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Central Sensitization and MAPKs are Involved in Occlusal Interference-Induced Facial Pain in Rats

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Abstract

We previously developed a rat dental occlusal interference model of facial pain that was produced by bonding a crown onto the right maxillary first molar and was reflected in sustained facial hypersensitivity that was suggestive of the involvement of central sensitization mechanisms. The aim of the present study was to investigate potential central mechanisms involved in the occlusal interference-induced facial hypersensitivity. A combination of behavioral, immunohistochemical, Western blot and electrophysiological recording procedures was used in 98 male adult Sprague-Dawley rats that either received the occlusal interference or were sham-operated or naive rats. Immunohistochemically labeled astrocytes and microglia in trigeminal subnucleus caudalis (Vc) showed morphological changes indicative of astrocyte and microglial activation after the occlusal interference. Prolonged upregulation of p38 MAPK and ERK was also documented in Vc after placement of the occlusal interference, and was expressed in both neurons and glial cells at time points when rats showed peak mechanical facial hypersensitivity. The i.t. administration of the p38 MAPK inhibitor SB203580 to the medulla significantly inhibited the occlusal interference-induced hypersensitivity, and the ERK inhibitor PD98059 produced an even stronger effect. Central sensitization of functionally identified Vc nociceptive neurons following placement of the occlusal interference was also documented by extracellular electrophysiological recordings, and i.t. administration of PD98059 could reverse the neuronal central sensitization. These novel findings suggest that central mechanisms including central sensitization of trigeminal nociceptive neurons and non-neuronal processes involving MAPKs play significant roles in the production of occlusal interference-induced facial pain.

Perspective—Central mechanisms including trigeminal nociceptive neuronal sensitization, non-neuronal processes involving glial activation and MAPKs play significant roles in occlusal

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interference-induced facial pain. These mechanisms may be involved in clinical manifestations of facial pain that have been reported in patients with an occlusal interference.

Keywords

Occlusal interference; Hypersensitivity; Trigeminal subnucleus caudalis; Central sensitization; Glia; Mitogen-activated protein kinases

INTRODUCTION

We have recently demonstrated³ that a structural change in the rat's dentition by placement of a dental occlusal interference produces a sustained masticatory muscle hyperalgesia with features mimicking several clinical characteristics of chronic orofacial pain conditions^{28,48,50,53}. In this model, rats manifest long-term mechanical facial hypersensitivity following the occlusal interference placement, and removal of the interference after 6 days does not relieve the hypersensitivity. However, systemic administration of the NMDA antagonist MK801 can dose-dependently attenuate the occlusal interference-induced hypersensitivity, raising the possibility that NMDA-related central sensitization mechanisms might be involved in the maintenance of the occlusal interference-induced pain³.

Central sensitization involving neuroplastic changes is considered to be an important mechanism related to chronic pain, and accumulating evidence also shows that glial cells play an important role in the development and maintenance of central sensitization and pain behavior in spinal nociceptive pathways^{18,37,42,47}. The glial involvement includes activation of mitogen-activated protein kinases (MAPKs) which are a family of signaling molecules that transduce extracellular stimuli into intracellular responses in a wide variety of circumstances. These signaling molecules include p38 MAPK, extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and ERK5²⁴. Also in the orofacial area, recent studies have indicated that glial mechanisms contribute to acute and chronic orofacial pain by influencing central sensitization processes in the trigeminal subnucleus caudalis (Vc)^{6,7,11,12,25,39,40,42,57,61–63}. These studies also suggest that activation of MAPKs in glial cells is essential in central mechanisms of orofacial pain^{15,17,25,30,55,61,64}. In the current study, we tested whether the placement of an occlusal interference induces neuroplastic changes reflecting central sensitization of Vc nociceptive neurons, and whether glial activation and changes in MAPK signaling pathways accompany the trigeminal central sensitization and associated facial hypersensitivity. A combination of behavioral, immunohistochemical, Western blot and electrophysiological recording procedures was used in rats receiving the occlusal interference and in sham-operated and naive rats. Data have been partly reported in abstract form⁴.

METHODS

Animals

The experimental protocol was reviewed and approved by Peking University and University of Toronto Animal Care and Use Committees. The methods used for animal preparation, manufacture and bonding of the crown to produce the occlusal interference have been described previously in detail³ and so are only briefly outlined here. Male adult Sprague-Dawley rats initially weighing 280–300g were used. All rats (n=98) were housed under a 12-hour light/dark cycle with food and water available ad libitum and randomly assigned to 1 of 3 different groups (occlusal interference, sham-operated and naive groups). Rats were anesthetized by i.p. injection of pentobarbital sodium (40mg/kg) or isoflurane (5% induction, 2~2.5% maintenance), and a crown with a thickness of 0.4mm (the minimal

occlusal alteration producing mechanical hypersensitivity in our previous study) was placed (for the occlusal interference group) or a band (for the sham-operated group) was bonded onto the right maxillary first molar with dental resin cement (Panavia F, Kuraray, Japan), or the mouth of the rat was held open for 3min (for the naïve group) to replicate the mouth-opening procedure. Compared to sham-operated group and naïve group, weight gains in the occlusal interference group over the observation period after adding a crown were similar, indicating that the procedure for producing the animal model did not influence the animals' general health. The baseline weights of the three groups were 312 ± 6.6 g for the occlusal interference group, 308 ± 4.4 g for the sham group, 303 ± 4.3 g for the naïve group, and on day 10 after treatment were 408 ± 11.8 g, 409 ± 7.8 g, and 412 ± 10.6 g, respectively.

Immunohistochemistry

Rats receiving the occlusal interference (n=21; three for each time point at 1, 3, 5, 10, 14, 21 and 28 days after occlusal interference placement) or sham-operated rats (n=3) or naïve rats (n=3) were deeply anesthetized with an overdose of pentobarbital sodium (100 mg/kg, i.p.) and perfused transcardially with 250ml body temperature 0.1M phosphate-buffered saline (PBS, pH7.4), followed by 300ml ice-cold 4% paraformaldehyde/4% sucrose in 0.1 M phosphate-buffer at pH7.4. After perfusion, a segment of brainstem (from 1mm rostral to 2mm caudal to the obex) was removed, postfixed in 4% paraformaldehyde fixative for 4 hours and then transferred to a 30% sucrose solution overnight at 4°C. Thirty-micron-thick brainstem sections were prepared for free-floating immunohistochemical staining. The sections were blocked with 4% normal goat serum (NGS) and then incubated for 48 hours at 4°C in the primary antibody of components of the phosphorylated MAPKs (anti-p-p38MAPK 1:200, anti-p-ERK 1:200, anti-p-JNK 1:200; Cell Signaling, Beverly, MA), glial fibrillary acid protein (GFAP, an astrocyte marker, 1:200; NeoMarkers, Fremont, CA) and OX-42 (CD11b, a microglia marker, 1:200; Serotec, Indianapolis, IN). The sections were then incubated for 90 min at room temperature with a corresponding FITC-conjugated secondary antibody (1:200, Jackson ImmunoResearch, West Grove, PA). As a control, the primary antibody was replaced by normal serum or PBS; this resulted in no staining in the brainstem tissue.

Double immunofluorescence was also carried out to determine if MAPK labeling occurred in neuronal or glial elements. Since p-JNK labeling was not increased following placement of the occlusal interference (see Results), tissues were incubated with a mixture of primary antibodies (anti-p-p38 MAPK, anti-p-ERK) with monoclonal neuronal-specific nuclear protein (NeuN, a neuronal marker, 1:5000; Chemicon, Temecula, CA), GFAP or OX-42. Following the incubation, brainstem sections were washed and incubated for 2 hours at room temperature in a mixture of FITC- and TRITC-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA). The stained sections were examined with an Olympus (BX51, Tokyo, Japan) fluorescence microscope, and images were captured with a CCD spot camera; image enhancement was performed by using Adobe Photoshop 10.0.

Astrocytic and microglial scoring of activation was based on morphological changes according to well-accepted scoring categories⁹. The following specific morphological changes were scored for GFAP and OX-42 labeled elements from the immunohistochemical sections: '0', unperturbed astrocytes/microglia with extensive ramifications, well-spaced, evenly distributed, GFAP/OX-42 immunoreactivity not apparent; '+', astrocytes/microglia still well ramified, less area between individual astrocytes/microglia, GFAP/OX-42 immunoreactivity becoming apparent; '++', astrocytes/microglia less ramified with shortened and thick processes and increased density, with occasional overlapping, prominent GFAP/OX-42 immunoreactivity; '+++ ', astrocytes/microglia extremely hypertrophic with few, short, thick processes, densely arranged/overlapping, intense GFAP/OX-42 immunoreactivity. The evaluator was blind to the animal's condition (occlusal interference

rat or sham-operated rat or naive rat) during scoring the sections. At least three brainstem sections on each side at comparable rostrocaudal levels were used for the scoring in each animal.

Western blot

Since no over-expression of MAPKs was observed in sham-operated rats by immunohistochemistry (see Results), rats receiving the occlusal interference (n=18; 3 for each time point at 1, 3, 5, 10, 14 and 21 days after occlusal interference placement) or naive rats (n=3) were deeply anesthetized with sodium pentobarbital (100 mg/kg, i.p.) then decapitated. A block of brainstem tissue (from 1mm rostral to 2mm caudal to obex) was removed. The tissue block was then turned coronally and the ventral portions of the Vc (where showed markedly over-expression of MAPKs, see results) was harvested bilaterally¹¹.

The tissues were homogenized in lysis buffer (20 mM Tris buffer, pH 7.6, containing 150 mM NaCl, 1% NP-40, 5% sodium deoxycholate, 1 mM EDTA, 2 mM sodium orthovanadate, 1 mM phenylmethanesulfonyl fluoride, phosphatase and protease inhibitor cocktail; Sigma). The homogenate was centrifuged at 15,000 g for 45 min at 4°C. The supernatant was removed, and the protein concentration of tissue lysates was determined with a BCA Protein Assay Kit (Pierce, Rockford, IL). Twenty µg aliquots were subjected to 12% SDS-PAGE, and proteins were transferred electrophoretically to polyvinylidene difluoride filters (Millipore, Bedford, MA). After blocking with 5% non-fat milk in Tris-buffered saline (TBS) containing 0.1% Tween-20 for 1 hour at room temperature, the membranes were incubated with antibody to p-p38 MAPK, p-ERK, p-JNK, p38 MAPK, ERK, JNK (1:1000, in 5% bovine serum albumin; Cell Signaling, Beverly, MA) overnight at 4°C; anti-β-actin (1:1000, in 5% BSA; Santa Cruz Biotechnology, Santa Cruz, CA) was used as internal control. After washing, the antibody-protein complexes were probed with HRP-conjugated secondary antibody (1:10000, Jackson), developed in enhanced chemiluminescence solution for 3 min, and exposed onto Kodak hyperfilms. The intensity of immunoreactive bands was quantified using NIH ImageJ 1.38 software, normalized to the density of the internal control (β-actin) and expressed as fold changes in the occlusal interference group as compared to the control (naive) group.

Intrathecal drug administration and behavioral testing

Behavioral tests for all occlusal interference rats (n=18), sham-operated rats (n=6) or naive rats (n=6) were conducted on 3 consecutive days before the treatment (as baseline), and subsequently on days 1, 3, 5, 7, 10 and 14; on days 1, 3 and 5, the behavioral tests were conducted before drug or vehicle delivery. The occlusal interference rats (n=18) receiving drug or vehicle administration were anesthetized with isoflurane (5% induction, 2~2.5% maintenance), and then a 10µl solution containing 10µg of the p38MAPK inhibitor SB203580 ([4-(4-fluorophenyl)-2-(4-methyl-sulfonylphenyl)-5-(4-pyridyl)-1H-imidazoles) or the ERK inhibitor PD98059 ([2-(20-amino-30-methoxyphenyl)-oxanaphthalen-4-one]) (Calbiochem, La Jolla, CA) or vehicle was delivered i.t. to the medulla and the catheter flushed with 10 µl of saline. SB203580 and PD98059 were dissolved in dimethylsulfoxide (DMSO), and just before their use in the experiment, they were diluted in saline. The vehicle (2% DMSO) or the 10µg/10µl SB203580 or PD98059 (shown to be an effective dose for attenuating neuropathic pain behavior without affecting mechanically evoked behavioral responses in naive rats^{30,64}) was first delivered i.t. at 1hr prior to the placement of the crown, and was then repeated once a day at 12 noon until day 6 (approximately 1 day after peak mechanical hypersensitivity occurs³).

The thresholds for mechanically evoked nociceptive behavioral responses before and after i.t. administration of the inhibitor or vehicle were measured as described previously³. Briefly, the rat was habituated to standing on its hindpaws and against the tester's gloved hand. Thresholds to mechanical stimulation of the left or right facial skin (overlying the masseter muscles) were measured by an electronic von-Frey anesthesiometer (IITC Life Science, CA, USA). The force in grams needed to elicit a head withdrawal indicative of a nociceptive response was recorded five times, first on one side of the face at 1-min intervals and then repeated on the contralateral side. The average of these five values on each side was used as the withdrawal threshold for that side. The observer was blind to the animal's condition.

Neuron Recording & Stimulation Procedures

The methods used for animal preparation, stimulation, neuronal recording and classification have been described previously in detail^{5,6,61} and so only will be briefly outlined here. Electrophysiological recording of Vc neuronal activity was conducted in animals with an occlusal interference and in sham-operated animals, on day 5–7 post-operation, i.e. around the time when peak mechanical hypersensitivity induced by the occlusal interference was observed. Occlusal interference rats (n=10) or sham-operated rats (n=10) weighing 300–350 g were anesthetized by a single injection (i.p.) of a mixture of α -chloralose (50 mg/kg) and urethane (1 g/kg). Then a tracheal cannula was inserted, and the left external jugular vein was cannulated. After the rat was placed in a stereotaxic apparatus, the medulla was surgically exposed, and the overlying dura and subarachnoid membrane were removed. Just before the neuronal recording session, a supplemental dose of urethane (200–300 mg/kg, i.v.) was administered, and the rat was then immobilized with pancuronium bromide [i.v. initial dose, 0.3–0.4 ml of 1 mg/ml solution, followed by a continuous intravenous infusion of a mixture of 70% urethane solution (0.2 g/ml) and 30% pancuronium solution (1 mg/ml) at a rate of 0.3–0.4 ml/h] and the rat was artificially ventilated throughout the whole experimental period. A deep level of anesthesia was confirmed periodically by the lack of spontaneous movements and responses to pinching the paw when the pancuronium-induced muscle paralysis was allowed to wear off. Heart rate, percentage expired CO₂, and rectal temperature were constantly monitored and maintained at physiological levels of 333–430 beats/min, 3.5–4.2%, and 37–37.5°C, respectively. Single neuronal activity in Vc was recorded extracellularly by means of an epoxy resin-coated tungsten microelectrode, at a Vc level where previous studies have characterized nociceptive neurons receiving cutaneous and deep orofacial afferent inputs^{1,2,16,26}. As the microelectrode was advanced with a rostral inclination of 23° into the rostral Vc from the obex caudal to 1.8 mm and 1.6–2.0 mm lateral to the midline, stimuli were applied to the orofacial tissues to search for nociceptive neurons receiving an orofacial sensory input (see below). Neuronal activity was amplified, displayed on oscilloscopes, and led to a window discriminator and an analog-to-digital converter (CED 1401 plus; Cambridge Electronic Design, Cambridge, UK) connected to a personal computer running Spike2 software (Cambridge Electronic Design), which digitized and stored the triggered action potentials. Data were analyzed off-line with this same software.

The neurons recorded in the study had a mechanoreceptive field (RF) on the facial skin which allowed for quantitatively testing of neuronal responses to a range of stimuli; additional inputs to these neurons from deep tissue were tested by palpation of the jaw muscles or temporomandibular joint. A wide range of mechanical (brush, pressure, and pinch), and noxious thermal (radiant heat; 51–53°C) stimuli were applied to the cutaneous RF to classify each neuron as wide dynamic range (WDR) or nociceptive-specific (NS)^{5,6,61}. The average frequency of any spontaneous activity (in Hz) of a nociceptive neuron was determined over the initial 3–5 min recording period. As outlined in our previous

studies^{5,6,61}, the cutaneous tactile RF of each WDR neuron was determined by brushing the skin with a force < 2 g; the cutaneous pinch/pressure RF of each NS or WDR neuron was determined through the use of a blunt probe and a pair of non-serrated forceps. A burst response consisting of at least two spikes during each stimulus trial was accepted as the criterion to determine the RF extent of the neuron tested. Noxious cutaneous stimulation was used sparingly so as to avoid damage to the skin and the production of peripheral sensitization. For NS neurons, the activation threshold to a mechanical stimulus applied to the orofacial RF was assessed by a pair of force-monitoring forceps, and as the mechanical force was gradually increased, the responses of the tested neuron were recorded by the use of the Spike2 program (CED 1401 plus). The neuronal responses to a mechanical pinch stimulus (100g for NS, 40g for WDR) were determined with the force-monitoring forceps applied to the neuronal orofacial RF. Responses evoked by graded pressure by means of von Frey monofilament applications (0.4g, 1g, 2g, 6g, 15g, 26g, 60g, 100g) delivered in ascending order (each for 2 s at an interval of >45s) to the RF were also determined, as described previously^{5,6,61}.

Since the ERK inhibitor PD98059 produced marked suppression of the occlusal interference-induced facial hypersensitivity whereas vehicle was ineffective and the behavioral threshold of sham-operated rats did not change compared to naive rats (see Results), the effects of PD98059 were tested on the neuronal RF and response properties in the occlusal interference group and in the sham group. The properties tested were spontaneous activity frequency (Hz), RF area (cm²), mechanical activated threshold (g), and responses to graded mechanical stimuli (sum of evoked spikes). After stable baseline RF and neuronal properties were obtained during the superfusion of saline over the exposed ipsilateral medulla, PD98059 (0.1mM, Calbiochem, La Jolla, CA) was continuously superfused (i.t.) over the medulla (at a rate of 0.6 ml/h). At 30 and 60 min after the PD98059 superfusion began, two assessments of the neuronal properties were carried out.

Statistical analyses

Data were tested for normality (Kolmogorov–Smirnov test) and equal variance. Data are reported as mean±SEM. Significance was calculated by using one-way ANOVA followed by the Bonferroni post-hoc test, for the Western blot analysis. In the behavioral tests, the changes of mechanical head withdrawal thresholds between the SB203580 group and vehicle group or between the PD98059 group and vehicle group were treated by two-way repeated measures ANOVA followed by the Bonferroni post-hoc test. For neuronal recordings, differences in spontaneous activity frequency, RF area, mechanical activation threshold and pinch/pressure-evoked responses between the baseline values and the values at different time points after PD98059 delivery in the occlusal interference group and the sham-operated group were treated by one-way ANOVA followed by the Bonferroni post-hoc test. The difference in stimulus-response function was treated by Univariate analysis. Differences were considered to be significant at a level of $p < 0.05$.

RESULTS

Experimental occlusal interference induces glial activation

We first examined whether the occlusal interference could induce activation of astrocytes and microglia in Vc. The immunofluorescence labeling for GFAP and OX-42 was tested in naive rats (n=3), sham-operated rats (n=3) and in occlusal interference rats at 1, 3, 5, 10, 14, 21 and 28 days (n=3 for each time point) after the placement of the occlusal interference.

In naive rats, GFAP and OX-42 immunoreactivities reflecting labeling of astrocytes and microglia, respectively, were homogeneously distributed, and astrocytes and microglia

appeared to be in a resting state. Astrocytes and microglia were extensively ramified and were evenly spaced (Figure 1A, C; 2A, C). No activation of astrocytes and microglia was apparent in sham-operated rats (Figure 1D, 2D). Astrocyte activation was detectable from day 3 after placement of the occlusal interference (Figure 1F, Table 1), with immunoreactivity and morphological changes apparent at all levels of Vc and especially in its most rostral part, the trigeminal subnucleus interpolaris/caudalis (Vi/Vc) transition zone; the most obvious changes were seen in the ventrolateral region. At day 5, in the same area, astrocytes became less ramified and exhibited thick processes, and the immunoreactivity was prominent (Figure 1G, Table 1). The most marked glial activation was observed at day 14 (Figure 1B, I, Table 1), at which time astrocytes had developed a round body with shortened and thick processes and showed intense GFAP immunoreactivity. In some parts of the ventrolateral region, there was densely overlapping of astrocytes (Figure 1I). At day 21 (Figure 1J), GFAP immunoreactivity appeared to decline and only slight labeling was apparent at day 28 (Figure 1K).

Activation of microglia was also observed at all levels of Vc and again especially in the ventrolateral region at the level of the Vi/Vc transition zone, from day 3 after the placement of the occlusal interference (Figure 2F, Table 1), with its peak at day 5 (Figure 2G, Table 1). The OX-42 labeled ramified cells had become hypertrophic, with shortened and thick processes and increased density of OX-42 staining. From day 10 to day 14, the microglia showed evidence of decreased activation and the OX-42 immunoreactivity had returned to basal levels by day 21 (Figure 2H–K, Table 1).

Experimental occlusal interference induces activation of MAPKs

The levels of p-p38 MAPK, p-ERK, and p-JNK in the rostral Vc and Vi/Vc transition zone were analyzed by immunofluorescence in naïve rats (n=3), sham-operated rats (n=3) and in occlusal interference rats at 1, 3, 5, 10, 14, and 21 days (n=3 for each time point). Expression of both p-p38 MAPK and p-ERK were markedly increased bilaterally (Fig 3). A few p-p38MAPK-immunoreactive cells were found in the dorsal portion of the rostral Vc in naïve rats (Fig 3), while there were many more p-p38 MAPK-positive cells at 3, 5 and 10 days after the occlusal alteration in the ventrolateral region (Fig 3). Similar changes were also observed for p-ERK immunofluorescence labeling: a few p-ERK immunoreactive cells were found in the naïve rats (Fig 3), and there were many more p-ERK positive cells at 1, 3, 5 and 10 days after the occlusal alteration, again mainly in the ventrolateral region (Fig 3). No over-expression of p-p38MAPK and p-ERK was observed in sham-operated rats (Fig 3).

To further explore the time course of p-p38 MAPK and p-ERK expression during the observation period, we also carried out Western blot analysis to quantify the phosphorylated protein level in the rostral Vc of naïve rats (n=3) and occlusal interference rats at 1, 3, 5, 10, 14, and 21 days (n=3 for each time point). Relative to the level (control) in naïve rats, an increase of p-p38 MAPK occurred that was significant at day 3 following the occlusal alteration, reached its peak by day 5, and had declined by day 14 (Fig 4). An increase of p-ERK was also apparent in occlusal interference rats; it was significant at day 1, remained at a high level until day 10, and had declined by day 14 (Fig 4). No significant change was observed for p-JNK immunofluorescence labeling and Western blot analysis at the same time points (Fig 4).

Analogous findings for both immunofluorescence and Western blot analysis occurred on the side contralateral to the occlusal interference. Examples of Western blot for contralateral p-p38MAPK, p-ERK and p-JNK expression are shown in Fig 5.

To identify the cell type that expressed p-p38 MAPK and p-ERK after the occlusal alteration, we carried out double immunofluorescence labeling for p-p38 MAPK and p-ERK

with several cell-specific markers; NeuN for neurons, GFAP for astrocytes, and OX-42 for microglia at day 5. The p-p38 positive cells were double-labeled primarily with the neuronal marker NeuN or the microglia marker OX-42, very few with the astrocyte marker GFAP (Fig 6). A similar pattern of co-localization of p-ERK with NeuN, OX-42 or GFAP was also observed (Fig 7).

Effects of p38MAPK and ERK inhibitors on occlusal interference-induced facial hypersensitivity

To investigate the participation of p38 MAPK and ERK pathways in our model, we tested the effects of i.t. administration of p38 MAPK and ERK inhibitors on the mechanical hypersensitivity. Behavioral tests were conducted in the naive group, sham-operated group, and occlusal interference groups receiving SB203580, PD98059 or vehicle (n=6 for each group) (Fig 8). No significant change of head withdrawal threshold was observed in sham-operated rats and in naive rats, but the occlusal interference produced a long-lasting bilateral hypersensitivity (Fig 8), consistent with our previous findings³. The vehicle did not affect the hypersensitivity, but the head withdrawal threshold increased significantly after i.t. of either PD98059 or SB203580, and was more marked and prolonged in the case of PD98059 (Fig 8).

Experimental occlusal interference induces central sensitization in functionally identified nociceptive neurons that can be suppressed by ERK inhibitor

Twenty functionally identified NS (n=10) and WDR (n=10) neurons were studied at post-operative day 5–7. All of these neurons were located in the deep laminae of the Vc, the average depth from the medullary surface was 896 μ m (min: 670 μ m, max: 1105 μ m). Ten neurons (five NS, five WDR) were from rats receiving the occlusal interference and were treated with PD98059 (Occlusal/PD98059 group); another 10 neurons (five NS, five WDR) were recorded in sham-operated rats and were also treated with PD98059 (Sham/PD98059 group). All 10 NS neurons (five from each group) had at baseline a cutaneous pinch/pressure RF that for most was located in the maxillary division of the trigeminal nerve (involving the vibrissal pad, medial facial skin or periorbital skin) and could also be activated by pressure stimulation of the jaw musculature or TMJ. All 10 WDR neurons (five from each group) also had at baseline a deep tissue input and an extensive cutaneous pinch/pressure RF involving the periorbital skin, vibrissal pad, medial or lateral facial skin, and a tactile RF located within the boundary of the pinch/pressure RF. Their baseline features are outlined in Table 2.

Comparison between the baseline values (values before PD98059 administration) of these Vc neuronal properties of the Occlusal/PD98059 group and Sham/PD98059 group showed significant differences for the NS and WDR neurons. The mechanical activation threshold of the NS neurons decreased significantly in the Occlusal/PD98059 group ($p=0.018$) (Table 2), and the responses to graded stimuli and the area of pinch RF increased significantly in the Occlusal/PD98059 group ($p=0.013$ and 0.015 respectively; Table 2). The stimulus-response function of the NS neurons also shifted upward significantly in this group (Univariate analysis, $F=46.8$, $p<0.001$) (Fig 10). None of the NS neurons in the Sham/PD98059 group had a baseline tactile RF, while 4 of 5 NS neurons from the Occlusal/PD98059 had a very localized tactile RF. No difference in NS neuronal spontaneous activity frequency (Hz) was observed between the two groups (Table 2). For the WDR neurons, the responses to graded stimuli also increased significantly in the Occlusal/PD98059 group compared to the Sham/PD98059 group ($p=0.002$) (Table 2). In addition, both the tactile and pinch RF areas were increased significantly (tactile: $p=0.002$; pinch: $p<0.001$) (Table 2) and the stimulus-response function of the WDR neurons shifted upward significantly in Occlusal/PD98059

group (Univariate analysis, $F=29.3$, $p<0.001$) (Fig 9). There was no difference between the two groups in WDR neuronal spontaneous activity frequency (Table 2).

In NS neurons of the Occlusal/PD98059 group, compared to baseline values, the i.t. administration of PD98059 significantly reversed the lowered mechanical activation threshold at 60min post-administration ($p=0.027$), and significantly reduced the elevated pinch/pressure-evoked responses at 30 and 60 min (baseline vs. 30min: $p<0.001$; baseline vs. 60min: $p<0.001$) (Table 2). Consistent with this finding was the stimulus-response function after PD98059 administration, which shifted downward significantly ($p<0.001$) (Fig 9), suggesting that the ERK inhibitor had depressed the evoked responses of the NS neurons. The stimulus-response function also shifted to the right (Fig 9), implying an increase of the response threshold consistent with the PD98059 effects on the mechanical activation threshold (see above). In 2 of 4 NS neurons, their tactile RF disappeared after administration of PD98059, while the increased area of the pinch RF documented in this group decreased significantly at 60min after PD98059 administration ($p=0.010$) (Table 2).

Similarly in WDR neurons from the Occlusal/PD98059 group, compared to baseline values, the i.t. administration of PD98059 significantly reduced the elevated pinch/pressure-evoked responses at 30 and 60 min (baseline vs. 30min: $p=0.002$; baseline vs. 60min: $p<0.001$) (Table 2), consistent with the finding that the stimulus-response function shifted downward and to the right significantly ($p<0.001$) (Fig 9). In addition, both tactile RF and pinch RF areas decreased significantly after PD98059 administration (baseline vs. 30min: tactile $p=0.013$, pinch $p<0.001$; baseline vs. 60min: tactile $p=0.004$; pinch $p<0.001$) (Table 2).

In the Sham/PD98059 group, no significant effects of PD98059 on NS and WDR neurons were observed (Fig 9, Table 2).

DISCUSSION

A major finding of the current study was that the occlusal interference induced-facial hypersensitivity was associated with enhanced excitability in functionally identified Vc nociceptive neurons, reflected in an enlarged RF, lowered activation threshold and increased responses to innocuous and noxious stimuli, which are characteristic parameters of Vc central sensitization^{5,16,20,44,49}. Other novel findings were that after placement of the occlusal interference both Vc astrocytes and microglia showed marked activation, especially those in the Vi/Vc transition zone where also members of the MAPK family were phosphorylated after the occlusal alteration and expressed in glial cells as well as neurons at time points when rats showed peak mechanical facial hypersensitivity. Further evidence of MAPK involvement came from our findings that the hypersensitivity and central sensitization could be reduced by i.t. administration of the MAPK inhibitors. These findings extend our previous data showing that an occlusal interference in rats produces a long-term bilateral facial hypersensitivity, which can be dose-dependently attenuated by the NMDA antagonist MK801³.

Peripheral and central mechanisms are involved in occlusal interference-induced facial pain

The occlusal interference-induced facial hypersensitivity has features which mimic some characteristics of human chronic facial pain conditions that manifest myofascial pain, some of which are characterized by pain and tenderness to palpation of the masticatory muscles, and also alterations in cutaneous sensitivity or jaw movements^{10,13,14,33,28,48,50,53}. However, despite extensive research in the past few decades, the pathophysiology of such myofascial pain conditions remains unclear. Several studies have demonstrated that myofascial pain patients exhibit significantly lower pressure pain thresholds in their

masticatory muscles than healthy controls as well as other features indicative of allodynia, hyperalgesia and pain spread^{4,10,28,33,41,45,48,50,53}. Local factors (e.g., occlusal alterations) as well as central (e.g. “psychological” or “psychophysiological”) processes have been implicated^{28,48,50,52,53}. The role of the dental occlusion as an etiological factor has been a controversial topic. Some clinical studies do not indicate that an occlusal interference is either predictive or causative of the pain^{8,27,58}, while others suggest occlusal factors (especially an acute occlusal change) may contribute^{29,31,34,36,46}.

The occlusal interference model used in the present study was one that has been shown³ in the rat to cause facial hypersensitivity reflecting masticatory muscle hyperalgesia. Although we cannot rule out the possibility that cutaneous receptors in the facial skin overlying the masseter muscle may also be involved in the behavior evoked by the mechanical stimulation of the skin overlying the masseter muscle, the high mechanical thresholds for evoking the withdrawal behavior in the present study in both naïve and experimental animals are consistent with our previous findings in this model³ and with the activation of receptors in deep tissues such as the masseter; much lower behavioral thresholds evoked by mechanical stimulation of facial cutaneous receptors have been reported with other orofacial pain models^{20,25,39, 44,57}. Thus it seems likely that activation of masseter muscle receptors at least contributed to the sensory input evoking the behavior. Facial cutaneous receptors may also have contributed, and the mechanisms could have involved peripheral processes also in other masticatory muscles, perhaps as a result of the occlusal alteration causing an altered muscular activity pattern that produces changes in the muscle or dental tissues, that include increases in substances such as bradykinin, lactate, ATP, phosphate, creatinine kinase, decreases in pH, and infiltration of neutrophils; these factors have been implicated in the sensitization of musculoskeletal nociceptive afferents^{28,35,48}. Besides these possible peripheral sensitization processes, central hyperexcitability in the trigeminal system may also be involved^{4,10,28,33,41,45,48–50,53}. The neuronal hyperactivity reflecting Vc central sensitization documented in the present study following the placement of an occlusal interference was evident at the peak period of facial hypersensitivity, and is indicative of alterations of the CNS processing of trigeminal nociceptive transmission. We cannot however be certain that stress related to the altered oral environment of the animals may have contributed to the effects of the occlusal interference, and further studies are needed to explore this possibility. Nonetheless, the findings are consistent with the proposition that the decreased activation threshold, increased responses to noxious stimuli and the expanded RFs of Vc nociceptive neurons in the central sensitization state are central mechanisms contributing to the allodynia, hyperalgesia, pain spread and referral that are clinical features typical of chronic facial pain conditions^{28,48,50,53}.

Vc glial activation is involved in central mechanisms of occlusal interference-induced facial pain

There is accumulating evidence that glial cells also play an important role in the development and maintenance of central sensitization in trigeminal nociceptive pathway^{7,42}. Glial cell activation in Vc has been reported in many trigeminal neuropathic pain models^{25,39,40,62,64}, such as orofacial cutaneous^{51,63} and deep tissue inflammatory models^{11,12}, experimental tooth movement model³², and acute and chronic tooth pulp inflammatory pain models^{6,19,57}. A different activation time course of astrocytes and microglia changes have been observed in many pain models. In some acute and chronic inflammatory pain states, astrocyte activation occurs before microglial activation, whereas microglial activation usually precedes astrocyte activation in neuropathic pain models^{9,40,43,54,62}. In the present study, there was evidence of astrocyte activation from day 3 after the placement of the occlusal interference, peaked at day 14, and markedly declined by day 28; while microglia activation was especially apparent at days 3 to 14. The relatively

late and long-lasting activation of both astrocytes and microglia in our model may be due to the constant placement of the occlusal interference throughout the observation period that produced a relatively mild but persistent stimulus compared to the short-lasting stimuli occurring in many other inflammatory pain models.

P38 MAPK and ERK upregulation but not JNK are involved in occlusal interference-induced facial pain

Although the participation of the MAPK family in CNS nociceptive processing in the spinal cord is usually accompanied by hyperalgesia and allodynia, comparatively little is known about its contribution to orofacial pain mechanisms. For instance, a subcutaneous injection of formalin or capsaicin into the perioral skin increases the number of activated ERK-immunoreactive neurons in the Vc of mice^{17,38}. ERK phosphorylation of Vc and C1-C2 neurons and astrocyte activation are involved in orofacial extraterritorial pain following upper cervical nerve injury²⁵, and p38 MAPK and ERK inhibitors can reduce facial mechanical hypersensitivity induced by chronic constriction injury of the rat infraorbital nerve³⁰. Also, i.t. application of the p38 MAPK inhibitor SB203580 attenuates the Vc central sensitization in an acute dental inflammatory pain model⁶¹. A recent study similarly showed that enhanced ERK phosphorylation and thermal hyperalgesia are simultaneously induced by glutamate injection into the tongue and vibrissal pad skin, which can be suppressed by the ERK inhibitor PD98059¹⁵. These various findings are consistent with the present observations of p38 MAPK and ERK activation in Vc following placement of an occlusal interference and that inhibitors of p38 MAPK (SB203580) or ERK (PD98059) can reverse the mechanical hypersensitivity and that PD98059 can also reverse the associated Vc central sensitization in this model. It has been reported that ERK in neurons may play a role in intracellular processes involving transduction of the glutamate signal from the cytoplasm to the nucleus where ERK regulates gene expression by phosphorylating nuclear transcription factors, and thus modulate neuroplasticity^{22,56,60}. In line with these data, the present electrophysiological and immunohistochemical results demonstrate that neurons in the rostral Vc, including the Vi/Vc transition zone, are centrally sensitized following placement of an occlusal interference and suggest that this activation may be regulated by ERK pathways. The Vi/Vc transition zone has been recognized as an important region of the trigeminal spinal tract nucleus for processing nociceptive inputs from orofacial deep tissues as well as from cutaneous tissues⁴².

In addition however, our double-labeling results demonstrate that activation of p38 MAPK and ERK, but not JNK, also occurs in glial cells of Vc following placement of an occlusal interference. The expression of p-p38 MAPK and p-ERK in glial cells has also been reported in other orofacial pain models. For example, p-p38 MAPK and p-ERK activations were found in microglia and astrocytes in the Vc in a lingual nerve injury model⁵⁵, and p-p38 MAPK is increased in Vc after inferior alveolar and mental nerve injuries and minocycline, an inhibitor of microglial activation, attenuates the development of neuropathic pain⁴⁰. Application to the medulla of the p38 MAPK inhibitor SB203580 or specific astroglial metabolic inhibitor methionine sulfoximine has also been shown to suppress Vc central sensitization induced in an acute dental inflammatory pain model^{6,61}. Also noteworthy are the present findings that PD98059 application did not affect the baseline RF and response properties of Vc nociceptive neurons in sham-operated rats, consistent with reports that PD98059 i.t. administration blocks mechanical hypersensitivity in an orofacial neuropathic pain rodent model, but not mechanical sensitivity in sham-operated or naive animals³⁰. Collectively, these results suggest that ERK activation is involved in the hyperexcitability state that is a feature of central sensitization in trigeminal nociceptive pathways but may not contribute to the normal baseline Vc nociceptive processing, consistent with findings in spinal nociceptive models^{21,23,59}.

Conclusions

We have previously established a rat model of long-lasting facial pain produced by an occlusal interference and reflected in facial hypersensitivity. In the present study, we further demonstrated that central mechanisms including Vc central sensitization of trigeminal nociceptive neurons and non-neuronal processes involving MAPKs play significant roles in the production of the occlusal interference-induced facial hypersensitivity. These mechanisms may be involved in clinical manifestations of facial pain that have been reported in patients with an occlusal interference.

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Figure 1.

GFAP-positive cells in the ipsilateral Vc following placement of occlusal interference. Low magnification photographs of naive rat (A) and rat on day 14 after occlusal interference placement (B). C–K showed high magnification photographs of the area encircled in (B) at different time points after occlusal interference placement. Astrocytes exhibited extensive fine processes and were well-spaced in naive rat (C). No activation of astrocytes was apparent in sham-operated rat (D). Activation of astrocytes was observed from day 3 after occlusal interference placement (F), peaked at day 14 (I). After that, GFAP immunoreactivity appeared to decline. Scale bar, 50 μ m.

Figure 2.

OX-42 positive cells in the ipsilateral Vc following placement of occlusal interference. Low magnification photographs of naive rat (A) and rat on day 5 after occlusal interference placement (B). C–K showed high magnification photographs of the area encircled in (B) at different time points after occlusal interference placement. Microglia exhibited extensive fine processes in naive rat (C). No activation of microglia was apparent in sham-operated rat (D). Activation of microglia was observed from day 3 after occlusal interference placement (F), peaked at day 5 (G). From day 10 to day 14 (H, I), microglia showed decreasing activation and returned to the basal level by day 21 (J). Scale bar, 25 μ m.

Figure 3.

Immunohistochemistry for p-p38 MAPK and p-ERK in ipsilateral Vc at different time points following placement of occlusal interference. A few p-p38 MAPK-immunoreactive cells were found in naive rats, while there were many more p-p38 MAPK-positive cells after occlusal interference placement in ventrolateral region. Very few p-ERK immunoreactive cells were found in naive rats, and there were more p-ERK positive cells after the occlusal interference placement, mainly in the ventrolateral region. High magnification photographs of the area encircled in yellow rectangle (ventrolateral region) are shown for day 5 and 10. No over-expression of p-p38 MAPK and p-ERK was observed in sham-operated rats.

Figure 4.

Western blot illustrating occlusal interference-induced increase in ipsilateral p-p38 MAPK, p-ERK and p-JNK. Examples are shown on the top, and the increased protein levels relative to control levels in naïve rats (fold changes) are shown in the bottom histogram. * $p < 0.05$ vs. naïve rats. Error bars represent SEM.

Figure 5.

Western blot of contralateral p-p38 MAPK, p-ERK and p-JNK following placement of occlusal interference. P-p38 MAPK and p-ERK showed similar expression on contralateral side. No significant change in p-JNK expression was observed.

Figure 6.

Colocalization of p-p38 MAPK with NeuN, GFAP, OX-42 at day 5 following placement of occlusal interference. The p-p38 positive cells were double-labeled primarily with the neuronal marker NeuN and microglia marker OX-42, very few with the astrocyte marker GFAP.

Figure 7. Colocalization of p-ERK with NeuN, GFAP, OX-42 at day 5 following placement of occlusal interference. The p-ERK positive cells were double-labeled primarily with the neuronal marker NeuN and microglia marker OX-42, very few with the astrocyte marker GFAP.

Figure 8.

The effects of i.t. administration of 10 μ g SB203580 or PD98059 or vehicle on occlusal interference-induced mechanical hypersensitivity. Mechanical head withdrawal thresholds were measured in naive group, sham-operated group, and occlusal interference groups received SB203580, PD98059 or vehicle at baseline and at post-operative days 1, 3, 5, 7, 10, 14. SB203580 and PD98059 significantly reversed the mechanical hypersensitivity in ipsilateral side and contralateral side. N=6 for each group. ++ p<0.01: occlusal interference + vehicle group vs. sham-operated group; ^^ p<0.01: occlusal interference + vehicle group vs. occlusal interference + SB203580 group; * p<0.05, ** p<0.01: occlusal interference + vehicle group vs. occlusal interference + PD98059 group; ## p<0.01: occlusal interference + SB203580 group vs. occlusal interference + PD98059 group. Error bars represent SEM.

Figure 9.

The stimulus-response function of NS and WDR neurons from Occlusal/PD98059 group and Sham/PD98059 group. The stimulus-response function shifted significantly following placement of the occlusal interference. (^: Comparison between baselines of Occlusal/PD98059 group and Sham/PD98059 group, Univariate analysis: NS, $F=46.8$, $p<0.001$; WDR, $F=29.3$, $p<0.001$). In the Occlusal/PD98059 group, the NS neuron stimulus-response function after PD98059 administration shifted significantly (* Univariate analysis: $F=13.45$, $p<0.001$). The shift of WDR neuron stimulus-response function was also significant (* Univariate analysis: $F=9.905$, $p<0.001$). PD98059 had no effect on stimulus-response function of either NS or WDR neurons in Sham/PD98059 group (Univariate analysis: NS, $F=0.747$, $p=0.476$; WDR, $F=1.476$, $p=0.233$).

Table 1

Glial responses following experimental occlusal interference

	Astroglia response			Microglia response		
	(GFAP)			(OX-42)		
	1	2	3	1	2	3
Naive	0	0	0	0	0	0
1d	0	0	0	0	0	0
3d	+	+	+	+	+	o
5d	+	+	+	++	+	+
10d	+	++	+	+	+	+
14d	++	++	++	+	+	+
21d	+	++	+	0	+	0
28d	0	+	+	0	0	0

Glial activation response scores were based on cell morphology, cell density, and intensity of immunoreactivity to GFAP and OX-42 (see methods). 1, 2, 3 represent the rat number. Scores: no response (0), mild response (+), moderate response (++), and intense response (+++).

Table 2

Baseline features and effects of i.t. superfusion of PD98059 on features of occlusal-induced central sensitization in nociceptive neurons

	Spontaneous activity frequency (Hz)	Tactile RF (cm2)	Pinch RF (cm2)	Mechanical activation threshold (g)	Responses to graded stimuli (sum of spikes)
Occlusal/PD98059					
NS neurons (n=5)					
baseline	0.01±0.01		3.8±0.5 [#]	22.0±3.3 [#]	149.2±18.8 [#]
30min	0		2.1±0.5	63.8±12.5	74.2±15.1 ^{**}
60min	0		1.6±0.4 [*]	78.6±16.5 [*]	41.8±15.3 ^{**}
WDR neurons (n=5)					
baseline	3.36±1.76	1.42±0.11 ^{##}	7.6±1.3 ^{##}		160.4±13.4 ^{##}
30min	2.11±0.92	0.63±0.04 [*]	3.3±0.5 ^{**}		46.0±9.3 ^{**}
60min	2.82±1.71	0.52±0.07 ^{**}	2.7±0.5 ^{**}		35.8±8.1 ^{**}
Sham/PD98059					
NS neurons (n=5)					
baseline	0.14±0.01		1.7±0.3	81.0±10.2	51.0±3.7
30min	0.01±0.01		1.4±0.4	84.4±12.4	49.2±17.9
60min	0.05±0.05		1.5±0.3	93.8±9.0	30.6±4.5
WDR neurons (n=5)					
baseline	2.25±1.44	0.44±0.07	2.7±0.4		46.2±10.4
30min	0.72±0.63	0.36±0.04	2.1±0.1		38.8±15.3
60min	3.46±3.43	0.31±0.04	2.0±0.1		38.0±10.5
Statistical comparisons					
NS neurons	ns		F=5.12;p=0.002	F=5.1;p=0.003	F=9.6; p<0.001
		F=36.5;			
WDR neurons	ns	p<0.001	F=11.1;p<0.001		F=18.3;p<0.001

All values shown as mean ± SEM.

Differences in spontaneous activity discharge frequency, RF area, mechanical activation threshold and pinch/pressure-evoked response between the different time points in two groups were treated by one-way ANOVA followed by Bonferroni post hoc-test, ns, Not significant.

* p<0.05

** p<0.01 for comparison between the baseline value and values at the different time points after PD98059 in each group;

p<0.05

p<0.01 for comparison between the baseline values of Occlusal/PD98059 group and Sham/PD98059 group.