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Role of medullary astroglial glutamine synthesis in tooth pulp hypersensitivity associated with frequent masseter muscle contraction

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Abstract

Background: The mechanisms underlying tooth pulp hypersensitivity associated with masseter muscle hyperalgesia remain largely underinvestigated. In the present study, we aimed to determine whether masseter muscle contraction induced by daily electrical stimulation influences the mechanical head-withdrawal threshold and genioglossus electromyography activity caused by the application of capsaicin to the upper first molar tooth pulp. We further investigated whether astroglial glutamine synthesis is involved in first molar tooth pulp hypersensitivity associated with masseter muscle contraction.

Methods: The first molar tooth pulp was treated with capsaicin or vehicle in masseter muscle contraction or sham rats, following which the astroglial glutamine synthetase inhibitor methionine sulfoximine or Phosphate buffered saline (PBS) was applied. Astroglial activation was assessed via immunohistochemistry.

Results: The mechanical head-withdrawal threshold of the ipsilateral masseter muscle was significantly decreased in masseter muscle contraction rats than in sham rats. Genioglossus electromyography activity was significantly higher in masseter muscle contraction rats than sham rats. Glial fibrillary acidic protein-immunoreactive cell density was significantly higher in masseter muscle contraction rats than in sham rats. Administration of methionine sulfoximine induced no significant changes in the density of glial fibrillary acidic protein-immunoreactive cells relative to PBS treatment. However, mechanical head-withdrawal threshold was significantly higher in masseter muscle contraction rats than PBS-treated rats after methionine sulfoximine administration. Genioglossus electromyography activity following first molar tooth pulp capsaicin treatment was significantly lower in methionine sulfoximine-treated rats than in PBS-treated rats. In the ipsilateral region, the total number of phosphorylated extracellular signal-regulated protein kinase immunoreactive cells in the medullary dorsal horn was significantly smaller upon first molar tooth pulp capsaicin application in methionine sulfoximine-treated rats than in PBS-treated rats.

Conclusions: Our results suggest that masseter muscle contraction induces astroglial activation, and that this activation spreads from caudal to the obex in the medullary dorsal horn, resulting in enhanced neuronal excitability associated with astroglial glutamine synthesis in medullary dorsal horn neurons receiving inputs from the tooth pulp. These findings provide significant insight into the mechanisms underlying tooth pulp hypersensitivity associated with masseter muscle contraction.

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Introduction

Although ectopic toothache is frequently associated with masseter muscle (MM) hyperalgesia, general dentists have often misdiagnosed non-odontogenic tooth pain as odontogenic.[1–4] To prevent such misdiagnosis, the mechanisms underlying the association between ectopic toothache and MM hyperalgesia must be clarified.

Previous studies have demonstrated that astroglial activation plays several important roles in the alteration of neuronal plasticity in the dorsal horn of the spinal cord following tissue injury and inflammation.[5–10] Furthermore, functional neuron-glia interactions in the trigeminal spinal subnucleus caudalis—also known as the medullary dorsal horn (MDH)—have been associated with the central sensitization of nociceptive neurons.[11] Additional studies have revealed that these neuron-glial cell interactions are fundamentally supported by astroglial glutamine synthesis.[12] During astroglial activation, excess extra-synaptic glutamate is taken up and synthesized into glutamine via glutamine synthetase (GS). The newly synthesized glutamine is then released from astrocytes to the extra-synaptic space, following which glutamine is taken up by primary afferent terminals via a neuronal glutamine transporter, thus replenishing the glutamate supply.[13,14] Several studies have indicated that neuron-glia interactions and the glutamate–glutamine shuttle play a critical role in the development and maintenance of chronic or persistent pain.[15–17] Therefore, astroglial glutamine synthesis may be involved in the development of tooth pulp hypersensitivity associated with MM hyperalgesia.

Extracellular signal-regulated protein kinase (ERK) is a member of the mitogen-activated protein kinase family. Animal experiments have revealed that ERK activation occurs within 10 min of calcium influx in the dorsal horn in response to a variety of noxious stimuli applied to the hind paw, such as chemical irritants (capsaicin), mechanical punctate stimuli (100 g for 2 min), heat (50°C for 1 min), or cold (4°C for 1 min).[18] Various research groups have observed somatotopic arrangement in the expression of phosphorylated ERK-immunoreactive (pERK-IR) cells within the MDH, and that such expression is proportional to and dependent on the intensity of the stimulus.[19–21] These findings strongly suggest that ERK phosphorylation in MDH neurons is a reliable indicator of the activation of nociceptive neurons following noxious stimulation of the orofacial region.

Therefore, we hypothesized that frequent contraction of the MM contributes to the extraterritorial spread of hyperalgesic state from the hypersensitive MM innervated by the third branch of the trigeminal nerve to the upper molar tooth pulp, which is innervated by the second branch. To examine the mechanisms underlying this potential association, we evaluated the expression of pERK-IR cells as a marker of activated neurons in the MDH following noxious stimulation of the tooth pulp in a rat model of frequent masseter muscle contraction (MMC).

Methods

Animals

The present study utilized a total of 145 male Sprague-Dawley rats (Japan SLC, Shizuoka, Japan) weighing between 250 g and 350 g. Animals were housed in a temperature-controlled room (23°C) under a 12-h light/dark cycle (light from 7:00 to 19:00). All rats were raised under pathogen-free conditions and given ad libitum access to food and water. Ethical approval was obtained from the Animal Experimentation Committee at Nihon University (animal protocol number: AP14D001–3). The present study was conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the guidelines of the International Association for the Study of Pain.[22] The number of rats used was calculated based on the minimum requirement for statistical analysis ($p < 0.05$).

MMC model and measurement of mechanical head-withdrawal threshold

Anesthesia was induced using 2% isoflurane (Mylan, Canonsburg, PA) in oxygen (2 L/min) and maintained via intraperitoneal administration of sodium pentobarbital (50 mg/kg; Schering Plough, Whitehouse Station, NJ). A pair of bipolar wire electrodes (enamel-coated stainless steel wire, interpolar distance: 10 mm; Narishige, Tokyo, Japan) was then inserted transcutaneously into the masseteric fascia, and the MM was electrically stimulated (10 Hz at 2V; pulse width: 200 μs) for 30 min/day for 14 days.[23,24] To verify if the constant

contraction of MM was caused by electrical stimulation of the MM, we measured the bite force during electrical stimulation of MM using bite force measurement instrument (MRU-1KN, Showa Measuring Instruments, Tokyo, Japan). The bite force was amplified (DC Strain Amplifier, San-ei Instruments Inc., Tokyo, Japan) and calculated (PowerLab and LabChart, ADInstruments, Nagoya, Japan). The constant contraction of MM was observed during MM stimulus with the lower stimulus intensity compared to that of previous studies.[23,24] The MM stimulation protocol has also been modified accordingly.

Rats of the sham group were subjected to the same protocol, without the administration of electrical stimulation. Mechanical stimulation of the lateral surface of the MM was applied using an electronic von Frey filament with a flat-head probe (BIO-EVF3, BIOSEB, France, probe diameter: 6.32 mm) to assess the mechanical sensitivity of the MM in conscious rats. The stimulus velocity was set to increase from 0 g to threshold at a speed of 20 g/s. The pressure value (in grams) required to elicit head withdrawal was defined as the applying to the lateral face over the MM to elicit head withdrawal was defined as the mechanical head-withdrawal threshold (MHWT). Stimulation was applied three times per minute, and the MHWT was calculated as the mean value. Baseline MHWT measurements were performed in intact rats prior to the MMC or sham operation.

Genioglossus electromyography following application of capsaicin to the tooth pulp

Following the final MMC stimulation on day 14 (MMC-14d), the tooth pulp of the upper first molar (M1TP) was exposed using a low-speed dental drill (OSADA, Tokyo, Japan) with a round tungsten carbide bur under running water. Rats were anesthetized with 2% to 3% isoflurane, following which a pair of bipolar wire electrodes (enamel-coated stainless steel wire, interpolar distance: 5 mm; Narishige, Tokyo, Japan) was inserted into the genioglossus muscle. A small piece of cotton saturated with isotonic physiological saline was placed on the surface of M1TP to prevent tissue drying. Following insertion of the electrodes and exposure of the tooth pulp, the isoflurane concentration was reduced to and maintained at 0.8% to 1.2% throughout the experiment. The heart rate, percentage of CO_2, and rectal temperature were continuously monitored and maintained at physiological levels of 333 to 430 beats/min, 3.5% to 4.2%, and 37°C to 37.5°C, respectively. Following surgery, a stable baseline level of genioglossus electromyography (G-EMG) activity was obtained following a resting period of 30 min. Then, a dental paper point (diameter, 0.15 mm; length, 1.5 mm; ISO size #15, Pro-Endo Paper Points, Yamahachi Dental, Aichi, Japan) saturated with capsaicin (0.3 nM, 30 nM and 3 μM: diluted with 7% Tween 80, 100% ethanol, and saline; Wako, Osaka, Japan) or the vehicle was applied to the M1TP, ipsilateral to the site of MM electrical stimulation. The G-EMG activity was measured for 20 min, both prior to and following capsaicin application. The amplification, rectification, and integration values of the EMG activity were then analyzed, and the area under the curve (AUC) of G-EMG activity was calculated using Spike 2 software (CED, Cambridge, UK). Before or after capsaicin application, the AUC value of G-EMG activity was averaged every 1 min. The mean value of G-EMG activity for each 1-min period was displayed on a graph. The baseline G-EMG activity was calculated from the average AUC value of G-EMG activity at 3 min before capsaicin application, and the baseline G-EMG activity was defined as 100%. The average G-EMG activity for 1 min was normalized at each time point.

Intra-cisterna magna administration of astroglial *GS* inhibitor

A midline skin incision (length: 2 cm) was made from the head to the neck, following which a midline opening was made in the caudal portion of the skull using a dental drill. A polyethylene tube (ID, 0.5 mm; OD, 0.8 mm; Imamura, Tokyo, Japan) was then inserted into the cisterna magna (i.c.m.).[25] The tube was connected to a mini-osmotic pump (Alzet model 2002, Alzet, Cupertino, CA, USA; total volume: 200 μL) filled with the drug or vehicle, and the pump was subcutaneously embedded in the animal's back, slightly caudal to the scapulae. The astroglial GS inhibitor methionine sulfoximine (MSO) or the vehicle was continuously administered for 14 days (0.5 μL/h, i.c.m.). The tip of the tube was placed at the obex, just above the surface of the medulla. The dosage (100 μM) and duration of drug infusion were determined based on the findings of previous reports.[12,15] Electrical stimulation of MM or the sham procedure was initiated on day 1 after placement of the pump in both the MSO and vehicle groups. The MHWT was then measured as described. G-EMG and pERK immunohistochemistry were performed in MSO-treated and control rats on MMC-14d. The mini-osmotic pump was removed at the end of each experiment, following which pump exhaustion was confirmed.

Immunohistochemistry

Astroglial activation following MMC was evaluated based on glial fibrillary acidic protein (GFAP) immunoreactivity. Rats were deeply anesthetized with sodium pentobarbital (50 mg/kg i.p.) for GFAP, neuronal nuclei (NeuN), or pERK immunohistochemistry.

Rats were transcardially perfused with 0.9% saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer on MMC-14d (for GFAP or pERK); or 5 min after the application of capsaicin to the M1TP on MMC-14d (for GFAP, NeuN, or pERK). The medulla and upper cervical spinal cord including the MDH were harvested, following which the tissue was soaked in the post-fixative solution (4% paraformaldehyde in 0.1 M phosphate buffer) for three days at 4°C. After post-fixation, the tissue was immersed in 0.1 M phosphate buffer containing 20% sucrose overnight at 4°C for cyto-protection. Coronal sections (thickness: 30 μm) were then obtained using a microtome, and immunohisto-chemistry was performed on every fourth selected section. The sections were soaked in 3% normal goat serum in 0.3% Triton-X 100 for 2 h at 22°C, following which they were treated with rabbit anti-GFAP (1:1000, Abcam, Cambridge, UK) or rabbit anti-phospho-p44/42 MAP kinase (1:1000, Cell Signaling, Beverly, MA) antibody for 72 h at 22°C. The sections were then treated with biotinylated goat anti-rabbit IgG (1:600; Vector Labs, Burlingame, CA, USA) for 2 h at 22°C, followed by peroxidase-conjugated avidin-biotin complex (1:100, ABC; Vector) for 2 h. Visualization of the antigen-antibody reaction was performed using 0.035% 3,3-diamino-benzidine-tetra-HCl (DAB, Sigma, St. Louis, MO, USA), 0.2% nickel ammonium sulfate, and 0.05% hydrogen peroxide in 0.05 M Tris buffer (pH 7.4) for 2 to 5 min.

After all sections were mounted on gelatin-coated slides and coverslipped (Sigma-Aldrich, St. Louis, MO, USA), the GFAP immuno products were measured using a computer-assisted imaging analysis system (ImageJ 1.37v; NIH) in a square grid (640×850 μm^2) of the MDH innervated by the second and third branches of the trigeminal nerve.[17,26,27] A light microscope (Optiphot-2, Nikon, Tokyo) with an attached camera lucida drawing tool (Neurolucida 2000, MicroBrightField, Nikon, Tokyo) was used to draw the pERK-IR cells. After counting the number of pERK-IR cells on every eighth serial section from rostral to caudal areas in the MDH, the mean number of pERK-IR cells was calculated for each animal.

Next, double immunostaining for either pERK and NeuN or pERK and GFAP were performed. After soaking in a blocking solution consisting of 3% normal goat serum in PBS for 2 h, the sections were treated with rabbit antiphospho-p44/42 MAP kinase antibody (1:300, Cell Signaling Technology) for 72 h at 4°C. The sections were then treated with mouse anti-NeuN monoclonal antibody (1:1000, Chemicon, Temicula, CA) for 1 h at 22°C or polyclonal mouse anti-GFAP antibody (1:1000, Sigma, St. Louis, MO) for 72 h at 4°C. Following immersion in Alexa Fluor 488 anti-rabbit IgG (1:1000 in 0.01 M PBS; Invitrogen, Paisley) and Alexa Fluor 568 anti-mouse IgG (1:1000 in 0.01 M PBS; Invitrogen) for 2 h at 22°C, the sections were rinsed with 0.01 M PBS and overlaid with a glass coverslip containing mounting medium (Thermo Fisher Scientific, Fremont, CA). The numbers of GFAP-, NeuN-, and pERK-IR cells were determined under a fluorescence microscope and images were analyzed using a BZ-9000 system (Keyence, Osaka).

Statistical analysis

Data are represented as the mean ± standard error of the mean. A two-way repeated measures analysis of variance (ANOVA) followed by Tukey's post hoc test was performed to analyze differences in the MHWT at each time point during treatment or to compare G-EMG activity among the groups (Figures 1(a), 1(b), and 3(b)). A two-way repeated measures ANOVA followed by a Tukey–Kramer post hoc test was used to compare the density of GFAP-IR cells among the groups (Figure 2(d)) or to compare G-EMG activity among the groups (Figure 3(c)). A one-way ANOVA followed by a Tukey–Kramer post hoc test was used to compare the density of GFAP-IR cells or the number of pERK-IR cells among the groups (Figure 3(a)). A one-way ANOVA followed by a Tukey post hoc test was used to compare the number of pERK-IR cells among the groups (Figure 5(e) and 5(f)). The level of statistical significance was set at $p < 0.05$.

Results

MM and M1TP hyperalgesia following MMC

The MHWT of the ipsilateral MM was significantly decreased in MMC rats on days 3 to 14 after electrical stimulation of the MM when compared to that of sham rats (Figure 1(a)). High G-EMG activity was observed following capsaicin application to the M1TP in sham or MMC-14d rats (inset box in Figure 1(b)). The relative AUC values of G-EMG activity were dose-dependently and significantly larger in MMC-14d rats following capsaicin application, relative to those observed in each groups (Figure 1(b)).

Astroglial cell activation in the MDH

The capsaicin application itself did not induce astroglial activation in the rats without MMC (Figure 2(a)). An increase in the number of GFAP-IR cells was observed in the MDH of MMC-rats on day 14, relative to that observed in sham rats (Figure 2(b) and (c)). Numerous GFAP-IR cells were distributed rostrocaudally in the MDH, and the distribution density of GFAP-IR cells was significantly higher in MMC-14d rats than naive

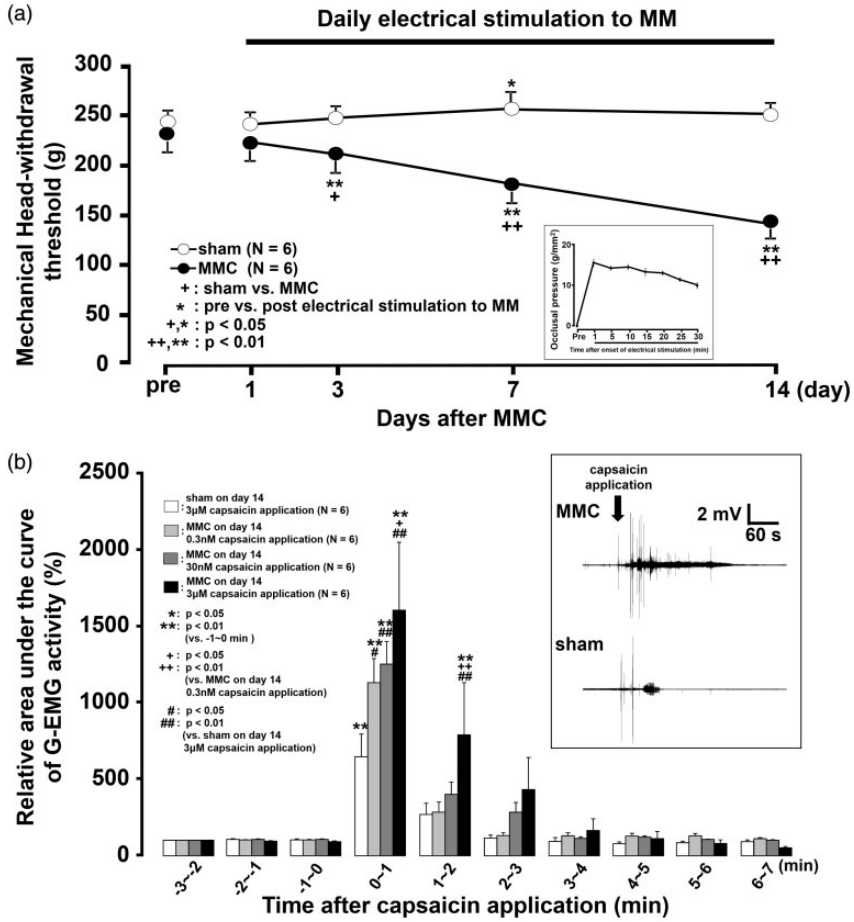

Figure 1. Masseter muscle (MM) hyperalgesia following electrical stimulation and tooth pulp hypersensitivity following masseter muscle contraction (MMC). (a) Change in mechanical head-withdrawal threshold (MHWT) of the MM following electrical stimulation. The inset graph in (a) is the change in the bite force after onset of the electrical stimulation. (b) The dose-dependent change in genioglossus electromyography (G-EMG) activity following capsaicin application to the tooth pulp of the upper first molar (M1TP) in MMC rats. The inset diagrams in (b) are examples of G-EMG recordings following capsaicin application to the M1TP. Data are represented as mean ± SEM.

or sham rats at 2160 μm ($p < 0.01$) and 2880 μm ($p < 0.05$) caudal to the obex (Figure 2(d)).

Effect of MSO administration on GFAP-positive IR cells, MHWT, and G-EMG activity

Administration of MSO produced no changes in the distribution density of GFAP-positive IR cells at 2160 μm and 2880 μm caudal to the obex in either PBS-treated rats on MMC-14d or sham rats (Figure 3(a)). In contrast, the MHWT was significantly higher in MSO-treated rats than PBS-treated rats on day 3 ($p < 0.01$) to day 14 ($p < 0.01$) following MMC (Figure 3(b)). The G-EMG activity following application of capsaicin to the M1TP was significantly lower in MSO-treated than PBS-treated rats ($p < 0.01$) on day 14 after MMC. MSO treatment itself produced no significant variation in the responses observed in sham rats (Figure 3(c)).

ERK phosphorylation in MDH neurons following M1 capsaicin application

While a few pERK-IR cells were observed in the MDH following MMC without capsaicin application (Figure 4(a)), an increased number of pERK-IR cells was observed in the superficial laminae of the MDH following the application of capsaicin to the M1TP in MMC rats (Figure 4(b)). The pERK-IR cells were visualized as dark soma with dendrites (Figure 4(c)). Most pERK-IR cells also exhibited NeuN immunoreactivity (Figure 4(d) to (f); arrows indicate pERK-IR and NeuN-IR cells), while no pERK-IR cells were colocalized with any GFAP-positive IR cells (Figure 4(g) to (i)). These observations indicated that pERK-IR cells present in the MDH represented neuronal cells rather than astrocytes, following application of capsaicin to the M1TP.

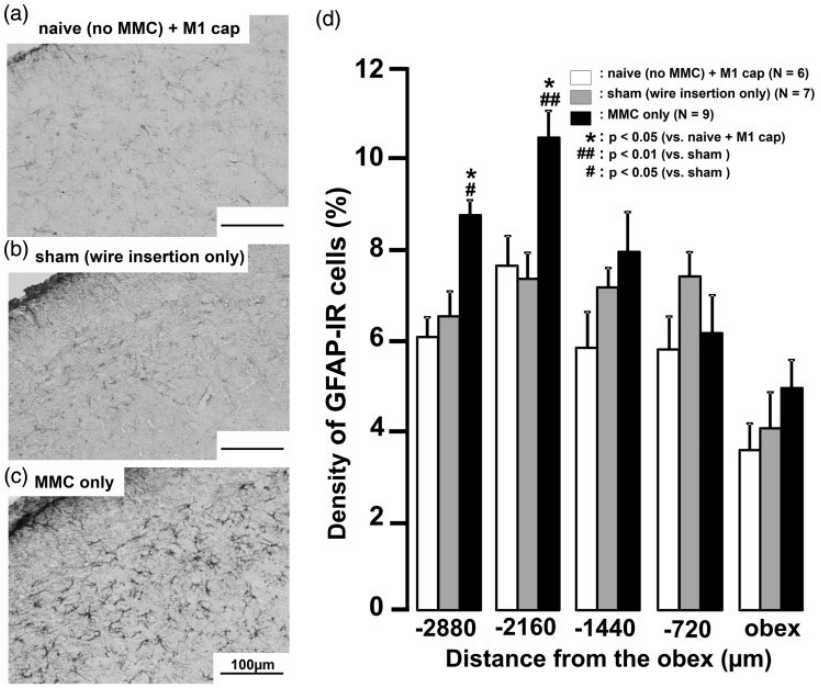

Figure 2. Astroglial activation in the medullary dorsal horn following electrical stimulation of the masseter muscle on day 14. (a) Glial fibrillary acidic protein (GFAP)-immunoreactive (IR) cells in the naive rats with the capsaicin application to the upper first molar tooth pulp (M1TP). (b) GFAP-IR cells in sham rats. (c) GFAP-IR cells in masseter muscle contraction (MMC) rats. Scale bars in panels indicate 100 μm. (d) Rostral-caudal distribution of GFAP-IR cells in naïve, sham, or MMC rats. Data are represented as mean ± SEM.

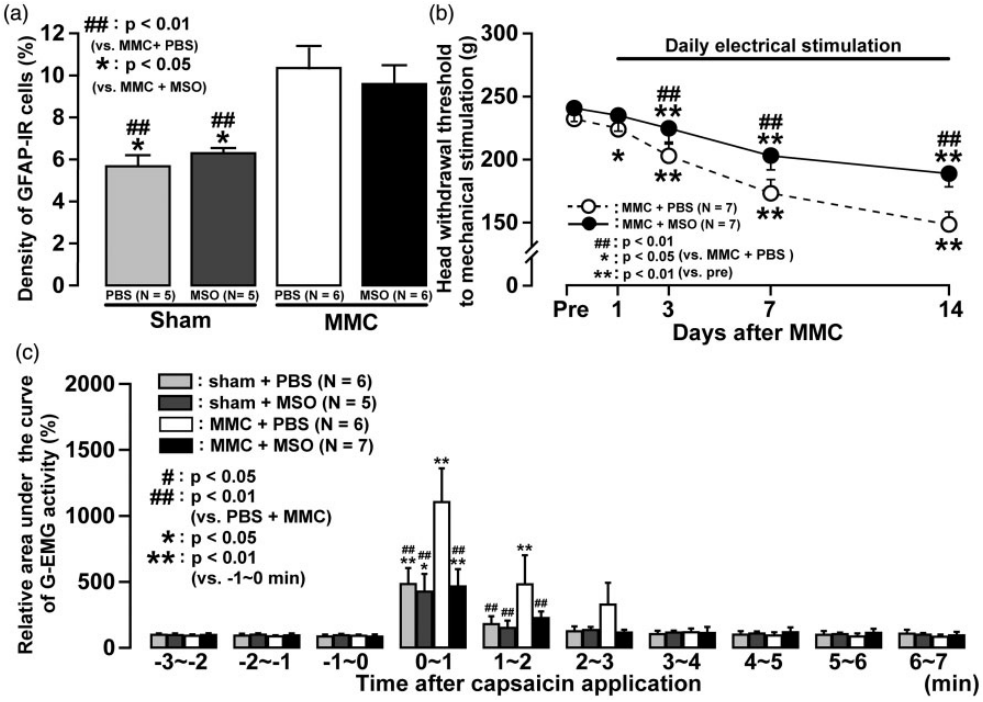

Figure 3. Effect of intra-cisterna magna (i.c.m.) administration of the astroglial glutamine synthetase inhibitor methionine sulfoximine (MSO) on glial fibrillary acidic protein (GFAP)-immunoreactive (IR) cell density, mechanical head-withdrawal threshold (MHWT), and genioglossus electromyography (G-EMG) activity in masseter muscle contraction (MMC) or sham rats. (a) Effect of MSO on the density of GFAP-IR cells on day 14 in MMC or sham rats. (b) Change in MHWT in MSO- or PBS-treated rats following MMC. (c) Change in G-EMG activity following capsaicin application to the tooth pulp of the upper first molar (M1TP) in MSO- or PBS-treated rats on day 14 after MMC or sham operation. Data are represented as mean ± SEM.

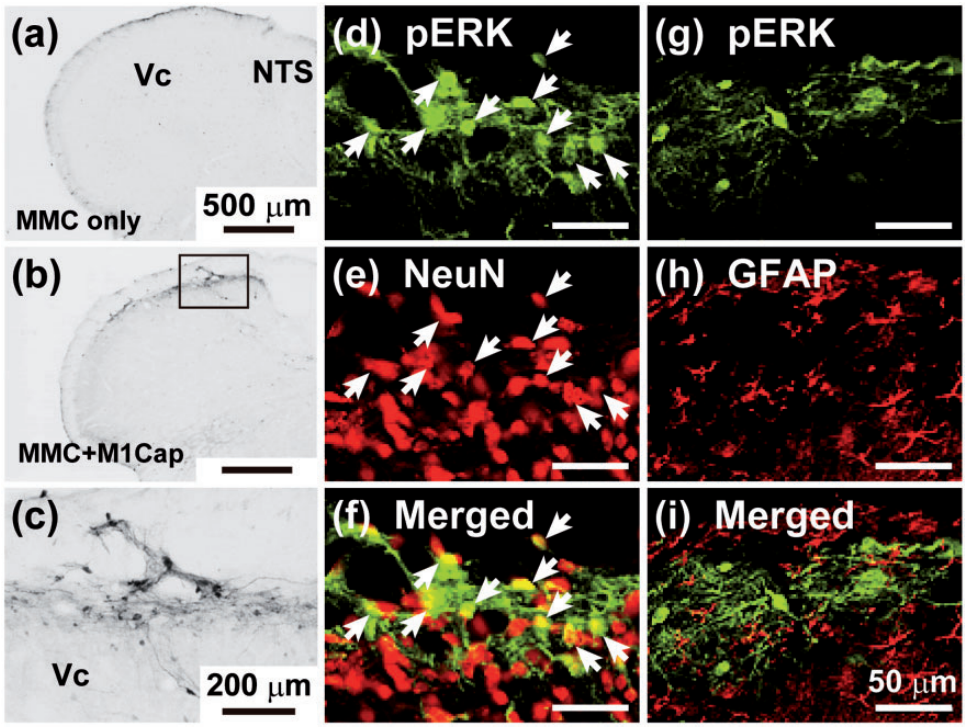

Figure 4. Photomicrographs of phosphorylated extracellular signal-regulated protein kinase (pERK)-immunoreactive (IR), neuronal nuclei (NeuN)-IR, and glial fibrillary acidic protein (GFAP)-IR cells following the application of capsaicin to the tooth pulp of the upper first molar (M1TP) on day 14 after masseter muscle contraction (MMC). (a) pERK-IR cell stained with nickel-conjugated DAB without capsaicin application in MMC rats ($n = 3$); (b) pERK-IR cell following capsaicin application in MMC rats; (c) pERK-IR cells stained with DAB and visualized brown in the nuclei and cytoplasm were regarded as positive; (d) pERK-IR cells; (e) NeuN-IR cells; (f) Merge of panels (d) and (e); (g) pERK-IR cells; (h) GFAP-IR cells; (i) Merge of (g) and (h). (d) to (f) The arrows indicate pERK-IR and NeuN-IR cells. (d) to (i) Scale bars in panels indicate 50 μm.

Effect of MSO administration on ERK phosphorylation

Although a few pERK-IR cells were observed in MMC-14d rats without capsaicin application, a large number of pERK-IR cells were observed in the MDH and upper cervical spinal cord of MMC-14d rats following the application of capsaicin to the M1TP. Moreover, pERK-IR cells were also observed in the solitariy nucleus (NTS) (Figure 5(a) to (d)). Figure 5((c, ipsilateral) and (d, contralateral)) depicts the rostral-caudal distribution of pERK-IR cells in the MDH and upper cervical spinal cord of MMC-14d rats without capsaicin application, and in PBS- or MSO-treated rats on MMC-14d following capsaicin administration. The pERK-IR cells were distributed rostrocaudally in vast areas of the MDH and upper cervical spinal cord, and the number of pERK-IR cells was lower at each distance in MSO-treated rats than in PBS-treated rats. The total number of pERK-IR cells in the ipsilateral or contralateral MDH significantly increased following capsaicin application in PBS- and MSO-treated rats following MMC, relative to that in rats without capsaicin treatment ($p < 0.01$). In the ipsilateral region, the total number of pERK-IR cells in the MDH was significantly decreased in MSO-treated rats relative to that in PBS-treated rats, following capsaicin application ($p < 0.01$). However, no significant differences were observed for the contralateral side ($p > 0.05$) (Figure 5(e) and (f)).

Discussion

In the present study, we aimed to determine whether MMC induced by daily electrical stimulation influences MHWT and G-EMG activity upon administration of capsaicin to the M1TP capsaicin. We further investigated whether astroglial glutamine synthesis is involved in M1TP hypersensitivity associated with MMC. Our results indicated some important points below. The MHWT of the ipsilateral MM was significantly decreased in MMC rats after electrical stimulation of the MM. The G-EMG activity was enhanced following capsaicin application to the M1TP in MMC-14d rats. The increment in expression of GFAP-IR cells was observed in the MDH of MMC-rats. Administration of GS inhibitor MSO produced no changes in the distribution density of GFAP-positive IR cells. In contrast, MSO administration increased MHWT in MMC rats. MSO administration decreased G-EMG activity

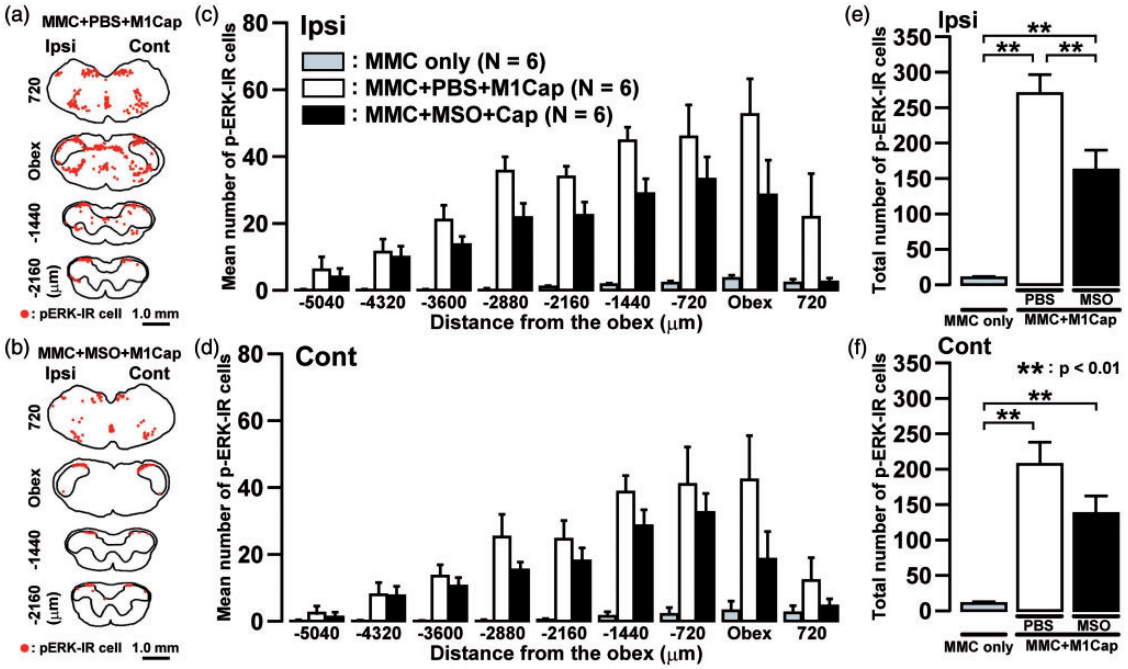

Figure 5. Rostral-caudal distribution of phosphorylated extracellular signal-regulated protein kinase (pERK)-imunoreactive (IR) cells following the application of capsaicin to the tooth pulp of the upper first molar (M1TP) on day 14 following masseter muscle contraction (MMC). (a) and (b) Neurolucida drawings of pERK-IR cells in PBS-treated (a) or MSO-treated (b) MMC rats. (c) and (d) Rostral-caudal distribution of pERK-IR cells in the medullary dorsal horn with or without capsaicin application in PBS- or MSO-treated rats after MMC. (e) and (f) Total number of pERK-IR cells in PBS- or MSO-treated rats with or without capsaicin application after MMC. Ipsi: Ipsilateral to M1TP capsaicin application; Cont: Contralateral to M1TP capsaicin application. Data are represented as mean ± SEM.

following application of capsaicin to the M1TP after MMC. A large number of pERK-IR cells were observed in the MDH of MMC-14d rats following the application of capsaicin to the M1TP. Together, these results suggest that astroglial activation and its glutamine synthesis associated with continuous MMC are involved in ectopic tooth pulp hypersensitivity.

MM hyperalgesia following MMC

Previous authors have reported that unilateral MM inflammation causes widespread mechanical allodynia and hyperalgesia in the face in rats.[28–31] In clinical practice, such intense inflammation has not been traditionally observed in the human MM. Rather, research has suggested that MMC does not induce inflammatory cell infiltration.[23,24] The MM hyperalgesia observed in MMC rats is believed to be associated with mechanisms similar to those underlying MM pain in humans.[32–34] Thus, we chose to examine MMC in a rat model of MM hyperalgesia. Previous studies have reported that NGF is released from muscle tissue following frequent muscle contraction, and that NGF is involved in the hyperactivation of small diameter primary afferent fibers.[35] In the present study, we observed a significant decrease in MHWT during the 14-day observation period following MMC, suggesting that non-inflammatory mechanisms are involved in MMC-induced MM hyperalgesia. We observed ERK phosphorylation in a few MDH neurons following electrical stimulation of the MM without M1 capsaicin application, indicating that electrical stimulus used in the present study activated small number of C-fiber and small diameter Aδ-fiber afferents despite the lower stimulus intensity compared to that of previous studies.[23,24] This observation suggests that MMC induced by electrical stimulation is mainly involved in MMC-induced MM hyperalgesia. However, we could not exclude the possibility that some of C-fibers and small diameter Aδ-fibers in the MM are involved in MM hyperalgesia.

G-EMG activity after capsaicin application following MMC

The digastric and MM are known to be activated by noxious stimulation to the TP.[36] In the present study, we observed dose-dependent increase in the G-EMG activity following increase in the concentration of capsaicin. This suggests that the increase in G-EMG activity after capsaicin application to the M1TP should be a result of the activation of intra-pulpal high-threshold afferents including C-fibers. Thus, we adopted G-EMG activity to define if M1 capsaicin application causes noxious responses in MMC rats. The EMG activity of the

digastric and MMs elicited by the application of capsaicin to the tooth pulp was observed for a longer duration in some previous studies, when compared to the findings of the present study.[37] However, we observed a shorter duration of G-EMG activity than has been reported in previous studies.[36] This discrepancy may be due to differences in the muscle from which the activity was recorded (genioglossus, diagastric, or masseter) or the depth of anesthesia. Indeed, research has demonstrated that reflex EMG activity is modulated by the depth of anesthesia, shortening in duration and decreasing in amplitude as the depth of anesthesia increases.[38] Thus, the relatively shorter duration of G-EMG activity observed in the present study may have been associated with the use of deep anesthesia.

Role of astroglial activation in tooth pulp hypersensitivity

Research has well established that central astroglial activation contributes to neuroplastic changes in neuronal circuits of the MDH and upper cervical spinal cord following inferior alveolar nerve transection.[17,39] Additional studies have revealed that blockade of astroglial activation via intrathecal administration of fluoroacetate or microinjection of fluorocitrate prevents both orofacial pain associated with inferior alveolar nerve transection and inflammatory MM hyperalgesia induced by injection of Complete Freund's Adjuvant.[16,17,29] In the present study, we observed significant activation of astrocytes at 2160 μm and 2880 μm caudal to the obex in MMC rats relative to sham rats, whereas the capsaicin application itself was not induced astroglial activation in the rats without MMC. ERK phosphorylation is known to occur within 5 min, following which the number of pERK-IR cells decreases.[27] Research has also demonstrated that neither trigeminal nerve injury nor orofacial inflammation alone results in ERK phosphorylation in MDH neurons.[40,41] In contrast, we observed that ERK phosphorylation was induced by MMC itself in some MDH neurons, suggesting that electrical stimulation of the MM itself causes ERK phosphorylation in a small number of MDH cells, contributing to MM hyperalgesia. We also observed that ERK phosphorylation in the MDH following capsaicin application to the M1TP in MMC rats was significantly suppressed upon administration of the GS inhibitor MSO. Taken together, these findings suggest that MMC may induce robust activation of astrocytes, resulting in enhanced excitability of neurons in the MDH and upper cervical spinal cord.

The major role of glutamate in nociceptive transmission has been well documented, though recent evidence indicates that the amount of glutamate in the synaptic cleft is adjusted by glutamate transporters present in presynaptic, postsynaptic, and astroglial cell membranes.[6,9] Activated astroglial cells release glutamine, which is then taken up at the presynaptic terminals via glutamine transporter, resulting in enhanced glutamate release from the presynaptic terminals. Commonly referred to as the glutamate-glutamine shuttle, this system is believed to be essential in the maintenance of glutamate homeostasis and pain processing at synaptic sites.[11,12] In the present study, we observed no significant changes in astroglial activation following administration of MSO in MMC-14d rats relative to controls. These results suggest that astroglial activation and the associated inflammation are not affected by GS inhibition or MSO-induced inhibition of glutamine generation.

Taken together, these findings indicate that inhibition of astroglial glutamine synthesis acts to suppress MMC-induced M1TP hypersensitivity. Such results further suggest that adjustment of glutamine release in the affected terminals may aid in the modulation of neuronal excitability in the MDH and upper cervical spinal cord, which receive input from the MM and M1TP.

Spread of astroglial cell activation

Previous studies have reported that connexin 43 (Cx43) gap junctions and hemichannels are critically involved in the development and maintenance of chronic pain, that astrocytes upregulate the expression of Cx43 following traumatic spinal cord injury, and that exposure to several types of astroglial mediators (e.g., cytokines, chemokines, and growth factors) induces astrocyte-astrocyte communication via gap junctions.[42,43] Widely distributed expression of GFAP-IR cells has also been observed within the portion of the MDH receiving afferent inputs from both the second and third branches of the trigeminal nerve at 7 days after inferior alveolar nerve transection.[17] We thus hypothesized that frequent contraction of the MM contributes to the extraterritorial spread of hyperalgesia from the hypersensitive MM—which is innervated by the third branch of the trigeminal nerve—to the tooth pulp of the upper molar—which is innervated by the second branch. Consistent with our hypothesis, we observed astroglial activation at 2160 μm and 2880 μm caudal to the obex in the MDH of MMC-rats, indicating that astroglial activation may have spread to the area of the second branch of trigeminal nerve. When taken with the findings of previous studies, our results suggest that MMC induces astroglial activation, and that this activation may spread within the MDH and upper cervical spinal cord via connexin43-related mechanisms, resulting in enhanced neuronal excitability within the regions of the MDH and upper cervical spinal cord receiving input from the tooth pulp. Such processes may thus represent the basis of tooth pulp hypersensitivity associated with MMC. Some of the previous studies have demonstrated that

intrathecal administration of MSO causes direct effect on DH neurons.[44] Therefore, the further detailed study may be necessary to clarify if MSO affects neuronal activity as well as astroglial cell activation.

Conclusions

The present study is the first to provide evidence that astroglial glutamine synthesis is involved in ectopic tooth pulp hypersensitivity associated with continuous MMC. Our findings suggest that astroglial activation is enhanced by continuous MMC, resulting in hypersensitivity of the MM and tooth pulp. We further observed that the GS inhibitor MSO suppressed ERK phosphorylation in the MDH following the application of capsaicin to the M1TP. These findings support our hypotheses that continuous MMC induces astroglial activation in the MDH, and that glutamine synthesis associated with such activation results in hypersensitivity of the tooth pulp to pain via increased excitability of MDH neurons.

Author contributions

TW performed the electrophysiological, behavioral, and immunohistochemistry experiments and analyzed data. KS conceived and designed the experiment, supervised research, and wrote the manuscript. NN, KHa, KHo, YT, and AK assisted in the pharmacological testing and helped to write the manuscript. KO, KK, and HK assisted in the recording of electrophysiological data. MS, BO, and KI conceived the research and helped to write the manuscript. All authors have read and approved the final manuscript.

Declaration of Conflicting Interests

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