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# Involvement of medullary dorsal horn glial cell activation in mediation of masseter mechanical allodynia induced by experimental tooth movement

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## ARTICLE INFO

### Article history:

Accepted 26 September 2009

### Keywords:

Experimental tooth movement  
Allodynia  
Minocycline  
Microglia  
Medullary dorsal horn

## ABSTRACT

**Objective:** To investigate the involvement of microglial and astrocytic activation in the medullary dorsal horn (MDH) during the mediation of masseter area allodynia induced by experimental tooth movement (ETM).

**Design:** Five groups of adult Sprague–Dawley rats ( $n = 60$ ) were divided into control (CON), minocycline (MIN), ETM, and 10 mg/kg or 30 mg/kg MIN plus ETM (METM) groups. The upper-first-molar was moved mesially for rats in ETM and METM groups. Rats were pre-injected with minocycline in the MIN (30 mg/kg) and METM (10 mg/kg or 30 mg/kg) groups. Pressure pain threshold (PPT) in masseter area was tested from day 0 to 14 for all 5 groups. Immunohistochemistry against OX42 (microglial marker) or GFAP (astrocytic maker) in the MDH was examined at days 1, 3, 7 and 14 for CON, MIN and 30 mg/kg METM groups.

**Results:** Baseline PPT was expectedly seen in either CON or MIN groups, masseter mechanical allodynia was detected in the ETM group from day 4 to 13 ( $P < 0.05$ ). OX42 expression level at days 1, 3 and 7, and GFAP expression level at days 3, 7 and 14 were higher in ETM ( $P < 0.05$ ), but not in 30 mg/kg METM, than in CON group. Minocycline reduced activation of microglia and astrocytes, and significantly attenuated the development of masseter mechanical allodynia in this model.

**Conclusions:** These results indicate that mechanical allodynia in the masseter area induced by ETM can be attenuated by minocycline. Activation of microglia, possibly together with subsequent activation of astrocytes, seems to contribute to masseter mechanical allodynia.

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## 1. Introduction

Patients who receive orthodontic treatment always experience pain and discomfort not only in teeth but also in the masseter area, and often suffer from headaches.<sup>1</sup> Clinical

experiments show that even short-term orthodontic separation can induce masticatory muscle pain.<sup>2</sup> Neuroplastic changes and neuro-anatomic convergence of trigeminal noxious input may account for the local tenderness, spread, and referral of pain.<sup>2</sup> Previous researches disclosed that

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doi:10.1016/j.archoralbio.2009.09.006

increased Fos immunoreactive neurons in the superficial layer of medullary dorsal horn (MDH), especially in ipsilateral MDH, can be induced by experimental tooth movement in rats.<sup>3-5</sup> Moreover, Fos immunoreactivity in the trigeminal sensory nuclear complex can be inhibited by MK-801, an inhibitor of N-methyl-D-aspartate (NMDA) receptors, with experimentally moved molars, suggesting that NMDA receptors of neurons in the MDH can be activated by tooth movement.<sup>5</sup> Central sensitization is an NMDA receptor-dependent process.<sup>6,7</sup> Therefore, both the clinical and animal experiments referred to above indicate that tooth movement induces sensitivity of neurons in the MDH.

Central sensitization is considered to be a major mechanism underlying allodynia.<sup>6</sup> Central glia, including microglia and astrocytes, are considered to be superimposed on NMDA receptors to drive neuronal changes in central sensitization.<sup>6,7</sup> Recent reports concluded that glia have a role in orofacial pain.<sup>6,8-10</sup> Not only do they release a host of substances that can influence the excitability of neurons involved in nociceptive transmission in the CNS, such as interleukin-1 $\beta$ , interleukin-6, tumour necrosis factor- $\alpha$ , prostaglandins, adenosine triphosphate (ATP) and excitatory amino acids, but they also have receptors including NMDA, AMPA, ATP, and CGRP receptors and ion channels sensitive to neurotransmitters and neuromodulators.<sup>6,7,11</sup> For the propagation of nociception, microglia may be involved in the initial induction. Then, activated microglia may lead, in turn, to astrocytic activation.<sup>7</sup>

Mechanical sensitivity of the orofacial region has been reported in pressure pain threshold (PPT) values of the masseter area in an inflammatory rat model.<sup>12,13</sup> Thus, we assumed that in the experimental tooth moving rat there is a behavioural change in the masseter area similar to that reported by Yang et al.,<sup>14</sup> which accompanies the activation of microglia with or without the activation of astrocytes. Activation of astrocytes and microglia can be determined immunohistochemically, respectively, by glial fibrillary acidic protein (GFAP) and by OX42, an antibody against CD11b of microglia.<sup>7,15-18</sup> Minocycline, a semisynthetic second-generation tetracycline that possesses superior penetration into CNS via the brain-blood barrier and selectively disrupts microglial activation without directly affecting neurons or astrocytes<sup>7,19-22</sup> was selected to be used as an antagonist to the early activation of microglia. The purpose of the present study was to document the early time course of masseter mechanical allodynia by measuring PPT values and changes in the activation of microglia and astrocytes in the MDH by assessing immunoactivation by OX42 and GFAP in experimental tooth movement (ETM) rats.

## 2. Materials and methods

### 2.1. Animals

All experiments were performed in accordance with the Committee of Animal Use for Research and Education of P.R. China and conformed to the National Institute of Health Guide for the Care and Use of Laboratory animals (NIH Publications No. 80-23). Sixty adult male Sprague-Dawley (SD) rats, weighing 200-250 g, were obtained from the animal centre

of Fourth Military Medical University. Rats were housed with a 12 h light-dark cycle (lights from 08:00 to 20:00) with readily available tap water and food.

Rats were randomly divided into a control group (CON, N = 10), minocycline group (MIN, N = 5), experimental tooth movement group (ETM, N = 20), 10 mg/kg (N = 5) and 30 mg/kg (N = 20) minocycline plus ETM group (METM). ETM was created in both ETM and the two METM groups by the method described below. In the ETM group rats were pretreated with saline (0.9%), whilst those in MIN and METM groups were pretreated with different doses of minocycline as described below. No treatment was administered to the rats in the CON group. Behaviour tests were carried out in CON, MIN, ETM, 10 mg/kg and 30 mg/kg METM groups from day 0 to day 14. Rats in the ETM and 30 mg/kg METM groups were sacrificed on days 1, 3, 7 and 14 (N = 5 per time point, each group), whilst rats in CON, MIN and 10 mg/kg METM groups were sacrificed on day 14.

### 2.2. Experimental tooth movement

Experimental tooth movement was carried out according to Waldo's method.<sup>23</sup> Under light anaesthesia with a gas mixture of ethyl ether and oxygen, a piece of elastic band (3 M Unite, 1/8<sup>#</sup>) was inserted between the left upper first and second molars, with the aim to move the first molar mesially. The elastic band outside of the dentition was carefully cut off to make sure that the elastic band ends did not interrupt any movement of the surrounding soft tissues. This procedure lasted 1-2 min. A gap was created between the left upper first and second molars and reached a maximum distance of about 0.8 mm at day 3. The elastic rubber band was kept in the same place to keep the gap until the end of the experiment.

### 2.3. Minocycline and saline treatment

Minocycline (Sigma-Aldrich, USA) was freshly dissolved daily in 0.9% sterile, isotonic saline. Minocycline, 30 mg/kg in MIN and 30 mg/kg METM groups and 10 mg/kg in 10 mg/kg METM group, and saline vehicle (1 mL/kg) in ETM group, was administered by i.p. injection 1 h before the application of elastic rubber bands, and daily for the following 14 days. The selection of minocycline and the rationale for the dosing regime was selected to be within the range at which these doses have been reported to be neuroprotective in rodents.<sup>8,19</sup>

### 2.4. Behavioural assessment

Behaviour tests were carried out from 4 days before to 14 days after application of the tooth movement between 9:00 a.m. and 12:00 a.m. in a quiet room. All behavioural experiments were performed by one of the investigator (XD) who was blinded to the experimental conditions. According to Ren's method,<sup>12,13</sup> pressure pain threshold (PPT) was measured with von Frey filaments (Semmes-Weinstein monofilaments, North Coast Medical, Inc., USA). Mechanical stimulation points were chosen as the skin site above bilateral masseter areas, 1 cm below and 1 cm caudal to the midpoint of the eye-ear line. An ascending series of the filaments were used with bending force of 25.6 g (5.46 marking), 63 g (5.88 marking) and

118 g (6.10 marking). The bending force of starting filament was 25.6 g, and the bending force of ending filament was 118 g. Each filament was applied 5 times at intervals of 5 s. Head flinching, characterized as sudden quick head withdraw, or vocalization/crying, was considered to be positive pain responses. PPT was defined as the lowest bending force of the filaments that produced at least three positive responses in five trials. In the preliminary experiment, we found that rats usually responded to the 118 g stimulation for 1–2 times, but seldom responded to the 15 g stimulation before tooth movement. So, we excluded the rats that either responded to the 15.0 g stimulus (>3 out of 5 trials) or did not respond to the 118 g stimulus (<1 out of 5 trials) from the behavioural assessment group during the 4 days before application of tooth movement.

### 2.5. Immunofluorescent staining

Under deep anaesthesia with sodium pentobarbital (60 mg/kg, i.p.), animals were perfused transcardially with 150 mL heparinized saline, followed by 400 mL 40 g/L paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The medulla oblongata was isolated and immersed in 0.1 mol/L PBS (phosphate buffer saline) containing 200 g/L sucrose overnight at 4 °C. Serial frozen transverse sections (30 µm thickness) were cut through the caudal medulla on a freezing microtome (Cryostat; Leitz, Wetzlar, Germany). Free-floating sections were incubated for 30 min with 3% H<sub>2</sub>O<sub>2</sub> in 0.1 M PBS to remove endogenous peroxidase activity, washed in PBS, and then incubated in a blocking solution containing 2% bovine serum albumin and 0.03% Triton X-100 for 1 h at room temperature. Sections were incubated with mouse anti-OX42 monoclonal antibody (1:500, Chemicon) or mouse monoclonal anti-GFAP antibody (1:1000, Sigma) for 48 h at 4 °C, respectively. After washing in 0.01 mol/L PBS, sections were incubated with secondary antibodies combining FITC (fluorescein isothiocyanate) conjugated goat anti-mouse IgG (1:500, Sigma) for 2 h, rinsed in PBS again, and mounted on gel-coated slides with ProLong AntiFade Kit (Molecular Probes).

Images were obtained with a confocal laser scanning microscope (FV300, Olympus Japan). For semi-quantification, the fluorescent brightness value of OX42 and GFAP immunoreactivity was measured in the same areas of the MDH using software for the FV300 confocal laser scanning microscope. Six randomly selected sections from 400 µm rostral to 600 µm caudal to the obex were collected for statistical analysis for each animal. The relative optical density (OD) of images was determined by subtracting the background density in each image.

### 2.6. Statistical analysis

The SPSS 11.0 package (SPSS Inc., Chicago, IL, USA) was used to analyze the difference between groups. PPT analysis was performed with the non-parametric Kruskal–Wallis *H*-test for multiple group comparisons and the Mann–Whitney *U*-test for two-group comparisons. Immunohistochemical analysis was performed by Student's *t*-test, with  $P < 0.05$  indicating significant difference.

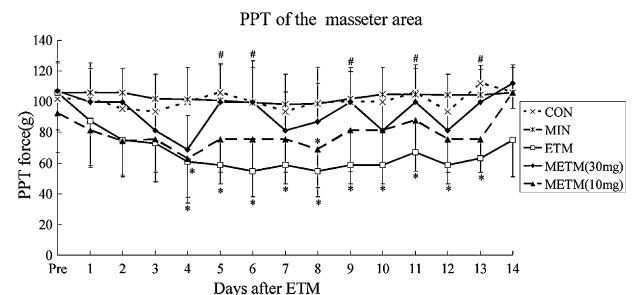
## 3. Results

### 3.1. Reversal of tooth movement induced masseter muscle allodynia by minocycline treatment

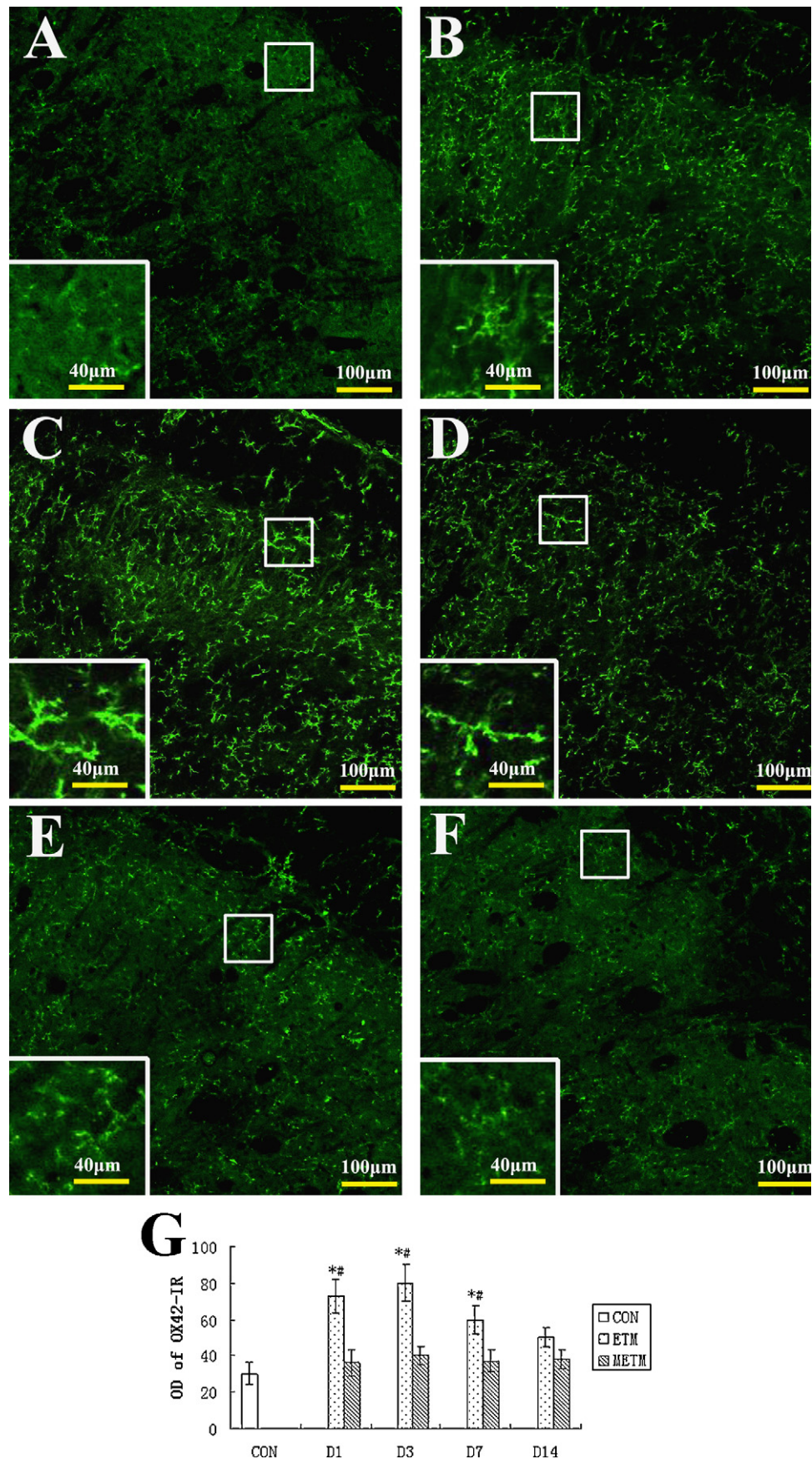
All animals experienced normal weight gain over the course of the experiment. The PPT baseline, values during the 4 days before the experiment, of all rats was similar ( $P > 0.05$ ) (Fig. 1). Animals in CON and MIN groups exhibited baseline behaviour throughout the observation period. A significant reduction of PPT was observed in ETM group from 4 to 13 days after the application of teeth movement compared to that before and to that in CON and MIN groups ( $P < 0.05$ ) (Fig. 1). In the 30 mg/kg METM group, values of PPT were not significant compared to those in the CON group ( $P > 0.05$ ), showing generally higher values than those in the ETM group, which were significant at days 5, 6, 9, 11 and 13 ( $P < 0.05$ ) (Fig. 1). In the 10 mg/kg METM group, values of PPT were similar to those in the ETM group ( $P > 0.05$ ). In the 10 mg/kg METM group PPT values were significantly lower than the values during the pre-treatment period in ETM group and the CON group values only on days 4 and 8 ( $P < 0.05$ ) (Fig. 1).

### 3.2. Tooth movement enhanced expression levels of microglial OX42 and astrocytic GFAP

In the CON group, very weak OX42 and GFAP immunoreactivity was observed in the MDH, which appeared as small cell bodies and thin processes (Figs. 2A and 3A). In the ETM group, OX42 immunoreactivity with many spines could be observed in the MDH (Fig. 2B–E). OX42 immunoreactivity was higher in the ETM group than that in the CON group at days 1, 3 and 7 ( $P < 0.05$ ), which was maximal on day 3, but was not significantly different at day 14 (Fig. 2G). In the ETM group, GFAP immunoreactive astrocytes in the MDH were more hypertrophic in cell bodies and had thicker processes than in



**Fig. 1** – Changes of pressure pain threshold (PPT) on the masseter area in the control (CON) group, minocycline (MIN) group, experimental tooth movement (ETM) group, 30 mg/kg and 10 mg/kg minocycline plus ETM (METM) groups, indicated in the figure as METM (10 mg/kg) and METM (30 mg/kg). In the ETM group, PPT decreased on day 4 to day 13 compared to that of pre-ETM and CON group ( $P < 0.05$ ). In the 10 mg/kg METM group, PPT decreased on days 4 and 8 compared with the pre-ETM and CON group ( $P < 0.05$ ). In the 30 mg/kg METM group, PPT was similar to the CON group ( $P > 0.05$ ), but was higher on days 5, 6, 9, 11 and 13 than the ETM group ( $P < 0.05$ ) (\* $P < 0.05$  vs. pre-ETM and CON group; # $P < 0.05$  vs. ETM group).



**Fig. 2** – Immunohistochemical staining of OX42 in the MDH. (A) Control (CON) group. (B–E) Day 1, 3, 7 and 14, respectively, in the experimental tooth movement (ETM) group. (F) Day 3 in the 30 mg/kg minocycline plus ETM (30 mg/kg METM) group. (G)

the CON group (Fig. 3B–E), but appeared later than OX42 immunoreactivity at days 3, 7 and 14 and were maximal on day 7 ( $P < 0.05$ ), but were not apparent at day 1 (Fig. 3G).

### 3.3. Minocycline treatment inhibited OX42 and GFAP increase induced by tooth movement

In the 30 mg/kg METM group (Figs. 2F and 3F), the number and density of OX42 and GFAP immunoreactive cells were similar to the level in the CON group ( $P > 0.05$ ) (Fig. 3G). Compared to the ETM group, OX42 immunoreactivity was lower on days 1, 3 and 7, and GFAP immunoreactivity was lower on days 3, 7 and 14 in the 30 mg/kg METM group ( $P < 0.05$ ) (Figs. 2G and 3G).

## 4. Discussion

In the present study, the experimental tooth movement rat model was used to investigate the masseter area allodynia using pressure pain threshold (PPT) values, which could be attenuated by minocycline. Moreover, activation of glial cells in the MDH, as observed by higher immunohistochemical reactivity of OX42 and GFAP, was also identified and found to be inhibited by minocycline. The present model of masseter area allodynia appeared later than the response to pain observed during other orthodontic therapies and in other animal models.<sup>1,14,24,25</sup> Differences in the tooth-moving model, as well as in the level of force, may explain this discrepancy.

Activated microglia change from resting and ramified shapes into an active and amoeboid morphology. OX42 antibodies recognize the upregulation of the expression of surface molecules such as complement 3 receptors, also known as cluster determinant (CD) 11b, which are associated with adhesion, migration and phagocytosis.<sup>18</sup> Activated microglia have been reported to release proinflammatory cytokines and other algescic substances, which may activate astrocytes and enhance neuronal transmission of nociception, leading to exaggerated pain.<sup>7–9,11</sup> The present results of early microglial activation, as seen by the significant increase in OX42 in MDH on days 1, 3 and 7, is consistent with the development of behavioural hypersensitivity. Alternatively, the significant up regulation of astrocytic GFAP expression in the present results is observed on days 3, 7 and 14, which is later than OX42 immunoreactivity, indicating a nociceptive facilitation by astrocytes. Activation of astrocytes is morphologically characterized by hypertrophy and increased production of the intermediate filament protein GFAP, vimentin and/or nestin.<sup>7</sup> The magnitude of the increase in GFAP staining appears to correlate with the degree of allodynia,<sup>16</sup> which makes sense because NMDA receptors are, in part, responsible for GFAP expression.<sup>15</sup> Although it is unclear whether upregulation of GFAP is required or/and sufficient to induce chronic pain sensitization, mounting evidence indicates that persistent activation of spinal astrocytes, i.e., GFAP upregula-

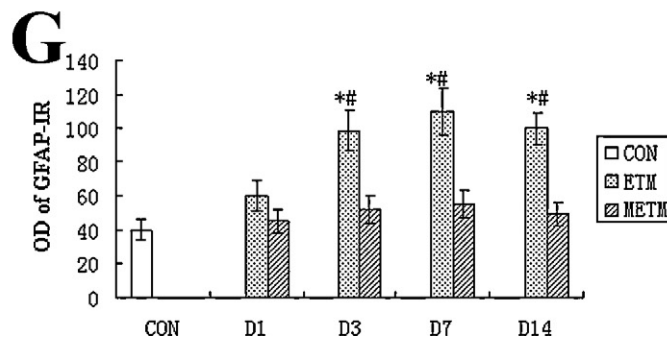
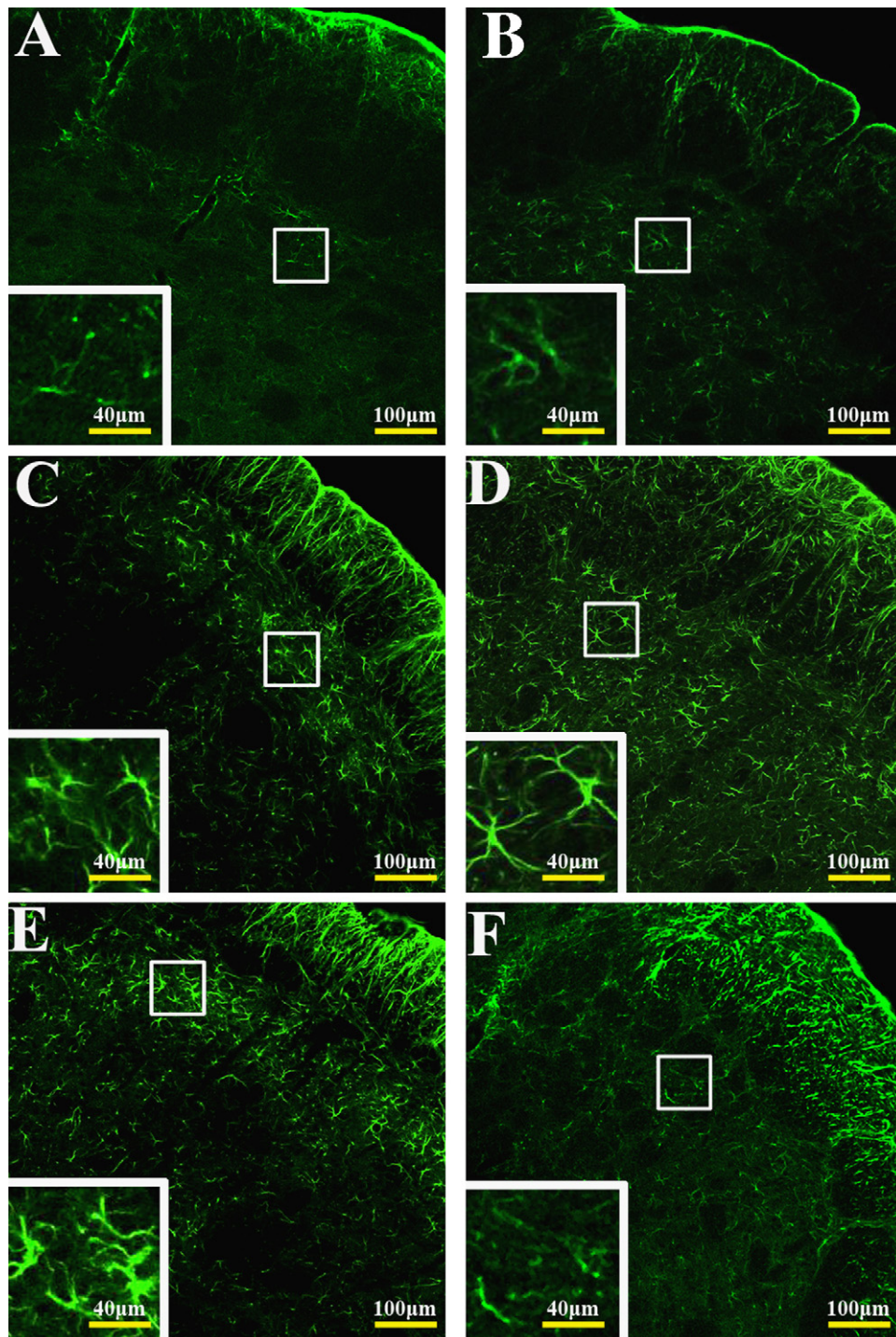
tion, is a unique feature of chronic pain in different animal models.<sup>17</sup>

Minocycline is a microglial inhibitor that prevents/delays chronic pain development, but does not change acute pain or baseline nociception.<sup>7,19–22</sup> The present results indicate that 30 mg/kg minocycline completely attenuated the hypersensitivity induced by moving molars, whilst 10 mg/kg had a partial effect, indicating a dose-dependent effect of minocycline on masseter hypersensitivity. This evidence provides further support that allodynia of the masseter area in this rat model is related to microglial activation. Furthermore, the present data demonstrated that minocycline pretreatment blocked activation of not only microglia, but also astrocytes because of the reduced GFAP expression in the MDH. Similar reports in animal studies show that preemptive treatment with minocycline suppressed GFAP expression in the lumbar spinal cord.<sup>12,19</sup> Recently, studies indicated that satellite glial cells and neurons in the sensory ganglion also play important roles in allodynia under peripheral inflammatory or neuropathic conditions.<sup>14,26–28</sup> Yang et al.<sup>14</sup> observed that P2X3 receptors in trigeminal ganglion are important in ETM pain. Thus, minocycline may inhibit astrocytic activation, and this effect is assumed to be mediated by blocking microglial activation. However, this hypothesis requires further investigation for confirmation. Further investigations concerning the observation of changes in trigeminal ganglion neurons and glia are also required.

Orthodontic pain is caused by a process of pressure, ischaemia, inflammation and oedema of the periodontal area.<sup>29</sup> Studies have indicated that pain caused by orthodontic tooth movement correlates to the presence of substances such as substance P, prostaglandins and calcitonin gene-related peptide (CGRP).<sup>30</sup> Orthodontic force wielded by Waldo's method can be regarded as an extremely noxious stimulus to the neural elements in the periodontal ligament.<sup>31,32</sup> In these rats, inflammation was often found in the regions beneath the insertion of the elastic band between the first and second molars, as previously reported.<sup>32</sup> It seems possible that periodontal nerve injury and inflammation, as the nociceptive sources, may have a role in the activation of spinal glia and neurons. Furthermore, occlusion trauma can activate astrocytes in the parabrachial nucleus.<sup>33</sup> The orthodontic force wielded by the elastic band in the present study creates an occlusion since the medially moved first molar contacts its original opposing molar but does not maximally intercuspace it. Whether this kind of occlusion alteration contributes to the masticatory muscle hypersensitivity needs further study.

Evidence indicates that central glial activation depends on nerve inputs from the site of injury and the release of chemical mediators.<sup>6,7,34</sup> Previous research has found that experimental tooth movement induced Fos immunoreactive neurons in the MDH as early as 1–4 h and reached a maximum at 2 h.<sup>3–5</sup> C-fos plays a key role in the regulation of transcription of several genes including tumour necrosis factor (TNF)- $\alpha$ , which is believed to activate glial cells.<sup>8,35</sup> We speculate that glial cells can receive

**The optical density (OD) of OX42 immunoreactivity in the MDH at days 1 (D1), 3 (D3), 7 (D7) and 14 (D14) after mesially moving the left maxillary first molar. OX42 immunoreactivity is weak in both control and 30 mg/kg METM groups, but intense in the ETM group on days 1, 3, and 7 with peak levels on day 3, which became weak on day 14. \* $P < 0.05$  vs. CON group; # $P < 0.05$  vs. 30 mg/kg METM group.**



information from both primary afferent terminals and pain transmission neurons.<sup>6,7</sup> This bidirectional neuron–glia signaling plays a key role in glial activation, cytokine production and the initiation and maintenance of allodynia.<sup>6,7,34</sup> Activated microglia and astrocytes release proinflammatory substances that can influence the excitability of neurons involved in nociceptive transmission in MDH,<sup>6,7,11</sup> which may be the mechanism of masseter allodynia in this model.

## 5. Conclusion

The present results indicate that mechanical masseter area allodynia can be induced by tooth movement and effectively suppressed by minocycline administration. Activation of microglia and subsequent astrocytic cells in the MDH contributes to masseter area allodynia. Blocking activation of central glia as a potential treatment to attenuate mechanical hypersensitivity during orthodontic procedures, and different modes and forces of tooth movement should be further investigated in the laboratory and clinic.

## Acknowledgements

Authors thank Dr. Shibin Yu, Xiaohan Du and Shuang Chen for their technical assistance. This work was supported by grants from the National Natural Science Foundation of China (No. 30540130469, 30772429 and 30801303).

Competing interests: The authors have no conflict of interest to disclose.

Ethical approval: All experimental procedures and the care administered to the animals were approved by the University Ethics Committee, and all procedures were performed according to institutional guidelines.

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**Fig. 3 – Immunohistochemical staining of GFAP in the MDH. (A) Control (CON) group. (B–E) Day 1, 3, 7 and 14, respectively, in the experiment tooth movement (ETM) group. (F) Day 7 in the 30 mg/kg minocycline plus ETM (30 mg/kg METM) group. (G) The optical density (OD) of GFAP immunoreactivity in the MDH on days 1 (D1), 3 (D3), 7 (D7) and 14 (D14) after ETM. GFAP immunoreactivity is very weak in control and 30 mg/kg METM groups, yet intense on days 3, 7, and 14 with peak level on day 7, and weak again by day 14 in the ETM group. \*P < 0.05 vs. CON group; #P < 0.05 vs. 30 mg/kg METM group.**

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