ERK POTENTIATES P38 IN CENTRAL SENSITIZATION INDUCED BY TRAUMATIC OCCLUSION

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Abstract—This study was to investigate the role of p38 activation via ERK1/2 phosphorylation in neurons and microglia of the spinal trigeminal subnucleus caudalis (Vc) in the promotion of orofacial hyperalgesia induced by unilateral anterior crossbite (UAC) traumatic occlusion in adult rats. U0126, a p-ERK1/2 inhibitor, was injected intracisternally before UAC implant. The effects of the U0126 injection were compared to those following the injection of SB203580, a p-p38 inhibitor. Mechanical hyperalgesia was evaluated via pressure pain threshold measurements. Brain stem tissues were processed for a Western blot analysis to evaluate the activation of ERK1/2 and p38. Double immunofluorescence was also performed to observe the expression of p-ERK1/2 and p-p38 in neurons (labeled by NeuN) and microglia (labeled by OX42). The data showed that UAC caused orofacial hyperalgesia ipsilaterally on d1 to d7, peaking on d3 (P < 0.05). An upregulation of p-ERK1/2 was observed in the ipsilateral Vc on d1 to d3, peaking on d1. An upregulation of p-p38 was also observed on d1 to d7, peaking on d3 (P < 0.05). p-ERK1/2 primarily co-localized with NeuN and, to a lesser extent, with OX42, while p-p38 co-localized with both NeuN and OX42. Pretreatment with U0126 prevented the upregulation of both p-ERK1/2 and p-p38. Similarly to an intracisternal injection of SB203580, U0126 pretreatment attenuated the UAC-induced orofacial hyperalgesia. These data indicate that UAC caused orofacial hyperalgesia by inducing central sensitization via the activation of ERK1/2 and p38 in both neurons and microglia in the Vc, potentially impacting the effects of p-ERK1/2 during p38 activation. © 2016 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: occlusal trauma, P38 mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK), microglia, neuron, hyperalgesia.

INTRODUCTION

Orofacial pain is a widespread chronic problem (Ohrbach and Dworkin, 1998; Khan et al., 2013) in which occlusal trauma plays a role (Le Bell et al., 2002). It has been shown that occlusal changes may induce transient symptoms of pain, headache, and tenderness in the jaw muscles (Michelotti et al., 2006). Studies have demonstrated that central sensitization significantly contributes to pain in patients with osteoarthritis, musculoskeletal disorders with generalized pain hypersensitivity, headache, temporomandibular joint disorders, dental pain, neuropathic pain, visceral pain hypersensitivity disorders and postsurgical pain (Woolf, 2011). Central sensitization, characterized by enhanced neuronal activation in the spinal or medullary dorsal horn, is a key process underlying the genesis of pathological pain (Sessle, 2007; Woolf, 2011). Glia, microglia and astrocytes may be involved in spinal and medullary dorsal horn central sensitivity (Sessle, 2007; Itoh et al., 2011). Not only do glia have receptors, including NMDA, AMPA, adenosine triphosphate (ATP) and CGRP receptors, and ion channels that are sensitive to neurotransmitters and neuromodulators, but they also release a host of substances that influence the excitability of neurons involved in nociceptive transmission in the CNS, such as proinflammatory cytokines, prostaglandins, ATP and excitatory amino acids (Watkins and Maier, 2003; Wiese-ler-Frank et al., 2005; Sessle, 2007).

Animal studies indicate that occlusal trauma leads to masticatory muscle sensitivity via central sensitization (Liu et al., 2009; Cao et al., 2013). Accumulating evidence demonstrates that the extracellular signal-regulated prokine...
kinase (ERK) 1/2 and p38 mitogen-activated protein kinase (MAPK) pathways are involved in mechanical hypersensitivity and central sensitization (Xie et al., 2007; Cui et al., 2008; Ji et al., 2009; Worsley et al., 2014). Activation of ERK1/2 is often observed in neurons. For example, phospho-ERK (p-ERK) 1/2 was selectively induced by noxious stimuli that may cause persistent pain sensitivity (Ji et al., 1999; Gao and Ji, 2009). Inhibition of p-ERK1/2 can attenuate trigeminal hyperalgesia (Kohno et al., 1991; Piao et al., 2006; Lim et al., 2007; Xie et al., 2007). Increased activation of ERK1/2 in dorsal horn neurons was observed in animals that received injections of capsaicin or formalin in the hind limbs. p-ERK1/2 increases were also observed in cells other than neurons during different stages depending on the nature of the stimulus (inflammatory or nerve injury) and the region affected (the limb or orofacial area) (Zhuang et al., 2005; Worsley et al., 2014). In an occlusal interference animal model, p-ERK1/2 was found in both neurons and glial cells (Cao et al., 2013). Activation of microglia and mechanical hypersensitivity were accompanied by p-p38 in the Vc after injection with Complete Freund’s Adjuvant (CFA) in the orofacial area, which was suppressed by the administration of the p-p38 inhibitor SB203580 (Kiyomoto et al., 2015). In addition, the presence of p-p38 was also observed in neurons of pain model animals, such as bee venom-induced pain (Cui et al., 2008), postoperative pain (Huang et al., 2000), adjuvant-induced arthritis (Boyle et al., 2006) and temporomandibular joints (TMJs) inflammation (Cady et al., 2010). However, the relationship between p-ERK1/2 and p-p38 in orofacial pain remains obscure.

Recently, we developed a traumatic occlusion model, termed unilateral anterior crossbite (UAC), which induced rat TMJ OA lesions (Zhang et al., 2013, 2015). The role of ERK1/2 and p38 activation in the trigeminal subnucleus caudalis (Vc) in orofacial hyperalgiesia and the relationship between p-ERK1/2 and p-p38 in different cells within the Vc are questions worth investigating in the UAC rat model. In the present study, we assessed behavior, detected molecules via immunohistochemistry and Western blot and assessed biological agent intervention by injecting ERK and p38 inhibitors in our UAC rat model. We hypothesized that central sensitization is engaged in UAC-induced orofacial hyperalgiesia via the progressive activation of ERK and p38 in both neurons and microglia of the Vc.

**EXPERIMENTAL PROCEDURES**

**Animals and operation**

All experiments were performed according to the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) and the International Association for the Study of Pain and Education of P.R. China. Ninety 6-week-old female Sprague–Dawley rats (weight 180–220 g) were provided by the animal center of the Fourth Military Medical University. The animals were housed in groups of 3–4 per cage (measuring 50 × 40 × 25 cm) and acclimatized to the laboratory conditions (12-h light/dark cycle; 22 ± 1 °C room temperature) 1 week before experimentation. The animals had free access to food and water. All sections of this report are in accordance with the ARRIVE reporting guidelines. Efforts were made to minimize animal suffering and the number of animals. All experimental and animal care procedures were approved by the Ethics Committee of the Fourth Military Medical University and performed according to institutional guidelines.

**Group assignment**

The rats were randomly divided into the behavior group (n = 10), immunofluorescence group (n = 15), Western group (n = 15), and drug delivery group (n = 50). The behavior group was further divided into the control subgroup (n = 5) and the UAC subgroup (n = 10). The immunofluorescence group and Western group were also divided into control (n = 3) and UAC subgroups (n = 12). The drug (U0126 or SB293580) delivery group was further divided into the behavior subgroup (n = 20), immunofluorescence subgroup (n = 15) and Western subgroup (n = 15).

The rats were anesthetized with 40 mg/kg of pentobarbital sodium. In the experimental group, UAC was established as a type of dental occlusal trauma using methods described in our previous reports (Wang et al., 2014; Yang et al., 2015). Briefly, a section of metal tubing cut from a 25 gauge needle (Shinva Ande, Shangdong, China; length = 2.5 mm, inside diameter = 3 mm) was bonded to the left maxillary incisor. A 4.5 mm long, 2.5 mm diameter 20 gauge needle was bent 135 degrees labially and bonded to the left mandibular incisor to create UAC on the left incisors. The surgery was completed within 5 min in each rat. The placement of the metal tubing remained stable throughout the experiment.

**Drug delivery**

A polyethylene (PE) 10 tube (Xintai, Xi’an, China) was inserted intracisternally into the anesthetized rats 3 days prior to injection. Briefly, a limited skin incision was made below the occipital bone. The atlanto-occipital membrane was exposed by retracting a part of the muscle away from the occipital bone. Using a 27 gauge needle, a tiny opening was made in the dura. The tip of the cannula was inserted through the opening at the level of the obex and secured in place with nes. The PE tube was threaded subcutaneously to the top of the skull and fixed onto the skin. Each tube was sealed shut using a flame to prevent cerebrospinal fluid leakage. Either the vehicle or the SB203580 p-p38 and p-ERK1/2 inhibitor (10 µg/rat/day, Sigma, St. Louis, MO, USA) and U0126 (1 µg/rat/day, Sigma) diluted in 10 µl of 10% DMSO were first injected and then flushed with 10 µl of saline immediately before the UAC implant. This procedure was repeated once a day until d3 with the first injection 1 h before. The inhibitor dosages were determined following preliminary experimentation and previous studies (Ji et al., 2002; Jin et al., 2003; Cui et al., 2008; Lim et al., 2007; Xie et al., 2007).
et al., 2008; Lee et al., 2010). Behavioral testing was carried out in rats after the UAC procedure, or approximately 1 h after the drug delivery. On d0, Pressure pain threshold (PPT) was measured followed by the delivery of either the drugs or vehicle 1 h prior to the UAC implant (Fig. 1A).

**Pressure pain threshold (PPT) measurement**

The behavioral test was carried out according to methods described in our previous study (Liu et al., 2009). Briefly, after a 30-min acclimation period, the PPT was measured using an electrical machine equipped with a connecting rod with a testing end 2 mm in diameter (HANDPI, HP-5, Yue qing Handpi Instruments Co., Ltd, Zhejiang, China). The skin overlying the bilateral masseter areas, 1 cm below and 1 cm caudal to the midpoint of the eye-ear line were chosen as the mechanical stimulation points. The measurements were carried out repeatedly on the ipsilateral side 5 times in 1-min intervals. The tip of the testing end was smooth and blunt. The test bar was carefully held vertically before pressing. Head flinching, characterized as sudden and quick withdrawals of the head, is a sign of a below threshold PPT measurement. Head flinching was used to identify a below threshold PPT measurement.

![Diagram](image)

**Fig. 1.** Changes in the pressure pain threshold (PPT) and the expression levels of p-ERK1/2 and p-p38 in rat Vc on d0 and d1, d2, d3, d7, and d14 after UAC stimulation. (A) Diagram of PPT, UAC and drug delivery. Black arrow showed that PPT was carried out from d3 before implanting the UAC to obtain a baseline measurement (d-3 to d0) and on d1, d2, d3, d5, d7 and d14. Blue arrow showed the UAC implant, and red arrow showed that the drug or vehicle on d0, d1, d2 and d3. (B) The PPT was lower on the side ipsilateral to UAC when compared to the contralateral side and controls on d0, d1, d2, d3, d7, and d14. (C) Representative p-ERK1/2 immunofluorescent staining of the ipsilateral Vc. (D) Representative p-p38 immunofluorescent staining of the ipsilateral Vc. (F and G) Western blotting and quantitative analysis of the western blotting results of p-ERK1/2 (E) and p-p38 (F). Ipsilateral vs. contralateral side: *P < 0.05; **P < 0.01. UAC ipsilateral side vs. control group: #P < 0.05; ##P < 0.01. Scale bars = 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
head, or vocalizations were considered to be nociceptive responses. The PPT was defined as the average (gram) value of the output that evoked responses within 5 trials. The behavioral assessment was carried out on d3 before implanting the UAC to obtain a baseline measurement (d3 to d0) and on d1, d2, d3, d5, d7 and d14 (Fig. 1 A).

Immunofluorescence

The rats were transcardially perfused with 150 mL of normal saline, followed by 400 mL of 40 g/L paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) under deep anesthesia with pentobarbital sodium (60 mg/kg, i.p.). After perfusion, the caudal medulla was dissected and postfixed in the same fixative overnight at 4 °C and dehydrated by immersion in 20% sucrose in 0.1 M PB at 4 °C overnight.

Serial frozen transverse sections (30 μm thickness) were made through the brain stem below the obex and collected in cold PBS. After blocking with 2% normal goat serum containing 0.3% Triton X-100 for 1 h at room temperature, the sections were incubated with a mixture of antibody against p-p38 (Cell Signaling Technology; 1:200) or p-ERK1/2 (Cell Signaling Technology; 1:200) together with one of the following monoclonal antibodies against neuronal nuclei (NeuN, a neuronal marker; Cell Signaling Technology; 1:1000), glial fibrillary acidic protein (GFAP; an astrocytic marker; Cell Signaling Technology; 1:2000) or OX42 (a microglial marker; Millipore; 1:500) for 24 h at 4 °C, followed by a mixture of Alexa Fluor 488 or Alexa Fluor 594 conjugated secondary antibodies (Molecular Probes; 1:500) for 2 h at room temperature. Then, the double-stained sections were viewed using an Olympus fluorescence microscope. The images were obtained using a confocal laser scanning microscope (FV1000, Olympus, Japan).

Cell counting

Five sections were selected every 120 μm from 1050 μm rostral to 1650 μm caudal to the obex. The author JL was blinded to the group assignments when analyzing the data. The mean of these 5 sections represented the number of immunoreactive double stained cells/section. Each group contained 3 rats. The sections were examined using an Olympus fluorescence microscope (FV1000, Olympus, Japan). The scale bars of the sections represent 100 μm.

Western blot

The animals were deeply anesthetized and then sacrificed. The medulla oblongata was quickly removed and the dorsal half was separated and frozen on dry ice. A 3-mm segment of the medulla oblongata dorsal horn caudal to the obex was homogenized using a hand-held pestle in SDS sample buffer (10 mL/mg tissue) containing cell lysis buffer mixed with protease inhibitor cocktail. The protein samples (30 μg) were resolved on 10% SDS–PAGE gels, transferred onto nitrocellulose membranes (Bio-Rad) at 4 °C and 300 mA for 2 h and then blocked with 5% nonfat milk for 1 h.

The blocked membranes were incubated overnight with primary antibody against p-p38 (Cell Signaling Technology; 1:5000) and p-ERK1/2 (Cell Signaling Technology; 1:200) and then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad Company, USA; 1:5000) for 2 h. Immunoblotting was detected using enhanced chemiluminescence (Amersham Bioscience) according to the manufacturer’s instructions. To quantify the relative levels of protein expression, the images were analyzed using the Total Lab software (Amersham Scientific) image analysis program. To analyze the protein expression levels of p-p38 and p-ERK1/2, the expression of β-actin (measured using the same methods) was used as the loading control.

Quantification and statistical analyses

The SPSS 17.0 package (SPSS Inc., Chicago, IL, USA) was used to analyze the between group differences. The PPT values were analyzed using the 2-way ANOVA method followed by the Bonferroni post hoc test. The Western blots were analyzed using a one-way ANOVA. The unpaired Student’s t-test was used to compare the percentages of p-ERK1/2 and p-p38-positive cells and the number of positive neurons labeled with p-ERK1/2 and p-p38 between sides ipsilateral and contralateral to the UAC, and between the UAC and control groups. The data from the left side of the control group were used when a side difference was detected. Differences were considered to be statistically significant at P < 0.05.

RESULTS

The body weights of the control rats increased with age. The body weights of the UAC rats decreased from d1 but then began to recover from d3 on. Although the body weights of the UAC rats recovered over time, they remained lower than the age matched controls until d14 (d1 to d7; P < 0.01, data not shown).

UAC induced short-term orofacial hyperalgesia

The baseline PPT in the region of the masseter muscle was similar in all rats. There were no significant differences between the right and left sides of the control rats (P > 0.05, Fig. 1B). However, the PPT values in the ipsilateral side of UAC rats were significantly lower than in controls (P < 0.05, Fig. 1B). The ipsilateral values remained low from d1 to d7 with the lowest values observed on d3 (P < 0.05, Fig. 1B). The PPT values measured in the contralateral side did not differ significantly from the control group (Fig. 1B).

UAC activated ERK1/2 and p38 sequentially in the Vc

The p-ERK1/2 protein was only weakly expressed in the control group (Fig. 1C). However, ipsilateral to the UAC, the expression of p-ERK1/2 was enhanced in the Vc from d1 to d7 (Fig. 1C, E) and peaked on d1 (P < 0.05, Fig. 1E). There were no differences between the
contralateral side in UAC rats and the control group
(P > 0.05, Fig. 1E).

Similarly, the expression of the p-p38 protein in the Vc was weak in the control group (Fig. 1D). Contralateral to the UAC, p-p38 expression was enhanced on d1 to d7 (peaking on d3) (Fig. 1D). These changes in expression paralleled changes in PPT in the UAC rats (P < 0.05, Fig. 1F). These differences were not observed between the controls and the UAC group on the contralateral side (Fig. 1D).

UAC enhanced the expression of p-ERK1/2 or p-p38 co-localized with both NeuN and OX42

Double immunofluorescence showed that p-ERK1/2 immunoreactive cells in the superficial layer were mostly labeled with NeuN and to a lesser extent with OX42 (Fig. 2A, C), but none were labeled with GFAP (data not shown). The p-ERK1/2 and NeuN double-positive cells were round in both the ipsilateral and contralateral sides, while the p-ERK1/2 and OX42 double-positive cells were ramified in the ipsilateral side but were dot-like in the contralateral side (Fig. 2A, C). The number of p-ERK1/2 and NeuN double-positive cells increased significantly in the ipsilateral side compared to the contralateral side from d1 to d7, and were larger than those in the control group on d1 and d3 (P < 0.05, Fig. 2B). The number of p-ERK1/2 and OX42 double-positive cells was higher in the ipsilateral side than in the contralateral side or the control group from d1 to 7 (P < 0.05, Fig. 2D). There were very few OX42 and p-ERK1/2 double-labeled cells in the control group.

Similarly, the p-p38 immunoreactive cells in the superficial layer co-localized with OX42 or NeuN (Fig. 3A, C), but none with GFAP (data not shown). Ipsilateral to the UAC, the p-p38 and NeuN double-positive cells were round, while the p-p38 and OX42 double-positive cells were ramified in the ipsilateral side or ameboid (Fig. 3A). In the contralateral side, however, p-p38 and NeuN double-positive cells were dot-shaped or round, and the p-p38 and OX42 double-positive cells were ramified (Fig. 3C). On d1 though d7, there were more p-p38 and NeuN double-positive cells in the ipsilateral side than in the contralateral side. There were more p-p38 and NeuN double-positive cells in the ipsilateral side than in the control group from d1 to d14 (P < 0.05, Fig. 3B). The number of p-p38 and OX42 double-positive cells was higher in the ipsilateral side than in the contralateral side or bilaterally in the control group on d3 (P < 0.05, Fig. 3D).

U0126 inhibited not only p-ERK1/2 but also p-p38 expression in neurons and microglia

Similar to findings using the p-p38 inhibitor SB203580 (data not shown), pre-treatment with U0126, a p-ERK1/2 inhibitor, suppressed not only p-ERK1/2 expression as expected (data not shown) but also p-p38 in the Vc (Fig. 4A, B). Double immunofluorescence confirmed that U0126 reduced the number of p-p38 and NeuN double-positive cells and the number of p-p38 and OX42 double-positive cells (Fig. 4C–F).

Inhibition of either p-p38 or p-ERK1/2 attenuated hyperalgesia in the masseter muscle region

Pre-treatment with either the p-ERK1/2 (U0126) or the p-p38 (SB203580) inhibitor attenuated the reduction of the PPT values on d1, d2 and d3 though none of them reached the levels observed in controls (P < 0.05, Fig. 5A). Withdrawing either U0126 or SB203580 on d3 abolished this attenuating effect such that the PPT values were no longer increased compared to those measured in the vehicle treatment group (P > 0.05, Fig. 5A).

DISCUSSION

The current data indicated that orofacial hyperalgesia was induced by the occlusal trauma of UAC from d1 to d7, with a peak on d3. During that period, ERK1/2 was activated in the ipsilateral Vc, which peaked on d1. P38 was also activated in the ipsilateral side and peaked on d3. The expression of p-ERK1/2 was greatest in neurons. The expression was also observed in the microglia, although to a lesser extent, while p-p38 was almost equally expressed in both neurons and microglia. The inhibition of p-ERK1/2 via a U0126 pretreatment attenuated the expression of both p-ERK1/2 and p-p38 and suppressed UAC-induced hyperalgesia. The p-p38 inhibitor (SB203580) produced similar effects in terms of both extent and time. Combined, these data indicate that UAC stimulated orofacial hyperalgesia via central sensitization by activating both the ERK1/2 and p38 pathways in both neurons and microglia. Additionally, p-ERK1/2, expressed mainly in neurons and to a lesser extent in microglia, promoted this effect by potentiating p38 (Fig. 5B).

Central sensitization plays a role in orofacial hyperalgesia (Sessle, 2007). The MAPKs are critical for neural plasticity (Svensson et al., 2003; Zhuang et al., 2005; Piao et al., 2006; Ji et al., 2009; Kiyomoto et al., 2013; Suzuki et al., 2013). The activation of ERK1/2 and p38 in different animal models varies in terms of cell type and length of time (Huang et al., 2000). In the nociceptive model, the chronic constriction injury model of the infraorbital nerve, the CFA model, capsaicin and formalin injection inflammatory models (Ji et al., 1999; Shimizu et al., 2006; Gao and Ji, 2009; Kiyomoto et al., 2013; Suzuki et al., 2013), p-ERK1/2 was observed mainly in neurons from several minutes to 1 h after the procedure. In the cancer-induced bone pain model, however, p-ERK1/2 was observed in microglia, but not in neurons (Wang et al., 2012). In the spinal nerve ligation model, transient expression of p-ERK1/2 was observed in neurons (<6 h) while short term expression was observed in microglia (1–3 days) (Zhuang et al., 2005). In general, p38 is activated after ERK1/2. In the nerve injury model, p-p38 increases in the spinal cord and medullary dorsal microglia (Piao et al., 2006; Ji et al., 2009). In the spinal nerve ligation animal model, activation of p38 began to increase at 12 h, peaked at 3 days and was maintained at elevated levels even after 3 weeks (Jin et al., 2003). In a rat model with disordered posterior occlusion, p38 and ERK1/2 were activated in microglia...
and neurons in the Vc. ERK1/2 was activated before (on d1 to d10) p38 which was activated on d3 to d10 (Cao et al., 2013). Similarly, in the UAC model, p-ERK1/2 peaked earlier than p-p38 in both neurons and microglia in the Vc. Using the current technique, we cannot determine whether the p-ERK1/2-positive cells (neurons and microglia) and the p-p38-positive cells are the same or are separate subgroups, or whether ERK1/2 activation preceded p38 activation within the same group or in different groups of cells.

Astrocytes are another group of cells found in central tissues. It has been reported that only ERK1/2 was significantly activated in astrocytes in the lingual nerve injury model (Terayama et al., 2011). In a cancer pain model, p-ERK1/2 was found to be expressed in both astrocytes and microglia (Wang et al., 2012). The activation of p38 in spinal astrocytes contributes to nociceptive behaviors in a nociceptive substance pain model (Nemoto et al., 2015). However, in the present study, there was no obvious activation of ERK1/2 and p38 in the Vc astrocytes.

The differences in side-specific activation of ERK1/2 and p38 were more likely influenced by the different types of stimuli. Unilateral acute pulpal stimulation with capsaicin elicited bilateral expression of p-ERK1/2 in the Vc (Shimizu et al., 2006). Chronic pulpal stimulation induced bilateral activation of ERK1/2 and p38 in the Vc (Worsley et al., 2014). In the posterior dental occlusal interference model, p-p38 and p-ERK1/2 were observed in ventrolateral Vc bilaterally (Cao et al., 2013). However, inferior alveolar nerve and mental nerve transaction were reported to activate p38 in the microglia of the ipsilateral

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**Fig. 2.** Representative p-ERK1/2 immunoreactive cells. p-ERK1/2 (green) in the rat Vc double-labeled with NeuN (red) or OX42 (red) ipsilateral (Ipsi) and contralateral to UAC (Contra) after UAC stimulation. (A) On d1, p-ERK1/2 immunoreactive cells in the ipsilateral and contralateral sides labeled with neuron appear as double-labeled (yellow). (B) p-ERK1/2 and NeuN double-positive cell counts. (C) In the ipsilateral and contralateral sides on d1, p-ERK1/2 immunoreactive cells labeled with microglia appear as double-labeled (yellow). (D) p-ERK1/2 and OX42 double-positive cell counts. UAC ipsilateral side vs. UAC contralateral side, *P < 0.05; **P < 0.01. UAC ipsilateral side vs. control group, #P < 0.05; ##P < 0.01. Scale bars = 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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Ligation of lingual nerve activated ERK1/2 and p38 in the ipsilateral trigeminal sensory nuclear complex (Terayama et al., 2011). Our experimental posterior disordered occlusion which was created by pushing the molar(s) away from its original location using orthodontic string stimulated the activation of bilateral microglia and astrocytes (Liu et al., 2009). This bilateral mechanical hyperalgesia induced by unilateral stimuli may be clarified with an orofacial ectopic pain model (Kiyomoto et al., 2015). The present UAC stimulation differed from our experimentally induced disordered occlusion created by the orthodontic strings and exhibited side-related differences during elicited orofacial hyperalgesia (Liu et al., 2009).

Central sensitization characterized by enhanced neuronal activation in the spinal dorsal horn or medullary dorsal horn (Piao et al., 2006). Ligation of lingual nerve activated ERK1/2 and p38 in the ipsilateral trigeminal sensory nuclear complex (Terayama et al., 2011). Ample evidence shows that neuronal activity is regulated by microglia and neuron may be involved in central sensitization in the UAC animal model. The involvement of molecules from activated neurons, such as fractalkine (CX3CL1), CCL2, neuregulin-1 (NRG-1), ATP and colony-stimulating factor 1 (CSF1) have been reported in the neuropathic pain model and the inflammatory animal model (Grace et al., 2014; Guan et al., 2016). In particular, microglia contribute to the transition from acute pain to chronic pain, as the inhibition of microglial signaling reduces pathologic pain in the inflammatory and nerve injury animal models (Piao et al., 2006; Fang et al., 2016).

Fig. 3. Representative p-p38 immunoreactive cells. p-p38 (green) in the rat Vc double-labeled with NeuN (red) or OX42 (red) in the side ipsilateral to UAC (Ipsi) and contralateral to UAC contralateral (Contra) after UAC stimulation. (A) In the ipsi and contra sides on d3, p-p38 immunoreactive cells labeled with neuron appear as double-labeled (yellow). (B) p-p38 and NeuN double-positive cell counts. (C) In the ipsi and contra sides on d3, p-p38 immunoreactive cells labeled with microglia appear as double-labeled (yellow). (D) p-ERK1/2 and OX42 double-positive cell counts. UAC ipsilateral side vs. UAC contralateral side, *P < 0.05; **P < 0.01. UAC ipsilateral side vs. control group, #P < 0.05; ##P < 0.01. Scale bars = 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
Inhibition of microglial signaling effectively reduces inflammatory and neuropathic pain (Berta et al., 2016). Importantly, activated microglia enhance pain states via the secretion of proinflammatory mediators, such as TNF-α, interleukin-1β (IL-1β) and 18, and brain-derived growth factor (BDNF) (Kawasaki et al., 2008; Berta et al., 2016). Suppression of p-38 and ERK attenuates inflammatory pain and neuropathic pain by controlling glial activation and the production of proinflammatory cytokines and chemokines in the spinal dorsal horn or medullary dorsal horn (Sessle, 2007; Taves et al., 2015). Therefore, we proposed that subsequent interactions between neurons and microglia contribute to pain hypersensitivity in the UAC model.

Fig. 4. The expression of p-p38 on d3 in the spinal trigeminal subnucleus caudalis (Vc) of rats exposed to UAC stimulation with and without (UAC + U0126) injection. (A) The immunofluorescent staining of p-p38. (B) Western blot and quantitative analysis of p-p38. (C) Number of p-p38 and NeuN double-positive cells. (D) The number of p-p38 and OX42 double-positive cells. (E) In the side ipsilateral to UAC (ipsi), many p-p38 immunoreactive cells are labeled with neuron and appear double-labeled (yellow); few p-p38 immunoreactive cells are labeled with neuron in the contralateral side (contra) and bilaterally in UAC rats with U0126 injection. (F) In the ipsilateral side (ipsi), many p-p38 immunoreactive cells are labeled with OX42 and appear double-labeled (yellow); few p-p38 immunoreactive cells are labeled with OX42 in the contralateral (contra) and bilateral sides of UAC rats with U0126 injection. Ipsi: ipsilateral side to UAC; Contra: contralateral side to UAC. *P < 0.05; **P < 0.01. Scale bars = 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2015; Berta et al., 2016). Inhibition of microglial signaling effectively reduces inflammatory and neuropathic pain (Berta et al., 2016). Importantly, activated microglia enhance pain states via the secretion of proinflammatory and nociceptive mediators, such as TNF-α, interleukin-1β (IL-1β) and 18, and brain-derived growth factor (BDNF) (Kawasaki et al., 2008; Berta et al., 2016). Suppression of p-38 and ERK attenuates inflammatory pain and neuropathic pain by controlling glial activation and the production of proinflammatory cytokines and chemokines in the spinal dorsal horn or medullary dorsal horn (Sessle, 2007; Taves et al., 2015). Therefore, we proposed that subsequent interactions between neurons and microglia contribute to pain hypersensitivity in the UAC model.
CONCLUSIONS

This study indicated that UAC traumatic occlusion evoked orofacial hyperalgesia by inducing central sensitization via the activation of ERK1/2 predominantly in neurons and to a lesser extent in microglia of the Vc. The activation of p38 in both neurons and microglia of the Vc. Activated ERK1/2 also affected p-p38 stimulation in neurons and microglia, which potentiated the UAC-elicted p-p38 expression and enhanced orofacial hyperalgesia.

AUTHOR CONTRIBUTIONS

All experiments were conducted by Mei-qing Wang and Xiao-dong Liu. Lei Jing, Hong-xu Yang assisted in performing experiments. Mei-qing Wang, Lei Jing, and Xiao-dong Liu designed the experiments. All authors assisted in interpretation of data, editing the manuscript and final approval of the manuscript.

CONFLICTS OF INTEREST

The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

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