of their afferent nerves ranged from Aδ- to C-fibers; (2) some of these fibers were capsaicin sensitive as well as noxious heat sensitive, and the former effect was blocked by pretreatment with a VR1 blocker, capsazepine; (3) the immunoreactivity for the VR1 was expressed in the small diameter TRG neurons innervating the ipsilateral MM; and (4) activation of these fibers evoked by a chemical inflammatory stimulant (MO) caused a reversible change in the mechanical receptive field properties in the facial skin. These results suggest that there may be the convergence of noxious afferent inputs from the MM and TP afferents on the same C_1 neurons in the rats, and that the functional VR1 expressed in the afferent nociceptive fibers may contribute to the hyperalgesia and/or referred pain associated with the TMJD.

Nociceptive afferent input from MM to C_1 neurons

It is generally known that information on muscle pain is transmitted to the central nervous system through nonmyelinated and myelinated fibers because in humans the muscle pain induced by pressure on the receptive field (Simone et al. 1994)or by intramuscular injection of capsaicin is mediated by group III and IV fibers

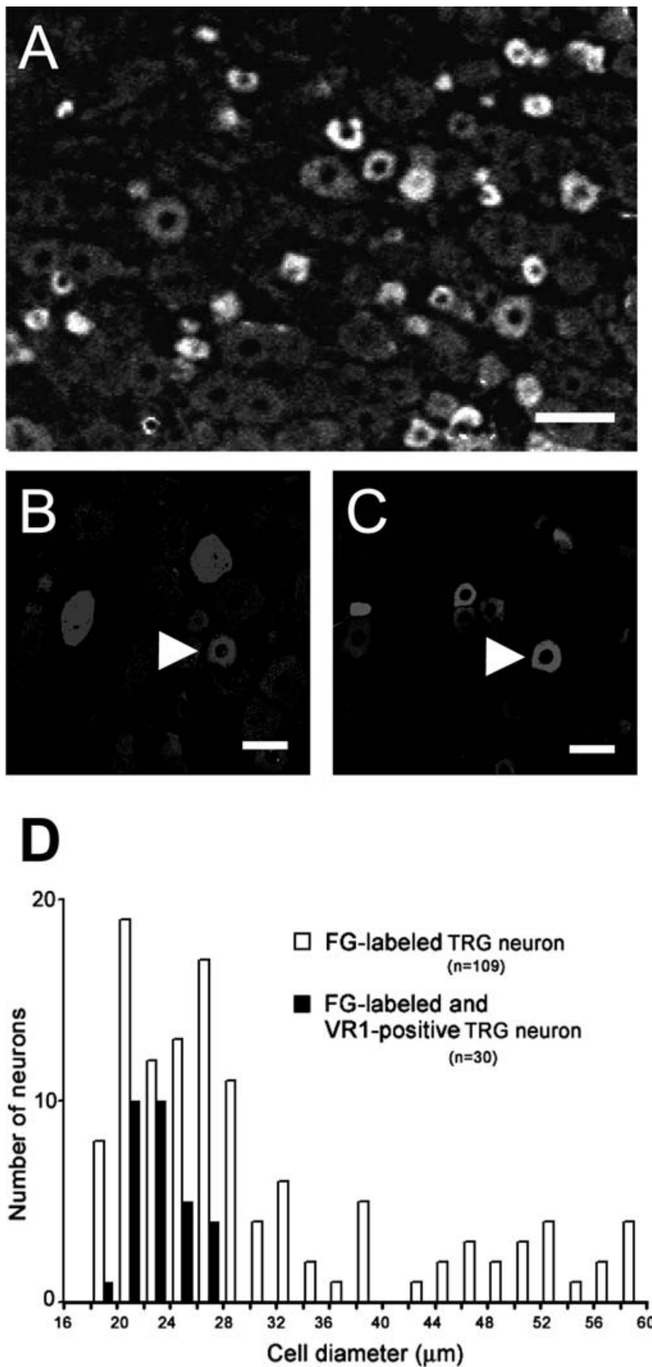

Fig. 5a–d VR1 immunoreactive TRG neurons that innervated the MM were retrogradely labeled with FG. **a** Example of VR1 immunoreactive TRG neurons. Scale bar: 100 µm. **b, c** Fluorescent photomicrographs shows FG-labeled TRG neurons (**b**). VR1 immunoreactive TRG neurons in the same section (**c**). *Triangle* shows a small-diameter FG-labeled/VR1 positive TRG neuron. Scale bar: 50 µm. **d** Size frequency distribution of FG-labeled and FG-labeled/VR1 immunoreactive TRG neurons

(Marchettini et al. 1996). In the present study, approximately 84% of the C_1 neurons responding to TP were also excited by the ipsilateral MM stimulation. In these neurons, 31 out of 51 units (62%) were considered to be afferent input from activated Aδ-fibers and the remaining units (20/51, 39%) were C-fibers, based on the calculated nerve conduction velocity.

Razook et al. (1995) have shown that C_1 spinal neurons may contribute to the pain referred to in the neck and jaw regions, because the neurons which responded to electrical stimulation of the ipsilateral and contralateral phrenic nerves above the heart are also excited by noxious stimulation of somatic receptive fields involving the neck and jaw regions. It was further confirmed that most C_1 spinal neurons responding to TP received afferent inputs from ipsilateral phrenic nerves (Matsumoto et al. 1999), suggesting that there may be a convergence of face, neck, jaw, TP and phrenic afferents on the same C_1 spinal neurons in rats. There is a report of the convergence of trigeminal inputs from with visceral and phrenic inputs on the primate C_1-C_2 spinothalamic tract neurons (Chandler et al. 1999). Moreover, it was also found that stimulation of cardiac afferent fibers excites C_1-C_2 spinothalamic neurons and superficial and deeper C_1-C_2 spinal neurons (Chandler et al. 2000; Qin et al. 2001). Taken together, Foreman (2000) suggested that neurons in the upper cervical segments play an important role in the integration of convergent inputs from somatic and visceral organs. In this study, most of the neurons (90%) responding to TP and MM stimuli were located in laminae I–III, and remaining neurons were located in the lamina IV (Chandler et al. 1999, 2000; Foreman 2000; Qin et al. 2001; Razook et al. 1995), while in the studies from Foreman's laboratory, most of neurons were located in lamina V and deeper, but a few neurons were found in the superficial laminae. Although the precise reason of the difference in the locations is unclear, one of the reasons may be due to a difference in the origin of convergent afferent inputs onto the C_1 neurons between present study (trigeminal and trigeminal afferent inputs) and Foreman's group studies (trigeminal and vagal/phrenic afferents inputs). On the other hand, location of recorded C_1 spinal neurons were consistent with those of c-fos immunohistochemistry after TMJ injury (Bereiter and Bereiter 2000; Bereiter et al. 2002a). Takeshita et al. (2001) suggested that neurons in the lamina I–II in the SpVc/C2 region play an important role in mediating pain sensation in the TMJD. This idea was supported by evidence that the evoked increase in glutamate in the Vi/Vc-vl transition region after TMJ injury was prevented by prior blockade of the most caudal portion of the SpVc and Vc/C_2 junction region, by using microinjection of a $GABA_A$ agonist (Bereiter et al. 2002b). They pointed out that the nociceptive input from receptors in deep craniofacial tissues was relayed to the SpVi/Vc-vl through the SpVc/C_2 junction region. Collectively, our results led us to suggest that C_1 spinal neurons play an important role in the referred pain associated with the masticatory and dental pain. This was further supported by a clinical report showing acute pulpalgia as a contributing factor to TMD symptoms because after receiving endodontic treatment with tooth extracts, patients reported either complete or partial relief TMD symptoms (Wright and Gullickson 1996).

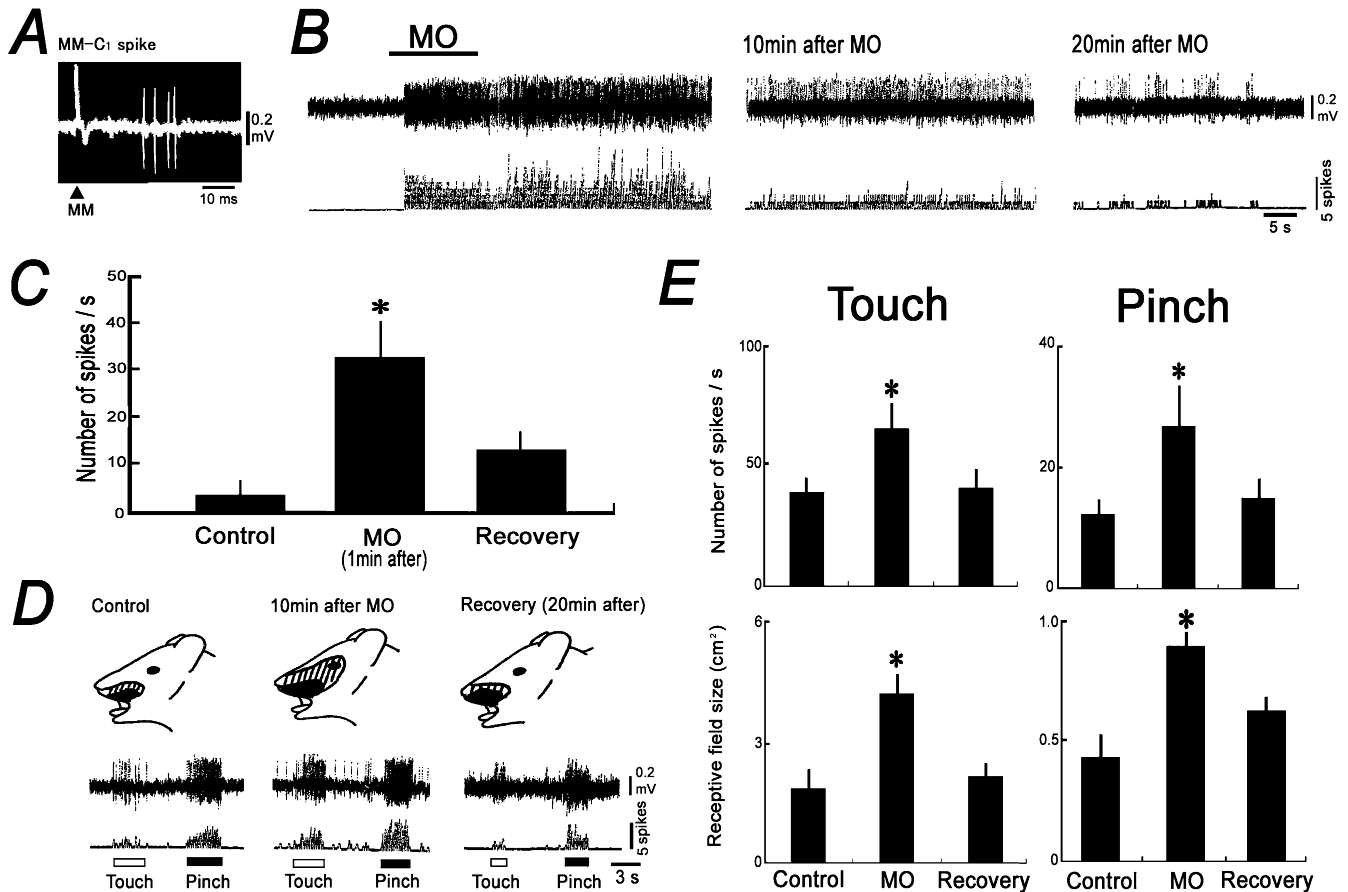

Fig. 6a–e Effect of intramuscular mustard oil (*MO*) injection on the C_1 neuronal activity and the mechanical receptive field properties. **a** Example of C_1 unit response to ipsilateral MM stimulation. **b** Responses elicited by intramuscular mustard oil injection (20 µl). **c** Summary of effect of intramuscular mustard oil injection on the C_1 neuronal firing frequency. * p <0.05. **c** Typical example of mechanical receptive field properties (after ipsilateral mustard oil injection). **d** Summary of changes in intramuscular mustard oil injection on the non-noxious (touch), noxious stimulation (pinch) -evoked response and size of receptive field. Each *column* represents the mean ± SE (n =5). * p <0.05 *Blacked area* indicates the location and size of receptive fields responding to pinch stimulation; *hatched area* indicates the location and size of receptive field responding to touch stimulation

Changes in orofacial mechanical receptive field properties induced by noxious chemical stimulation of MO

Previously it has been reported that SpVc neurons receive afferent inputs from the ipsilateral TMJ and MM (Kojima 1990). They also reported that afferent fibers projecting to SpVc neurons (69%) received nociceptive information from either the TMJ or MM (Kojima 1990). This finding is compatible with reports in the cat (Amano et al. 1986; Broton et al. 1998; Sessle et al. 1986) that SpVc neurons may be involved in the relay of orofacial deep pain. Hu et al. (1992, 1993) demonstrated that the increases in SpVc neuronal excitability and expansion of their mechanoreceptive field occurred after inflammatory irritant application (mustard oil) to the MM. Similarly, in the present study we found that the frequency of spontaneous firing gradually increased after MO intramuscular injection and these changes gradually decreased to the control level within 20–30 min. In 87% (13/15) of C_1 neurons tested, application of MO to the ipsilateral MM enhanced the activities of C_1 neurons induced by both touch and pinch stimuli and also increased expansion of their receptive field. Chiang et al. (1998) reported that the application of MO to the TP could induce significant neuroplastic changes in the SpVc neurons, and that a central N-methyl-D-aspartate (NMDA) mechanism may be involved in the appearance of these neuroplastic changes because intrathecal pretreatment with the NMDA receptor antagonist MK-801 could block neuroplastic changes occurring in the SpVc neurons. Similar neuropathic changes were seen in the spinal trigeminal oralis neurons (Park et al. 2001). We have already reported that the release of endogenous excitatory neurotransmitter was necessary for activation of C_1 spinal neuron that were associated with the transmission of nociceptive information, and that both NMDA and non-NMDA receptors contributed to the mechanism of excitation of TP-evoked C_1 neurons (Takeda et al. 1999). When taken together, it is more conceivable that in the C_1 spinal neurons, MO application into the ipsilateral MM, which enhanced the activities of C_1 neuron induced by both touch and pinch stimuli and which expanded their receptive field size, may promote the activation of NMDA receptors.

Functional implication of VR1 expressed afferent fiber for trigeminal referred pain

The regulator of nociceptive function of primary afferent fibers is involved in substance P, calcitonin gene related peptide (CGRP) and a neurotrophic factor (Bennett 2001). A specific population of nociceptors are also expressed by the VR1 (TRPV1), which is thought to be an important marker for nociceptors (Caterina and Julius 2001; Szallasi and Blumberg 1996). The VR1 is a member of the TRPV receptor family of nonselective cation channels that include several receptors to be gated by changes in temperature within a narrow range: VR1 (42–56°C), vanilloid receptor-like protein-1 (VRL-1) (>52°C), cold and menthol-sensitive receptor-1 (CMR1) (8–28°C), and TRPV3 (>39°C) (Benham et al. 2002; Gunthorpe et al. 2002). In the present study, we found that some of both TP and MM responsive units were also significantly excited by noxious heat stimulation (45–55°C). Because the activation threshold of C_1 neurons for noxious heat stimulation was within 43–47°C, it is more conceivable that afferent fibers project into the C_1 spinal region, which is activated by noxious heat stimulation that expressed the VR1. Although it is well known that capsaicin is a selective agonist for VR1, capsaicin application has two apparently contradictory effects: excitation and desensitization for nociceptors (Guo et al. 1999). In fact, we found in this study that intramuscular injection of capsaicin causes excitation followed by inhibition on the C_1 neuronal activity, after intramuscular injection of capsaicin. The effect of nociceptor desensitization is the reason for using capsaicin as an analgesic agent in the treatment of painful disorders (Szallasi and Blumberg 1996). The mechanism by which capsaicin desensitizes sensory neuronal activity has not yet been elucidated. Nevertheless, Liu et al. (2001) have demonstrated that capsaicin could desensitize capsaicin-sensitive trigeminal ganglion neurons by inhibiting the generation of action potential through the reduction in voltage-gated sodium currents. There is evidence that the immunoreactivity for the VR1 was expressed in the small- and medium-diameter TRG neurons (Caterina et al. 1997; Helliwell et al. 1998; Szallasi et al. 1995). Indeed, we found that intramuscular injection of capsaicin produced a reduction in MM-evoked C_1 neuronal activity in both Aδ- and C-fibers. The magnitude of the inhibitory effect on the C-fiber-evoked C_1 neuronal excitation was much stronger than that in Aδ-fiber input C_1 neuronal activity. Ma (2002) recently found that in DRG neurons, the VR1 is expressed by Aδ-fiber neurons as well as C-fiber neurons. In the present study, we have demonstrated retrograde-labeled TRG neurons derived from the MM using FG retrograde labeling tracer, and as a result, 24.4% of TRG neurons expressed FG. Also, we found that immunoreactivity for VR1 was expressed in the 37.5% small-diameter (<30 μm) FG-labeled TRG neurons. In the rat DRG neurons, there is a positive correlation between the neuronal cell size and the axonal conduction velocity for Aδ- neurons and for C-fiber neurons (Harper and Lawson 1985). Thus, FG-labeled VR1 positive TRG neurons are considered to be Aδ- and C-fiber neurons. Because the cell body diameter of Aδ-type neurons overlaps the border between small and medium diameters (<30 μm) (Harper and Lawson 1985). These results were consistent with the observation indicating that trigeminal spinal nucleus oralis neuronal activity was decreased after intracutaneous injection of capsaicin into the receptive field (Dallel et al. 1999). Therefore, our results suggest that a convergent input from Aδ- and C-afferent fibers expressing the VR1 to C_1 neurons may contribute to masticatory pain.

A role for the VR1 in neuropathic and inflammatory pain is supported by several recent studies indicating that the VR1 is up-regulated in both inflammatory and neuropathic conditions. Peripheral inflammation has been shown to increase the sensitivity of isolated dorsal root ganglion neurons to capsaicin (Nicholas et al. 1999). Moreover, the VR1 can be sensitized or activated by molecules such as prostaglandin E_2 and bradykinin in inflamed tissues (Premkummar and Ahern 2000; Vyklicky et al. 1998). From these observations, it is possible that the expression and functions of the VR1 are regulated under pathological conditions and may contribute to inflammatory-induced hyperalgesia. Accordingly, the results of this study suggest that the functional VR1 expressed in the afferent nociceptive fibers may produce a referred pain between TP and TMJ disorders, and that a vanilloid receptor agonist, capsaicin, may be useful for the treatment of hyperalgesia.

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