Original Article

Chemokine ligand 2 in the trigeminal ganglion regulates pain induced by experimental tooth movement

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ABSTRACT

Objective: To test the hypothesis that the chemokine ligand 2/chemokine receptor 2 (CCL2/CCR2) signaling pathway plays an important role in pain induced by experimental tooth movement.

Materials and Methods: Expression of CCL2/CCR2 in the trigeminal ganglion (TG) was determined by Western blotting 0 hours, 4 hours, 1 day, 3 days, 5 days, and 7 days after tooth movement. CCL2 localization and cell size distribution were revealed by immunohistochemistry. The effects of increasing force on CCL2 expression and behavioral changes were investigated. Furthermore, the effects of CCL2/CCR2 antagonists on these changes in pain behaviors were all evaluated. Exogenous CCL2 was injected into periodontal tissues and cultured TG neurons with different concentrations, and then the pain responses or c-fos expression were assessed.

Results: Experimental tooth movement led to a statistically significant increase in CCL2/CCR2 expression from day 3 to day 7, especially in small to medium-sized TG neurons. It also triggered an increase in the time spent on directed face-grooming behaviors in a force magnitude–dependent and CCL2 dose–dependent manner. Pain induced by experimental tooth movement was effectively blocked by a CCR2 antagonist and by CCL2 neutralizing antibody. Also, exogenous CCL2 led to an increase in c-fos expression in cultured TG neurons, which was blocked by CCL2 neutralizing antibody.

Conclusions: The peripheral CCL2/CCR2 axis is modulated by experimental tooth movement and involved in the development of tooth movement pain. (*Angle Orthod.* 2014;84:730–736.)

KEY WORDS: Experimental tooth movement; Pain; CCL2; Trigeminal ganglion

INTRODUCTION

Patients always perceive pain and discomfort during orthodontic treatments, especially at the initial clinical stage.[1] Orthodontic pain has been proved to be associated with substance P, calcitonin gene-related peptide, and up-regulation of c-fos in the trigeminal system, and it has been characterized as inflammatory pain,[2] which is innervated mainly by the primary sensory neurons of the trigeminal ganglion (TG).[3] However, orthodontic pain control is a great challenge clinically, largely as a result of the fact that the mechanism regulating tooth movement pain is still unclear.

Chemokine (C-C motif) ligand 2 (CCL2), also known as monocyte chemoattractant protein-1 (MCP-1), is a member of the β or C-C family of chemokines and is a ligand for chemokine receptor 2 (CCR2).[4,5] For a long time, inflammatory CCL2 was thought to be involved specifically in the hematological system and in immune responses.[6] Although it is almost absent from the normal central nervous system, expression of CCL2 in the spinal cord could be induced following ischemia, neural trauma, and inflammation.[7–11] Similar up-

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regulation of CCL2 has also been found in the dorsal root ganglion (DRG) subjected to peripheral nerve injury.[12–14] A growing amount of evidence has demonstrated that the CCL2/CCR2 axis might play an essential role in neuropathic pain.[15,16] However, the importance of CCL2/CCR2 signaling remains to be explored in a special animal model of orofacial pain associated with experimental tooth movement (ETM).

The present study is aimed at investigating the hypothesis that peripheral CCL2 plays an essential role in modulating ETM pain in rats by documenting changes in CCL2 expression and nocifensive behavior. Also, the effects of recombinant CCL2 and its antagonist on nociceptive behaviors were investigated.

MATERIALS AND METHODS

Animals

One hundred fifty-four male Sprague-Dawley rats weighing 200–300 g each were used in this study. They were housed in standard clear plastic cages with soft bedding, with access to food and water ad libitum. Studies were approved by the ethical committees of the Shanghai Ninth Hospital affiliated with Shanghai Jiaotong University. The guidelines for investigation of pain in animals given by the International Association for the Study of Pain were followed.[17]

Appliance for ETM in Rats

A fixed nickel-titanium alloy closed-coil spring appliance was connected between a maxillary first molar and the ipsilateral upper incisor, as described previously.[18–20] After an intraperitoneal injection of sodium pentobarbital at a dose of 40 mg/kg body weight, 40 g of force[19,20] was applied for 0 hours, 4 hours, 1 day, 3 days, 5 days, and 7 days. Sham-treated rats received the same procedures, but the springs in their mouths were not active. Forces of 10 g, 30 g and 50 g were applied respectively for 3 days.

Drug Application In Vivo and Behavioral Testing

Periodontal injection of exogenous recombinant CCL2 (10, 50, and 100 ng/μL, respectively; 3 μL in all, R&D Systems, United Kingdom) or 0.9% saline was done using a modified glass needle around the upper anterior incisors at six different sites (0.5 μL/site) in the sham-treated (spring inactive) rats under a short period of anesthesia induced by isoflurane.

To evaluate antagonist and the effects of increasing force, we chose day 3 as the appropriate time to assess behavior according to the change rhythms of CCL2 expression and nocifensive behavioral responses after ETM. The delivery of subarachnoid antagonists in the medullary region of rats was performed carefully on day 3 after ETM, according to procedures developed previously.[21] The CCR2 antagonist (10 μL of a 10 nM solution; Calbiochem, Darmstatt, Germany) or CCL2 neutralizing antibody (100 ng/μL,[22] 10 μL, Millipore, Billerica, MA, USA) was applied under a short course of anesthesia.

The rats were videotaped 30 minutes after drug application to record behavioral responses characterized by directed face grooming, which has been validated as a reliable behavior for assessment of tooth movement pain in rats.[19] According to the procedures developed in previous studies,[19] the rats were videotaped for 10 minutes each time, starting at least 15 minutes after placement in the cage. Each animal was videotaped two times at 10-minute intervals.[19] Videotaped behavior was analyzed offline by an investigator who was blinded to the information regarding the rats. Each rat was weighed on every interval day, and changes in body weight versus the previous weights were used for statistical analyses.

Immunofluorescence and Image Acquisition

The maxillary portion of the TG was selected for immunohistochemistry (IHC) study. Specimens were sectioned serially at a thickness of 8 μm with a cryostat along the axis. Three sections were selected randomly from among these serial sections in each specimen for IHC according to procedures described previously.[20] Mouse anti-CCL2 (1:100, Millipore) primary antibody was incubated overnight at 4°C. Hoechst was used for nuclear staining. Z-stacks of images taken at 1-μm intervals were acquired and photographed under the appropriate objective lenses of a Nikon A1 confocal microscope. To study the distribution of cell size, large (>40 μm), medium (30–40 μm), and small (<30 μm) neurons were carefully determined by scale bars, and the ratios of each type of neurons were calculated.

Western Blot

The corresponding maxillary portion of TG was collected for Western blot study according to the procedures described by previous studies.[18] Mouse anti-CCL2 (1:2000, Millipore) and goat anti-CCR2 (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA) were selected as primary antibodies. Actin was used as the internal control protein. Band intensity was assessed using Image Quant 5.2 software and protein levels were normalized to that of the 0-hour group in Figure 1, or 10-g group in Figure 3.

Trigeminal Neuron Cell Culture and Drug Treatment In Vitro

The TGs were removed from 2-month-old rats and cultured according to previous procedures.[22] In brief,

Table 1. Summary of Experiments, Animal Numbers, and Corresponding Results in the Present Studies

Figure	Experiments[a]	Number of Rats	Results[a]
Figure 1	WB	18 total, 3/group	CCL2/CCR2 increased following ETM
	IHC	9 total, 3/group	CCL2 was up-regulated in small to medium-sized neurons after ETM
Figure 2	Behavioral testing and body weight	60 total, 6/group; 30 in sham groups and 30 in experimental groups	Nocifensive behaviors increased and body weight decreased after ETM
Figure 3	WB and behavioral study	15 total, 5/group	CCL2 expression and behavioral responses increased in a force-dependent manner
	IHC	9 total, 3/group	CCL2-ir cell numbers increased in a force-dependent manner
Figure 4	Behavioral studies	35 total, 5/group	Exogenous CCL2 triggers increased nocifensive behaviors in a dose-dependent manner; ETM induced pain responses could be reduced by CCL2/CCR2 antagonists
	TG neuron in vitro	8 total	Exogenous CCL2 could induce c-fos expression in cultured TG neurons

[a] WB indicates Western blot; IHC, immunohistochemistry; TG, trigeminal ganglion; CCL2, chemokine ligand 2; CCR, chemokine receptor 2; ETM, experimental tooth movement; ir = immunoreactive.

they were incubated for 1 hour in 0.15% collagenase (Worthington type I, Scima, Templestow, Victoria, Australia), followed by an additional 1 hour of incubation in 0.15% collagenase and 0.25% trypsin (Sigma, St. Louis, MO, USA). After 2 days of incubation, the neurons were used for the in vitro assay.

Recombinant rat CCL2 (R&D Systems, United Kingdom) was added to the culture plates for 15 minutes in the appropriate concentrations. Each antagonist was added 30 minutes before the addition of CCL2.

Statistics

For statistical analyses, one-way analysis of variance (least significant difference) and Student's *t*-test were used (SPSS 11.5, SPSS Inc, Chicago, IL, USA). The level of statistical significance was set at $P < .05$.

RESULTS

The study and results are summarized in Table 1 and Figures 1 through 4.

Time Course of the Changes in CCL2/CCR2 Expression in the TG

We observed a gradual increase in CCL2 and CCR2 expression that reached a statistically significant value after day 3, peaked on day 5 (Figure 1A), and down-regulated significantly after day 7 (Figure 1B,C). Also, the number of CCL2-immunoreactive (ir) neurons was statistically significantly increased on days 3 and 7 (Figure 1D,E). Further analysis showed that most of the ir cells were found in the small to medium-sized trigeminal neurons (Figure 1D,F), which are thought to play an important role in pain mechanisms.

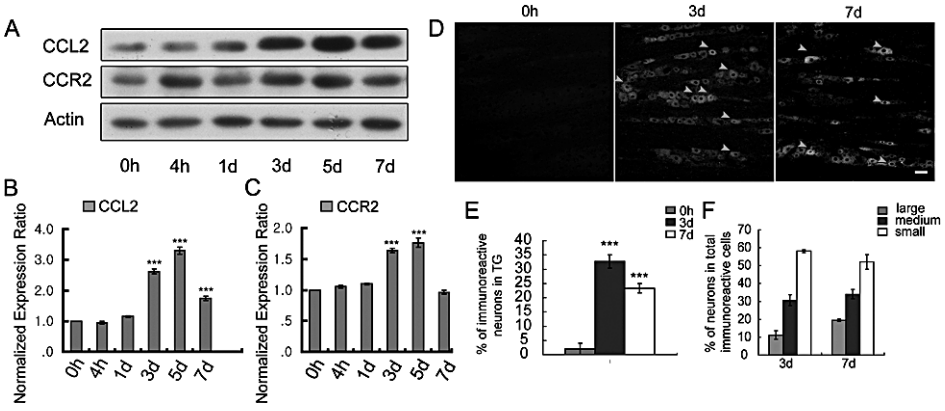

Figure 1. Changes in CCL2/CCR2 expression in TG (mean relative ratio ± standard error of the mean [SEM], n = 3/group). Scale bar: 50 μm; ***$P < .001$. (A,B,C) Western blot showed the changes in expression in time course of CCL2/CCR2 following ETM, which were statistically significantly up-regulated 3 days after ETM. (D,E,F) ETM-induced CCL2-ir cells showed a statistically significant increase on days 3 and 7. Analysis of cell size distribution showed that the majority of CCL2-ir cells were located in small to medium-sized neurons. Arrowheads indicate representative immunostained neurons.

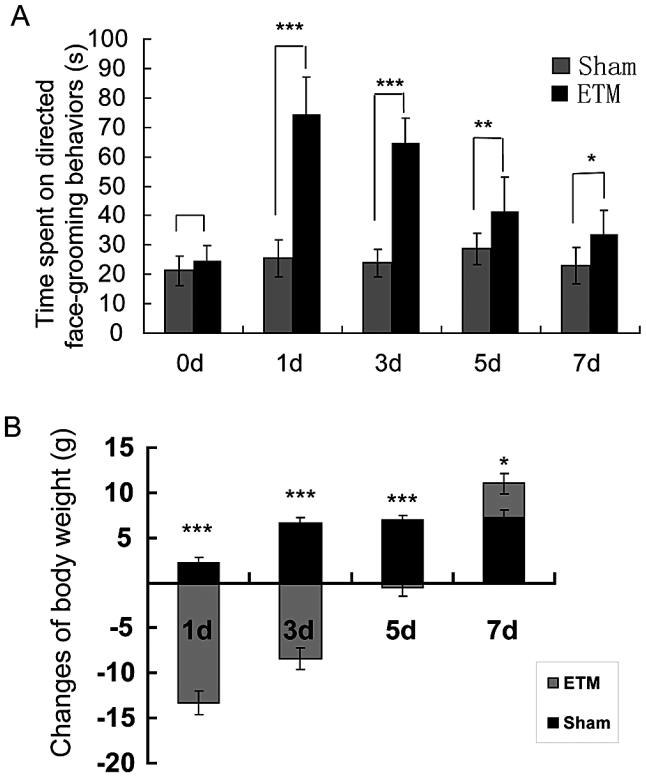

Figure 2. Changes in nocifensive behaviors and body weight after ETM in rats (mean ± SEM, n = 6/group). *$P < .05$, **$P < .01$, ***$P < .001$. (A) Time spent on directed face-grooming activities was statistically significantly increased from day 1 to day 7, with a peak on day 1 following ETM. (B) Body weight decreased at the early stage and increased at the later stage on day 7.

Nociceptive Behavioral Responses Following ETM

ETM triggered a significant increase in nocifensive behavior on days 1, 3, 5, and 7. The amplitude of this nociceptive-like behavior seemed to reach a maximum on days 1 to 3 and decreased significantly on day 7 (Figure 2A).

We further evaluated the changes in body weight after ETM as an indirect reflection of pain perception in the rats. As expected, body weight increased in the sham group and decreased in the experimental group until 5 days after ETM (Figure 2B).

The Effects of Increasing Force on the Nocifensive Behavioral Responses and CCL2 Expression in TG

To further validate our hypothesis, we tested the effect of increasing force on the changes in behaviors and CCL2 expression. As we expected, we found that both CCL2 expression intensity and positive cells in TG increased in a force magnitude–dependent manner (Figure 3A–D). Also, nocifensive grooming activities increased correspondingly in the same manner (Figure 3E).

Effects of Exogenous Recombinant CCL2 and Its Antagonists on Nocifensive Behavioral Activities and C-Fos Expression

To illustrate CCL2's role in the trigeminal pain mechanism directly, exogenous CCL2 (3 μL of 10-, 50-, and 100-ng/μL solutions) was injected into periodontal tissues. This led to a significant increase in nociceptive-like behavioral responses in a dose-dependent manner (Figure 4A).

In addition to the study of change in function, we also evaluated the pain behavioral responses after inhibiting CCL2/CCR2 signaling 3 days after ETM. Intrathecal application of CCR2 antagonist (10 μL of a 10 nM solution) or CCL2 neutralizing antibody (10 μL of a 100 ng/μL solution) decreased the nocifensive behavior significantly (Figure 4B).

We also applied exogenous CCL2 to cultured TG neurons in vitro. As expected, exogenous CCL2 led to a marked increase of c-fos expression within 15 minutes in a dose-dependent manner; this could be blocked by CCL2 neutralizing antibody (Figures 4C,D).

DISCUSSION

In the current study we observed a marked increase of CCL2/CCR2 signaling in the TG after ETM. The increase in directed face-grooming behaviors, indicative of pain triggered by ETM, correlated well with CCL2 expression. Both the changes in CCL2 expression and nociceptive behavioral responses were modulated in a force magnitude–dependent manner. Furthermore, exogenous recombinant CCL2 led to a marked increase in pain response in rats and c-fos expression in a dose-dependent manner. By contrast, knocking down of the CCL2/CCR2 signaling pathway via selective inhibitors relieved, at least in part, the pain behavior responses. This indicates that the CCL2/CCR2 signaling pathway may play a significant role in the mediation of pain caused by ETM.

The central importance of chemokines in the recruitment of leukocytes after tissue injury or inflammation is well known.[6] It was found that CCL2 and its high-affinity receptor CCR2 could be involved directly in nociceptive signal transduction in neurons or glia.[5] CCL2 could induce hyperexcitability of preinjured or cultured adult DRG neurons by causing the release of calcitonin gene-related peptide[23,24] or transactivation of the transient receptor potential cation channel subfamily V member 1[25] and other ion channels.[26] Therefore, the unique ability of the CCL2/CCR2 axis to simultaneously coordinate neuronal excitability and

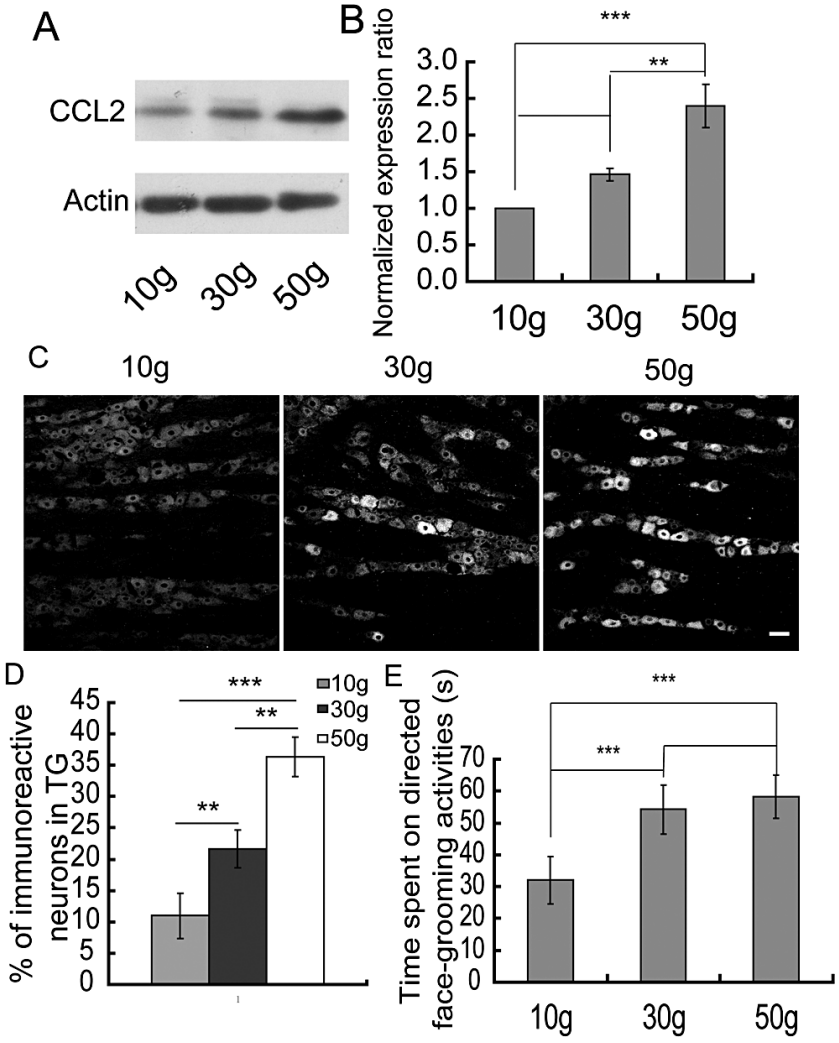

Figure 3. Effect of increased force on nociceptive-like behavioral responses and CCL2 expression in TG (mean ± SEM, n = 3 to 5/group). $^{**}P < .01$, $^{***}P < .001$. (A,B,C,D) CCL2 expression was markedly up-regulated in a force magnitude–dependent manner. Also, the number of CCL2-ir cells was increased obviously in a force-dependent manner, as revealed by IHC. (E) The correspondent nociceptive-like behaviors increased significantly in a force magnitude–dependent manner.

inflammation might be related to the initiation, development, and maintenance of ETM pain.

Our results indicated an increase in the expression of CCL2/CCR2 in TG 3 days following ETM. Similar up-regulation was found in previous studies using other rodent models for neuropathic pain in the DRG or TG. These models included unilateral application of glycoprotein 120 into the sciatic nerve,[27] chronic constriction injury,[28] partial ligation of the sciatic nerve,[29] focal nerve demyelination,[30] and experimental osteoarthritis.[16] However, the change rhythms in CCL2 expression were different in some cases. ETM-induced pain has been identified as noninfection inflammatory pain, which is different from previous bacterial inflammatory and neuropathic pain models. Thus, different pain models display different tissue damage and corresponding responses.

Moreover, the expression of CCL2 in TG and nocifensive grooming behaviors as well as c-fos expression in TG cultured neurons were modulated in a force magnitude–dependent manner. Similar nociceptive behavior responses were found with respect to capsaicin concentration.[31] Also, c-fos expression was reported to be correlated with force magnitude after ETM.[32] One possible interpretation is that heavier forces induce greater periodontal inflammation, leading to greater CCL2 release and correspondingly more dramatic behavior changes.

Furthermore, behavioral studies have shown that mechanical pain hyperalgesia is attenuated by treatment with CCL2 neutralizing antibody[23] or CCR2 antagonists[33] in other pain models. Our results were in agreement with those involving CCR2-null mice that showed an inhibition of mechanical pain responses

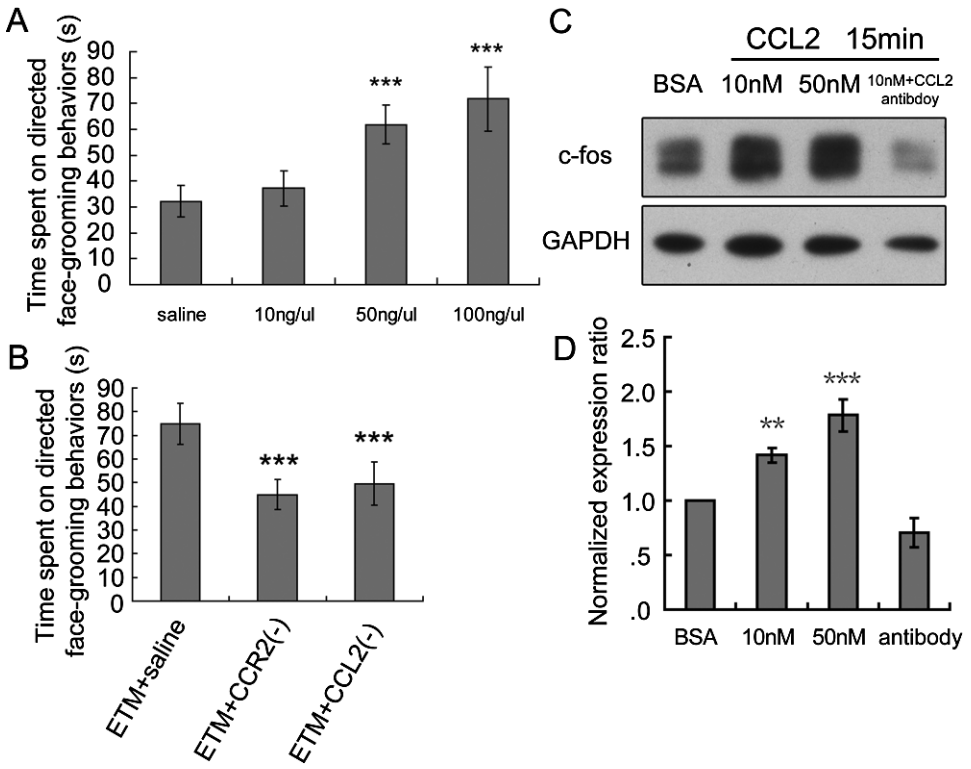

Figure 4. Exogenous CCL2 led to nocifensive behaviors and c-fos expression. $^{**}P < .01$, $^{***}P < .001$. (A) Peripheral injection of exogenous recombinant CCL2 (10, 50, or 100 ng/μL; 3 μL overall) led to a marked up-regulation of nocifensive grooming behaviors in a dose-dependent manner. (B) Intrathecal application of CCL2 neutralizing antibody and CCR2 antagonist attenuated nocifensive behaviors at 3 days after ETM (mean ± SEM, n = 5/group). (C,D) Recombinant CCL2 triggered c-fos expression in cultured TG neurons within 15 minutes in a dose-dependent manner, which was blocked by CCL2 neutralizing antibody.

after nerve injury or inflammation.[16,34] In addition, in the present study, peripheral application of recombinant CCL2 protein led to a pain response in a dose-dependent manner. Thus, the development of nocifensive behaviors due to ETM could be dependent on the CCL2/CCR2 signaling pathway.

It would be interesting to identify the source of CCL2 in peripheral sensory ganglia in the present pain model. CCL2-ir cells clearly increased in TG neurons. This is consistent with previous findings in DRG in models of neuropathic pain, whereas it differs with studies that showed increases in CCL2 in satellite cells in DRG following nerve injury.[12,13,27,29] This discrepancy may be attributed to the different pain models. Further analysis showed that the number of CCL2-ir cells increased significantly, mainly in small to medium-sized neurons, which implies that tooth movement triggers peripheral inflammation, leads to CCL2/CCR2 synthesis in pain-related TG neurons, and finally sends pain signals to the central nervous system.

In general, the current study was designed to unravel the molecular mechanism of pain triggered in a model of orthodontic treatment pain. The study demonstrated that the CCL2/CCR2 axis is actively linked to the development and maintenance of nociceptive behaviors in the context of a pain model for ETM associated with clinical practice. In this respect, it is a promising finding that blocking the CCL2/CCR2 signaling pathway may reverse the tooth movement–evoked pain.

CONCLUSION

- The CCL2/CCR2 axis was clearly activated in the TG by ETM and is essentially involved in ETM-induced pain mechanism.

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