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The $P2Y_{14}$ receptor in the trigeminal ganglion contributes to the maintenance of inflammatory pain

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ABSTRACT

P2Y purinergic receptors expressed in neurons and satellite glial cells (SGCs) of the trigeminal ganglion (TG) contribute to inflammatory and neuropathic pain. $P2Y_{14}$ receptor expression is reported in the spinal cord, dorsal root ganglion (DRG), and TG. In present study, the role of $P2Y_{14}$ receptor in the TG in inflammatory orofacial pain of Sprague-Dawley (SD) rats was investigated. Peripheral injection of complete Freund's adjuvant (CFA) induced mechanical hyperalgesia with the rapid upregulation of $P2Y_{14}$ receptor, glial fibrillary acidic protein (GFAP), interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), C–C chemokine CCL2, phosphorylated extracellular signal-regulated kinase 1/2 (p-ERK1/2), and phosphorylated p38 (p-p38) proteins in the TG. Furthermore, immunofluorescence staining confirmed the CFA-induced upregulation of $P2Y_{14}$ receptor. Double immunostaining showed that $P2Y_{14}$ receptor colocalized with glutamine synthetase (GS) and neuronal nuclei (NeuN). Finally, trigeminal injection of a selective antagonist (PPTN) of $P2Y_{14}$ receptor attenuated CFA-induced mechanical hyperalgesia. PPTN also decreased the upregulation of the GFAP, IL-1β, TNF-α, CCL2, p-ERK1/2, and p-p38 proteins. Our findings showed that $P2Y_{14}$ receptor in TG may contribute to orofacial inflammatory pain via regulating SGCs activation, releasing cytokines (IL-1β, TNF-α, and CCL2), and phosphorylating ERK1/2 and p38.

1. Introduction

Chronic pain of the orofacial region, including inflammatory pain, neuropathic pain, and cancer pain, is common in clinical practice, and its effective management is lacking due to its complicated mechanisms (Ji et al., 2013). Currently, accumulating evidence supports the participation of glial cell activation and neuroinflammation in the pathogenesis of chronic pain, including inflammatory pain (Ji et al., 2013; Ji et al., 2016; M. Takeda, Takahashi and Matsumoto, 2008a,b). Peripheral acute and chronic inflammation enhance the sensitivity of nociceptors on primary sensory neurons innervating injured muscle, skin, and joint tissues (Ji et al., 2016). The cell bodies of trigeminal ganglion (TG) neurons are enwrapped in satellite glial cells (SGCs), which modulate neuronal function via extracellular purine nucleotides, inflammatory cytokines, and growth factors, etc. (Ji et al., 2013). In addition, orofacial inflammatory pain is mediated by inflammatory cytokines and chemokines, which are released from neurons or SGCs via an autocrine/paracrine mechanism (Ji et al., 2013; Z. J. Zhang, Jiang and Gao, 2017a,b). For instance, inflammatory pain induced by CFA increased interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) protein expression in the TG (Lukacs et al., 2016; Spears et al., 2005). Furthermore, IL-1β and TNF-α could activate interleukin-1 type I receptors (IL-1RI) and TNF receptors (TNFR), respectively, to promote the development of inflammatory pain by enhancing neuronal excitability (Leo et al., 2015; Richter et al., 2010; M. Takeda, Kitagawa, Takahashi and Matsumoto, 2008a,b; M. Takeda, Takahashi, et al., 2008a,b). The TG plays a crucial role in transmitting and modulating orofacial pain from the peripheral nervous system to the central nervous system (Mamoru Takeda, Matsumoto, Sessle, Shinoda and Iwata, 2011a,b). Thus, it is crucial to understand the molecular and cellular mechanisms involved in peripheral sensitization of the TG.

P2 purinergic receptors have been reported to participate in the sensitization of the TG, the dorsal root ganglion (DRG), and the spinal cord, modulating acute and chronic pain through activating glial cells

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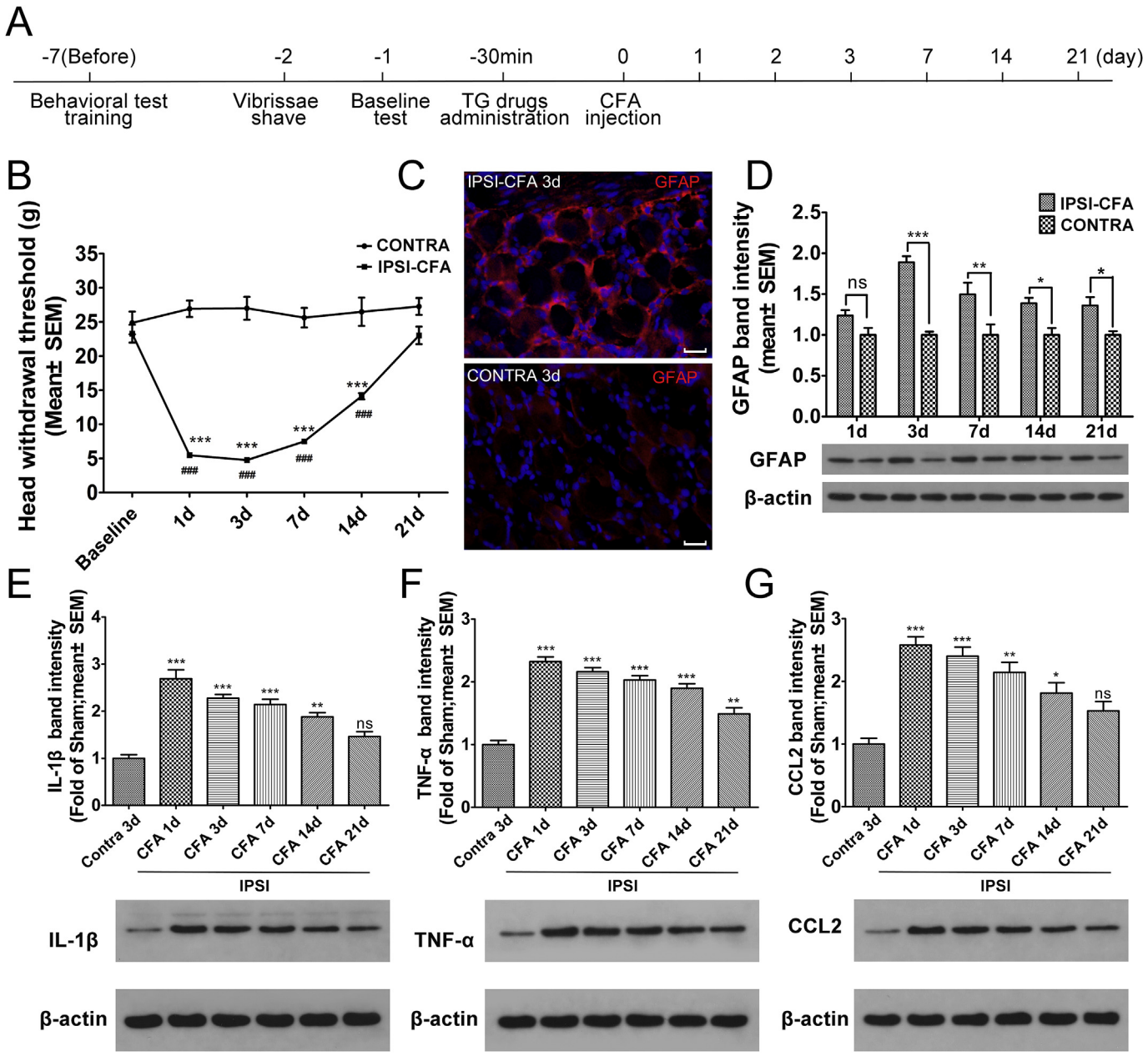

Fig. 1. CFA induced mechanical hyperalgesia, SGCs activation, and the release of proinflammatory cytokines (IL-1β, TNF-α, and CCL2). **(A)** The sequence of treatment in experiment. **(B)** CFA injection decreased the HWT from 1 d to 14 d. The HWT was not influenced by saline injection (n = 9 per group). Two-way RM ANOVA followed by Bonferroni test. **(C)** Immunofluorescence staining showed that CFA treatment upregulated GFAP protein after 3 d. Scar bar indicates 50 μm. **(D)** Western blot further demonstrated that GFAP protein was upregulated from 3 d to 21 d after CFA injection. Two-way RM ANOVA followed by Bonferroni test. **(E, F, G)** CFA induced the rapid upregulation of IL-1β, TNF-α, and CCL2 in the TG after 1 d, which was maintained for more than 14 d. Data are presented as the means ± SEMs (n = 3 per group); One-way RM ANOVA followed by Bonferroni test. $^{**}P < 0.01$, $^{***}P < 0.001$, compared to contra. $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, compared to the baseline mechanical threshold in CFA group.

and releasing inflammatory cytokines and chemokines (Katagiri et al., 2012; Kobayashi et al., 2012; G. Magni and Ceruti, 2014; G Magni et al., 2015; Tsuda, 2017). P2 purinergic receptors include seven inotropic P2X receptors and eight metabotropic P2Y receptors. Endogenous activators of P2Y receptors include adenosine triphosphate (ATP), adenosine diphosphate (ADP), uridine triphosphate (UTP), and uridine diphosphate (UDP) (Burnstock, 2017; G. Magni and Ceruti, 2013; Sak and Illes, 2005; Tsuda, 2017). As a novel member of the P2Y receptor family, the $P2Y_{14}$ receptor is activated by UDP and UDP-sugars (Lazarowski and Harden, 2015). The $P2Y_{14}$ receptor, which is expressed in glial cells (astrocytes, microglia, etc.), inflammatory cells (neutrophils, mast cells, etc.), aortic smooth muscle cells, osteoclasts, etc., plays a role in various physiological and pathological conditions, including its promotion of proinflammatory cytokines secretion (Azroyan et al., 2015; Barrett et al., 2013; Kinoshita et al., 2013; Lazarowski and Harden, 2015). Previous studies demonstrated an increase of the $P2Y_{14}$ receptor in the spinal cord after peripheral nerve injury, and $P2Y_{14}$ receptor inhibition attenuated mechanical hypersensitivity (Kobayashi et al., 2012). In addition, $P2Y_{14}$ receptor expression in the DRG was increased significantly after spared nerve injury (SNI) or CFA-induced inflammatory injury (Malin and Molliver, 2010; Vega-Avelaira et al., 2009). Increasing evidence suggests that $P2Y_{14}$ receptors are expressed in the TG (Ceruti et al., 2008; G. Magni and Ceruti, 2013). However, their function and behavior in orofacial inflammatory pain remain

unclear. Therefore, this study aimed to investigate the role of the $P2Y_{14}$ receptor in the TG and the effects of its antagonist on pain behaviors in CFA-induced inflammatory pain.

2. Materials and methods

2.1. Animals

Male Sprague-Dawley (SD) rats (200–300 g; male) were purchased from the Laboratory Animal Center of Sichuan University. Rats were housed in a controlled room (12 h light/dark cycle; temperature 23 ± 2 °C; relative humidity 50% ± 15%). All animals had unrestricted access to standard food and distilled water. Our experiments were approved by the Ethics Committee of the West China Hospital of Stomatology Sichuan University (WCHSIRB-D-2018-146). All animal procedures followed the Society for Neuroscience's policies on the Use of Animals and Humans in Neuroscience Research.

2.2. Induction of facial inflammation

CFA-induced inflammatory pain is commonly utilized to explore the mechanisms involved in acute or chronic pain conditions (G Magni et al., 2015; M. Takeda, Takahashi, et al., 2008a,b). To induce facial inflammation in rats under brief isoflurane anesthesia, complete Freund's adjuvant (CFA; 50 µl, 1:1 oil/saline suspension) was slowly injected into the right whisker pad area (ipsilateral) over 1 min (M. Takeda et al., 2007). The contralateral left whisker pad area of each rat was injected with an equal volume of normal saline (0.9%). According to previous studies (krzyzanowska, Martin, Avendaño, Piedras and Krzyzanowska, 2010; M. Takeda, Takahashi, Nasu and Matsumoto, 2011a,b), in some experiments (n = 3), the Evan's blue dye (50 mg/ml, 1 ml/kg) was injected in the lateral vein of the tail to verify CFA-induced facial inflammation. The whisker pad area treated with CFA was observed a strong blue colouring and the dye was accumulated in the injected facial skin in the postmortem examination, which demonstrated that inflammation induced plasma protein extravasation.

2.3. Behavioral analysis

According to a previously described protocol (Chaplan et al., 1994; G Magni et al., 2015), mechanical hyperalgesia in rats was measured with von Frey filaments (Stoelting Company, Wood Dale, USA). The sequence of experiment was shown in Fig. 1A. Briefly, animals were placed in a metal mesh cage (15 cm × 25 cm × 20 cm), which were trained to adapt the cage and the probe by von Frey filaments once a day for one week before CFA injection (G Magni et al., 2015). Then, the vibrissae in the test area were carefully shaved under isoflurane anesthesia one day before baseline testing. CFA and saline were injected one day after baseline testing. To test mechanical sensitivity, animals were allowed 30 min in a quiet environment at a temperature of 22 ± 1 °C for habituation before each behavioral testing. The filaments were used to perpendicularly probe the injected sites and surrounding orofacial skin of rats. The initial filaments used for the contralateral and ipsilateral applied a force of 10 g and 2 g, respectively. Each filament was tested for a few seconds, and 5 min elapsed between two filaments. The 50% HWT was calculated using Dixon's up-down method (Chaplan et al., 1994).

2.4. TG drug administration

Trigeminal injection was performed according to a previous study (Long et al., 2017). Following anesthesia with isoflurane, facial hairs between the ears and eyes were removed with clippers. This area was disinfected with 10% Betadine. The injection site was between the notch and tympanic bulla; more specifically, the injection site was 2 mm posterior to the most anterior point on the notch. After the notch was palpated between the ipsilateral angular process and the condylar process, the rats were injected by Hamilton syringe in the medial–superior direction (90° to the head midline and 15° to the coronal plane). The needles were inserted to a depth of approximately 9 mm. Then, drugs were injected over 1 min, and the needles were held at the final position for 1 min before their slow removal. Thirty minutes before CFA injection, the rats were treated with dimethyl sulfoxide (DMSO diluted to 0.05%, 0.05 µl/g body weight; Sigma–Aldrich) and PPTN (5 µM or 10 µM dissolved in 0.05% DMSO, 0.05 µl/g body weight; Tocris Bioscience, UK). The concentration and volume of PPTN were chosen according to previous studies (Azroyan et al., 2015; Long et al., 2017; Sesma et al., 2016). Thus, rats were also randomly divided into four groups: the control group (contralateral whisker pad area injected with saline), the CFA group (ipsilateral whisker pad area injected with CFA, inflamed rats), the CFA + DMSO group (inflamed rats with ipsilateral trigeminal injection of 0.05% DMSO), and the CFA + PPTN group (inflamed rats with ipsilateral trigeminal injection of PPTN).

2.5. Immunofluorescence

Under 10% chloral hydrate (30 mL/kg) anesthesia, animals were transcardially perfused with 4% formalin fixative according to a previous method (G Magni et al., 2015). Then, the TG of each rat was excised, fixed with 4% paraformaldehyde for 4 h (4 °C), and incubated in 30% sucrose for at least 48 h. After being embedded in Tissue-Tek (Sakura Finetek, USA) at −20 °C, each ganglion was cut on a cryostat microtome (Leica, Nussloch, Germany) into 10 µm-thick sections. TG sections were incubated in 0.25% Triton X-100 (Solarbio, Beijing, China) at room temperature (RT) for 15 min. Then, they were incubated in 10% goat serum (Solarbio, Beijing, China) at RT for 30 min. The TG sections were incubated overnight at 4 °C with the following primary antibodies: mouse anti-GFAP (1:500; Cat# Ab10062; Abcam, UK), mouse anti-GS (1:500; Cat# Ab73593; Abcam, Cambridge, UK), mouse anti-NeuN (1:500; Cat# Ab104224; Abcam, Cambridge, UK), and rabbit anti-$P2Y_{14}$ receptor (1:200; Cat# ARP-018; Alomone Labs, Jerusalem, Israel). After 3 washes with PBS, the sections were incubated with Alexa Fluor 488 (1:500; Cat# Ab150077, Abcam, UK) and Alexa Fluor 647 (1:500; Cat# Ab150115, Abcam, UK) for 1 h at RT. All antibodies were diluted in PBS containing 1% BSA. Cellular nuclei were subsequently stained with DAPI (Beyotime, Shanghai, China) for 5 min at RT. The sections were sealed with a nonfluorescent quencher (Beyotime, Shanghai, China) and then examined using a fluorescence microscope (Olympus, Tokyo, Japan).

2.6. Western blotting

Animals were anesthetized with 10% chloral hydrate (30 ml/kg), and then transcardially perfused with PBS at different time points after CFA injection. The isolated TGs were homogenized via mechanical disruption. Samples containing equal amounts of protein (40 µg) were separated by electrophoretic blotting procedures and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, USA). Nonfat dried milk (5%) was utilized to block the membranes at RT for 2 h. Then, the membranes were incubated at 4 °C overnight with the following primary antibodies: rabbit anti-GFAP (1:1000; Cat# 16825-1-AP, Proteintech, Wuhan, China), mouse anti-IL-1β (1:1000; Cat# 66737-1-Ig, Proteintech, Wuhan, China), rabbit anti-TNF-α (1:1000; Cat# bs-0078R, Bioss, Beijing, China), mouse anti-CCL2 (1:1000; Cat# 66272-1-Ig, Proteintech, Wuhan, China), rabbit anti-$P2Y_{14}$R (1:200; Cat# ARP-018; Alomone Labs, Jerusalem, Israel), and mouse anti-β-actin (1:500; Cat# BM0627, Boster, Wuhan, China). After 5 washes with TBST, the membranes were incubated with HRP-conjugated secondary antibody (goat anti-mouse IgG; Cat# BA1051; goat anti-rabbit IgG; Cat# BA1054; 1:50000, Boster, Wuhan, China) for 2 h at 37 °C. Signals were detected by enhanced chemiluminescence (Thermo, Waltham, MA, USA) and autoradiography, and the optical density ratio

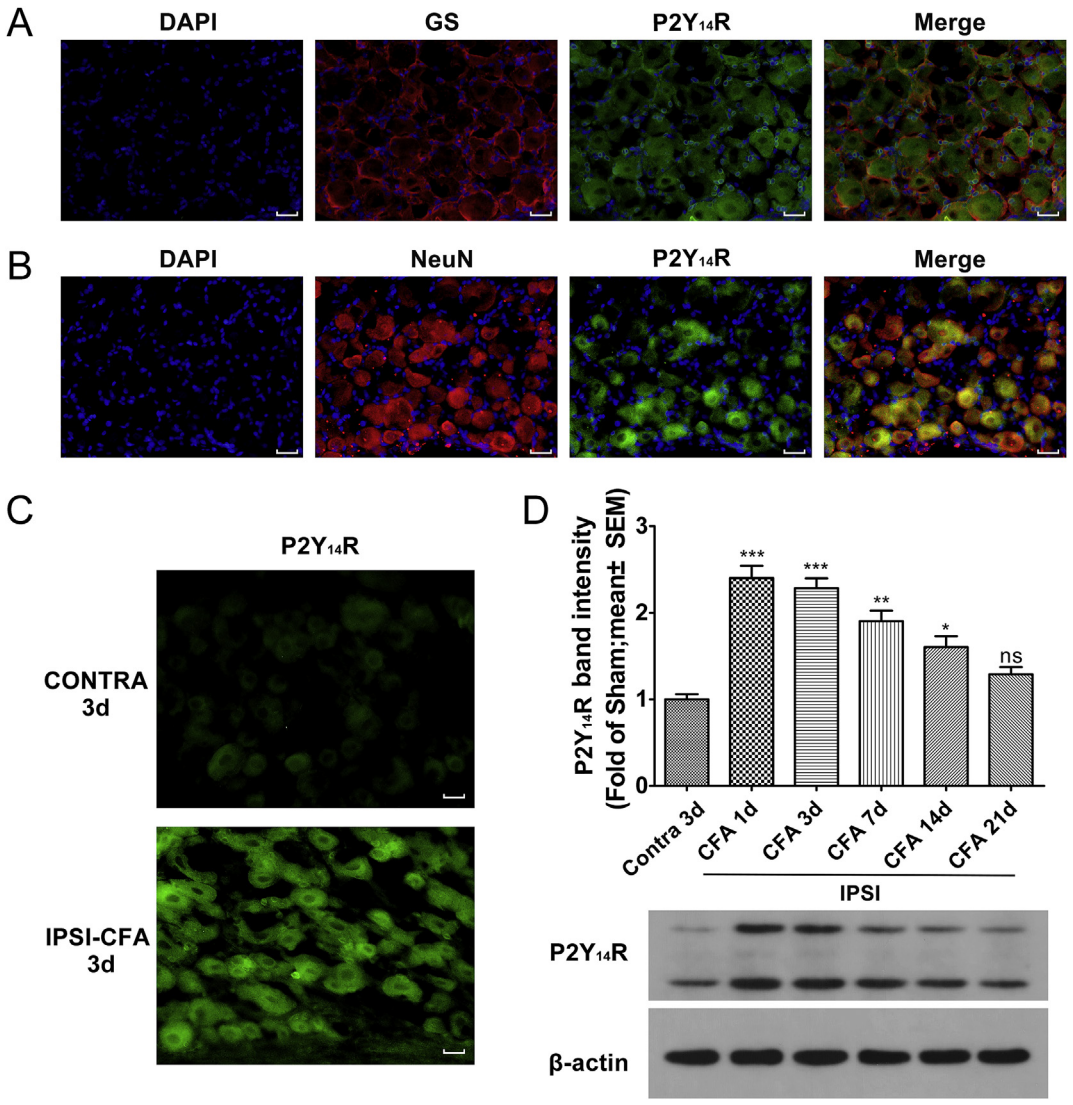

Fig. 2. The $P2Y_{14}$ receptor was upregulated in the TG after CFA injection. **(A, B)** As measured by immunofluorescence, the $P2Y_{14}$ receptor colocalized with GS **(A)** and NeuN **(B)**. **(C)** Immunofluorescence staining showed that CFA induced the upregulation of the $P2Y_{14}$ receptor after 3 d. **(D)** Western blot further showed that CFA induced the upregulation of the $P2Y_{14}$ receptor from 1 d to 14 d. Data are presented as the means ± SEMs (n = 3 per group). *P < 0.05, **P < 0.01, ***P < 0.001. One-way ANOVA followed by Bonferroni test. Scar bar indicates 50 μm.

of each band was quantified using BandScan software. Each sample was normalized to the corresponding β-actin content.

2.7. Data analysis

All data are expressed as the mean ± standard error of the mean (SEM). The data were analyzed by one-way ANOVA followed by Bonferroni posttest or two-way (or Repeated-Measure) ANOVA followed by Bonferroni posttest using SPSS version 18.0. The within-subjects factors included treatment and time, and the main effects of them and the interaction effects between them were analyzed in two-way Repeated-Measure ANOVA. Meanwhile, the between-subjects factors (treatment and time), the main effects, and interaction effects were analyzed in two-way ANOVA, where no within-subjects factors. Three degrees of significance were indicated by the following: *$P < 0.05$, $P < 0.01$, or $P < 0.001$*.

3. Results

3.1. CFA induced rapid mechanical hyperalgesia and SGCs activation

CFA-treated rats were intravenously injected with Evan's blue dye, and their ipsilateral facial skin turned blue 1 d after CFA injection, which indicated the successful induction of facial inflammation. In Fig. 1A, the sequence of treatments that animals received and time points tested were shown. The head withdrawal threshold (HWT) of the rats was tested by von Frey filaments. In Fig. 1B, compared to that in the contralateral, the HWT was significantly decreased 1 d ($P < 0.001$), 3 d ($P < 0.001$), 7 d ($P < 0.001$), and 14 d ($P < 0.001$) but not 21 d ($P > 0.05$) after CFA injection. Meanwhile, compared to the baseline HWT, the HWT was also significantly decreased 1 d ($P < 0.001$), 3 d ($P < 0.001$), 7 d ($P < 0.001$), and 14 d ($P < 0.001$) but not 21 d ($P > 0.05$) after CFA injection (n = 9 per group, two-way RM ANOVA followed by Bonferroni test) [the main effect of treatment ($F_{(1,8)} = 315.696$, $P < 0.001$), the main effect of time ($F_{(5,40)} = 22.192$, $P < 0.001$), and the interaction effect ($F_{(5,40)} = 25.208$, $P < 0.001$)]. The results above suggested the development of CFA-induced mechanical hyperalgesia.

Based on the behavioral data, we further examined the expression of glial fibrillary acidic protein (GFAP), a glial cell/SGC activation marker, in the TG (G Magni et al., 2015). In Fig. 1D, GFAP expression in contralateral and ipsilateral TG were detected by western blot at different time points. Compared to the contralateral, GFAP expression in the ipsilateral was significantly increased at 3 d ($P < 0.001$), 7 d ($P < 0.01$), 14 d ($P < 0.05$), and 21 d ($P < 0.05$) after CFA injection and peaked at 3 d (n = 3 per group, Two-way ANOVA followed by Bonferroni test) [the main effect of treatment ($F_{(1,20)} = 69.637$, $P < 0.001$), the main effect of time ($F_{(4,20)} = 3.868$, $P = 0.017$), and the interaction effect ($F_{(4,20)} = 3.868$, $P = 0.017$)]. As indicated by the immunofluorescence results in Fig. 1C, compared to that in the contralateral, GFAP expression in the ipsilateral TG was upregulated 3 d after CFA injection. These data indicated that SGCs in the TG were activated in CFA-induced inflammatory pain.

3.2. CFA induced IL-1β, TNF-α, and CCL2 upregulation in the TG

The expression of IL-1β, TNF-α, and CCL2 in the contralateral and ipsilateral TG was analyzed by western blot. In Fig. 1E, compared to the contralateral, IL-1β expression was significantly increased 1 d ($P < 0.001$), 3 d ($P < 0.001$), 7 d ($P < 0.001$), and 14 d ($P < 0.01$) but not 21 d ($P > 0.05$) after CFA treatment (n = 3 per group). Meanwhile, as indicated in Fig. 1F, compared to the contralateral, TNF-α expression in the ipsilateral TG was significantly increased 1 d ($P < 0.001$), 3 d ($P < 0.001$), 7 d ($P < 0.001$), 14 d ($P < 0.001$), and 21 d ($P < 0.01$) (n = 3 per group). Similarly, compared to the contralateral, CFA treatment significantly increased CCL2 expression after 1 d ($P < 0.001$), 3 d ($P < 0.001$), 7 d ($P < 0.01$), and 14 d ($P < 0.05$) but not 21 d ($P > 0.05$) (n = 3 per group, one-way ANOVA followed by Bonferroni test). Our results suggested that IL-1β, TNF-α, and CCL2 in the TG might participate in CFA-induced inflammatory pain.

3.3. CFA induced $P2Y_{14}$ receptor upregulation in the TG

Glutamine synthetase (GS) is a SGC marker, and NeuN is a neuron-specific nuclear protein (G Magni et al., 2015). In Fig. 2A, the $P2Y_{14}$ receptor and GS were coexpressed, which indicated that the $P2Y_{14}$ receptor was expressed in SGCs of the TG. As indicated in Fig. 2B, the clear colocalization of the $P2Y_{14}$ receptor and NeuN indicated the expression of the $P2Y_{14}$ receptor in TG neurons.

Based on the behavioral data, $P2Y_{14}$ receptor expression in the TG was investigated 1 d, 3 d, 7 d, 14 d, and 21 d after CFA injection. In Fig. 2D, $P2Y_{14}$ receptor protein expression was significantly increased 1 d ($P < 0.001$, vs. contra), 3 d ($P < 0.001$, vs. contra), 7 d ($P < 0.01$, vs. contra), and 14 d ($P < 0.05$, vs. contra) but not 21 d ($P > 0.05$, vs. contra) after CFA injection (n = 3 per group, one-way ANOVA followed by Bonferroni test). Immunofluorescence staining showed that $P2Y_{14}$ receptor expression in ipsilateral TG was higher than that in the contralateral at 3 d after CFA injection, further confirming the western blot results. These results implied that the $P2Y_{14}$ receptor is expressed in the SGCs and neurons of the TG, which are involved in CFA-induced inflammatory pain.

3.4. Inhibition of the $P2Y_{14}$ receptor in the TG attenuated CFA-induced mechanical hyperalgesia

A selective inhibitor of the $P2Y_{14}$ receptor, 4-[4-(4-piperidinyl) phenyl]-7-[4-(trifluoromethyl) phenyl]-2-naphthalenecarboxylic acid hydrochloride (PPTN), was diluted in 0.05% dimethyl sulfoxide (DMSO). DMSO (0.05%) and PPTN were injected into the TG 30 min prior to CFA treatment. In Fig. 3A, compared to mechanical hyperalgesia in the CFA group, DMSO administration had no effect on mechanical hyperalgesia at 1 d ($P > 0.05$), 2 d ($P > 0.05$), and 3 d ($P > 0.05$) (n = 9 per group) (two-way RM ANOVA followed by Bonferroni test) [the main effect of treatment ($F_{(3,24)} = 315.696$, $P < 0.001$), the main effect of time ($F_{(3,24)} = 22.192$, $P < 0.001$), and the interaction effect ($F_{(9,72)} = 25.208$, $P < 0.001$)]. However, compared to mechanical hyperalgesia in the CFA + DMSO group, the administration of 5 μM PPTN attenuated mechanical hyperalgesia at 1 d ($P < 0.001$), 2 d ($P < 0.001$), and 3 d ($P < 0.05$) (n = 9 per group), and the administration of 10 μM PPTN attenuated mechanical hyperalgesia at 1 d ($P < 0.001$), 2 d ($P < 0.001$), and 3 d ($P < 0.001$) (n = 9 per group). These results suggested that the inhibition of the $P2Y_{14}$ receptor in the TG might modulate CFA-induced inflammatory pain.

3.5. Inhibition of the $P2Y_{14}$ receptor in the TG decreased the CFA-induced upregulated expression of IL-1β, TNF-α, GFAP, and CCL2

Thirty minutes before CFA treatment, 0.05% DMSO and 10 μM PPTN were injected into the TG. The expression of the IL-1β, TNF-α, GFAP, and CCL2 proteins in the contralateral and ipsilateral TG was analyzed by western blot at 1 d after CFA injection. In Fig. 3C, D, E, and F, expression of the IL-1β, TNF-α, CCL2, and GFAP between the CFA group and the CFA + DMSO group were not significantly different ($P > 0.05$). As indicated in Fig. 3C, compared to the contralateral, IL-1β expression in ipsilateral was significantly increased by CFA treatment ($P < 0.001$). However, compared to CFA + DMSO group, 10 μM PPTN pretreatment reduced IL-1β expression ($P < 0.01$) (n = 3 per group). As indicated in Fig. 3D, the expression of TNF-α was significantly upregulated after CFA injection ($P < 0.001$, vs. contra). However, TNF-α was decreased by pretreatment with 10 μM PPTN ($P < 0.05$, vs. CFA + DMSO) (n = 3 per group). In Fig. 3E, GFAP expression was significantly increased by CFA treatment ($P < 0.001$, vs. contra) and then attenuated by 10 μM PPTN ($P < 0.01$, vs. CFA + DMSO) (n = 3 per group). The results in Fig. 3F indicated that CFA treatment significantly upregulated CCL2 expression ($P < 0.001$, vs. contra), which was decreased by 10 μM PPTN ($P < 0.05$, vs. CFA + DMSO) (n = 3 per group). The data above implied that $P2Y_{14}$ receptor inhibition might attenuate mechanical hyperalgesia through inhibiting SGCs activation and decreasing IL-1β, TNF-α, and CCL2 expression in the TG.

3.6. Inhibition of the $P2Y_{14}$ receptor decreased the CFA-induced phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2) and p38

The phosphorylation and activation of ERK1/2 and p38 are involved in inflammatory pain (Cady et al., 2014; Dong et al., 2014). After 0.05% DMSO and 10 μM PPTN were injected into the TG, the expression of p-ERK1/2 and p-p38 in the contralateral and ipsilateral TG was measured by western blot at 3 d after CFA treatment according to previous studies (Cady et al., 2014). In Fig. 4B and E, the integrated optical density (IOD) ratios of ERK1/2 to β-actin and p-38 to β-actin were not significantly different among the control group, the CFA group, the CFA + DMSO group, and the CFA + PPTN group ($P > 0.05$) (n = 3 per group). In Fig. 4C, CFA treatment enhanced the IOD ratio of p-ERK1/2 to ERK1/2 compared to that in the contralateral ($P < 0.01$) (n = 3 per group). The difference in the ratio of p-ERK1/2 to ERK1/2 between the CFA group and the CFA + DMSO group was not significant ($P > 0.05$). However, 10 μM PPTN significantly attenuated the ratio of p-ERK1/2 to ERK1/2 compared to that in the CFA + DMSO group ($P < 0.01$). In Fig. 4F, the IOD ratio of p-p38 to p38 was also increased after CFA injection ($P < 0.01$, vs. contra) (n = 3 per group). The ratio of p-p38 to p38 in the CFA group did not differ from that in the CFA + DMSO group ($P > 0.05$). However, the p-p38 to p38 ratio was significant lower following 10 μM PPTN pretreatment than in the CFA + DMSO group ($P < 0.05$) (n = 3 per group). These data suggested that the effects of the $P2Y_{14}$ receptor on CFA-induced mechanical hyperalgesia might include the phosphorylation of ERK1/2 and p38

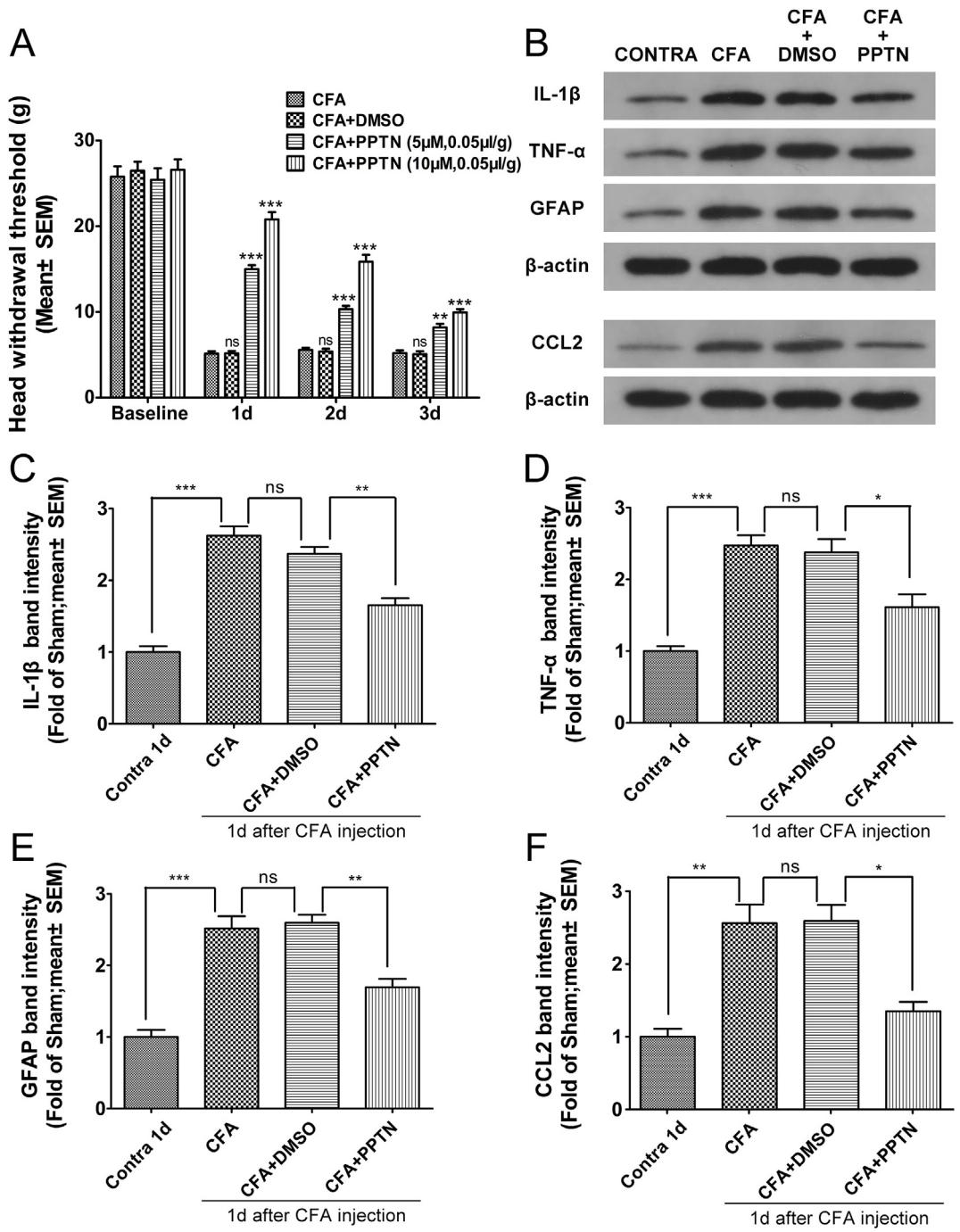

Fig. 3. Inhibition of the $P2Y_{14}$ receptor in the TG attenuated CFA-induced mechanical hyperalgesia and decreased the upregulation of IL-1β, TNF-α, GFAP, and CCL2. **(A)** A $P2Y_{14}$ receptor antagonist (PPTN) attenuated CFA-induced mechanical hyperalgesia from 1 d to 3 d. The HWT was not influenced by 0.05% DMSO. Two-way RM ANOVA followed by Bonferroni test. **(B, C, D, E, F)** Injection with 0.05% DMSO did not affect the expression of IL-1β **(C)**, TNF-α **(D)**, GFAP **(E)**, and CCL2 **(F)** 1 d after CFA injection. However, 10 μM PPTN significantly decreased the CFA-induced upregulation of IL-1β, TNF-α, GFAP, and CCL2. Data are presented as the means ± SEMs (n = 3 per group); One-way ANOVA followed by Bonferroni test. *P < 0.05, **P < 0.01, ***P < 0.001.

in the TG.

4. Discussion

The P2 purinergic receptors, including the inotropic $P2X_{1\text{-}7}$ receptors and metabotropic $P2Y_{1,2,4,6,11,12,13,14}$ receptors, have emerged as some of the most innovative targets in a variety of physiological processes, including pain (Cekic and Linden, 2016; Puchalowicz et al., 2014). The $P2Y_{14}$ receptor, a G-protein–coupled receptor, is regarded as a novel member of the P2Y receptor family. $P2Y_{14}$ receptor mRNA is expressed in the TG, the DRG, astrocytes, and microglia (Ceruti et al., 2008; Kinoshita et al., 2013; Lazarowski and Harden, 2015). With the confirmation of specificity of the $P2Y_{14}$ antibodies when staining of tissue slices, our immunofluorescence results further demonstrated that the $P2Y_{14}$ receptor colocalized with NeuN (a neuronal marker) and GS (a SGC marker), suggesting that the $P2Y_{14}$ receptor is expressed in both the neurons and SGCs of the TG. In addition, $P2Y_{14}$ receptor expression is upregulated in pathological pain conditions. For instance, SNI dramatically increased $P2Y_{14}$ receptor mRNA expression in the spinal cord after 3 d, and $P2Y_{14}$ receptor mRNA expression remained elevated after

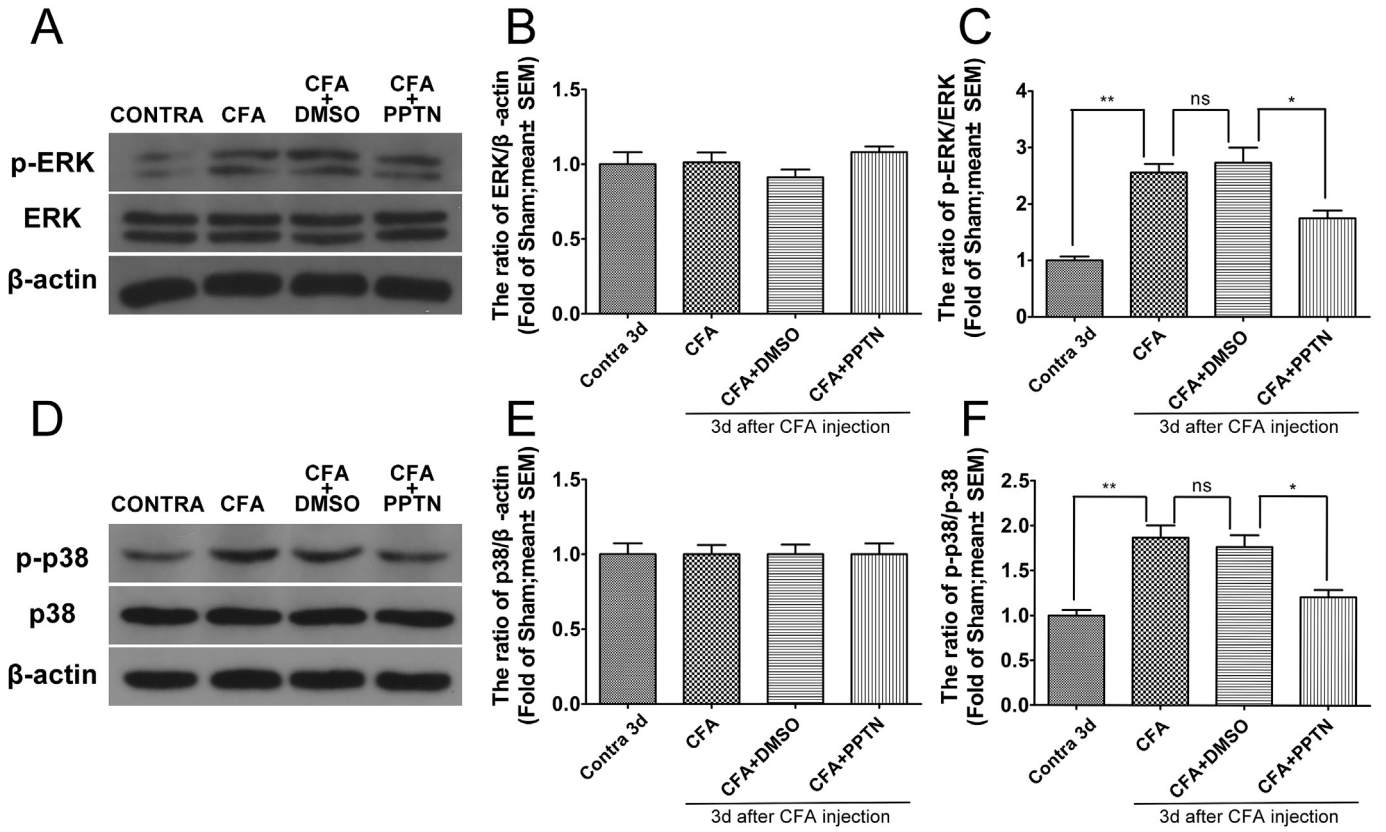

Fig. 4. Inhibition of the $P2Y_{14}$ receptor affected the phosphorylation of ERK1/2 and p38 in the TG. **(A) (D)** The expression of ERK1/2, p-ERK1/2, p38, and p-p38 was measured by western blot. **(B) (E)** The IOD ratios of ERK1/2 to β-actin and p38 to β-actin were not significantly different among four groups. **(C) (F)** Compared to those in the control group, CFA injection enhanced the levels of p-ERK1/2 and p-p38. The differences in the ratios of p-ERK1/2 to ERK1/2 and p-p38 to p38 between the CFA group and the CFA + DMSO group were not significant. However, 10 μM PPTN significantly decreased the ratios of p-ERK1/2 to ERK1/2 and p-p38 to p38. Data are presented as the means ± SEMs (n = 3 per group). *P < 0.05, **P < 0.01, ***P < 0.001. One-way ANOVA followed by Bonferroni test.

14 d (Kobayashi et al., 2012). Meanwhile, $P2Y_{14}$ receptor mRNA was also significantly increased in the DRG 7 d after SNI surgery (Vega-Avelaira et al., 2009). In our study, the $P2Y_{14}$ receptor protein in the TG detected by western blot was dramatically increased 1 d after CFA injection and remained elevated 14 d after CFA injection. Furthermore, immunofluorescence staining showed that $P2Y_{14}$ receptor protein was upregulated 3 d after CFA injection. These data suggest that the $P2Y_{14}$ receptor is involved in inflammatory pain.

Accumulating evidence has verified that SGCs activation plays a critical role in neuropathic pain (Ji et al., 2013). SGCs activation contributes to neuropathic pain and inflammatory pain, which is usually evaluated by GFAP expression (Donegan et al., 2013; Ying et al., 2017). Our results showed that GFAP protein was significantly upregulated 3 d after CFA injection and remained elevated 14 d after CFA injection. Our immunofluorescence results confirmed the increased GFAP protein level at 3 d after CFA injection. Similarly, GFAP protein was dramatically increased and remained elevated 10 d after CFA injection into the temporal-mandibular joint (TMJ) (G Magni et al., 2015). Meanwhile, previous studies reported that the proinflammatory cytokines IL-1β, TNF-α, and CCL2 play a critical role in neuropathic pain and inflammatory pain (Dauvergne et al., 2014; Kim et al., 2014; Rozas et al., 2016). IL-1β could enhance sodium currents and suppress potassium currents in TG neurons (M. Takeda, Kitagawa, et al., 2008a,b). TNF-α could activate TNFR in DRG neurons, which subsequently induced abnormal neuronal excitability (Leo et al., 2015). CCL2 could upregulate the current density and expression of TRPV1 channels and $Na_v1.8$ sodium channels in DRG neurons (Belkouch et al., 2011; Kao et al., 2012). Our data demonstrated that the IL-1β, TNF-α, and CCL2 proteins in TG were dramatically increased and remained elevated after CFA injection. Similarly, IL-1β protein in the TG was significantly increased in CFA-induced trigeminal inflammatory hyperalgesia (Lukacs et al., 2016; M. Takeda, Takahashi, et al., 2008a,b). Meanwhile, TNF-α protein in the TG was dramatically upregulated after CFA injection or inferior alveolar nerve transection (Batbold et al., 2017; Ito et al., 2018), and administration of TNFR inhibitor significantly reduced mechanical orofacial hyperalgesia (Lis et al., 2017). The CCL2 expression was increased in peripheral traumatic trigeminal pain and experimental tooth movement pain (Dauvergne et al., 2014; Yang et al., 2014). Furthermore, previous studies reported that CCL2 production is the downstream of IL-1β and TNF-α signaling (Guo et al., 2012; Lu et al., 2014; Rozas et al., 2016). Thus, CCL2 might be increased by the upregulated IL-1β and TNF-α in the TG. The results above indicate that upregulated IL-1β, TNF-α and CCL2 may be important in CFA-induced facial inflammatory pain.

P2 purinergic receptors were reported to regulate the release of cytokines in pathological pain conditions (Ji et al., 2013; Martucci et al., 2008). For instance, the $P2X_7$ receptor and $P2Y_{12}$ receptor in the DRG regulated the expression of TNF-α receptor and IL-1β in HIV gp120-associated neuropathic pain (Shi et al., 2018; Wu et al., 2017). In addition, a $P2X_7$ receptor antagonist attenuated allodynia/hyperalgesia and blocked the upregulation of TNF-α in the trigeminal sensory nuclear complex after transection of the mental nerve (Murasaki et al., 2013). In addition, the $P2X_7$ receptor, $P2Y_6$ receptor, and $P2Y_2$ receptor were reported to be involved in CCL2 production (Morioka et al., 2013; Shieh et al., 2014; Stokes and Surprenant, 2007). Our results showed that a selective $P2Y_{14}$ receptor antagonist (PPTN) attenuated CFA-induced mechanical hyperalgesia and the expression of IL-1β, TNF-α, and CCL2 at 1 d after CFA injection. Compared to CFA group, 0.05% DMSO solution injection had no significant effect on mechanical hyperalgesia and IL-1β, TNF-α, and CCL2 protein expression. Our findings were

consistent with the previous study, in which intrathecal injection of $P2Y_{14}$ antisense locked nucleic acids attenuated mechanical pain hypersensitivity (Kobayashi et al., 2012). Similarly, the $P2Y_{14}$ receptor on renal intercalated cells regulated the secretion of CCL2 to mediate sterile inflammation in the kidney (Azroyan et al., 2015). Therefore, the $P2Y_{14}$ receptor may upregulate the expression of IL-1β, TNF-α, and CCL2 in the TG to induce mechanical hyperalgesia in inflammatory pain.

Previous studies reported that the activation of P2 purinergic receptors is involved in the modulation of neuropathic pain via the phosphorylation of the MAPK signaling pathway (Wu et al., 2017; Xie et al., 2017; Xiong et al., 2017). The $P2Y_{14}$ receptor is a G-protein–coupled receptor coupled through natively occurring G_i heterotrimers, which can active MAPK signaling pathways (Fricks, Carter, Lazarowski, & T Kendall, 2009; Lazarowski and Harden, 2015). The MAPK family contains ERK1/2, p38, and c-Jun N-terminal kinases (JNKs). Mounting evidence has indicated that MAPK pathways participate in the intracellular signaling of neurons and glial cells, which are essential for the development and maintenance of chronic pain (Ji et al., 2013; Ji et al., 2009). The inhibition of ERK1/2 or p38 in the TG decreased mechanical hyperalgesia after partial infraorbital nerve ligation (Q. Zhang, Cao, Zhang, Jiang and Gao, 2016; Q. Zhang et al., 2017a,b). The present study showed that the IOD ratios of p-p38 to p38 and p-ERK1/2 to ERK1/2 were significantly enhanced following CFA treatment, suggesting that ERK1/2 and p38 phosphorylation in the TG were involved in CFA-induced hyperalgesia. The results are consistent with previous studies that CFA-induced TMJ inflammation activated ERK1/2 and p38 in the TG after 2 d (Cady et al., 2014). Meanwhile, the ratios of p-p38 to p38 and p-ERK1/2 to ERK1/2 in the CFA + PPTN group were significantly lower than those in the CFA + DMSO group, suggesting that the $P2Y_{14}$ receptor in the TG was involved in ERK1/2 and p38 phosphorylation. Similarly, it was reported that activation of the $P2Y_{14}$ receptor by UDP-glucose significantly increased ERK1/2 phosphorylation in multiple cell types, such as HEK293 cells, differentiated HL-60 cells, neutrophils, and MDCK-C11 cells (Azroyan et al., 2015; Fricks et al., 2009; Scrivens and Dickenson, 2006). In addition, activation of the $P2Y_{14}$ receptor by UDP-glucose or MRS2690 (diphosphoric acid 1-α-D-glucopyranosyl ester 2-[(2-thio) uridin-5″-yl] ester) significantly increased p38 phosphorylation in RBL-2H3 mast cells (Gao et al., 2010). Furthermore, p38 MAPK was also reported to be involved in the transcriptional regulation of the $P2Y_{14}$ receptor. A previous study demonstrated that a p38 inhibitor significantly suppressed $P2Y_{14}$ receptor mRNA expression in the spinal cord following SNI (Kobayashi et al., 2012). Thus, the relationship between the $P2Y_{14}$ receptor and the MAPK signaling pathway needs further study. In summary, our study demonstrated that the inhibition of $P2Y_{14}$ receptor decreased the phosphorylation of ERK1/2 and p38 in the TG, relieving mechanical hyperalgesia induced by orofacial inflammation.

5. Conclusion

In conclusion, in the present study, we demonstrated the expression of the $P2Y_{14}$ receptor in the TG and explored its role in CFA-induced inflammatory pain. CFA rapidly induced mechanical hyperalgesia and upregulated the expression of the $P2Y_{14}$ receptor, IL-1β, TNF-α, GFAP, CCL2, p-ERK1/2, and p-p38 in the TG. We found that the $P2Y_{14}$ receptor was expressed in both neurons and SGCs of the TG. Furthermore, specific inhibition of the $P2Y_{14}$ receptor in the TG attenuated CFA-induced mechanical hyperalgesia and the upregulated level of IL-1β, TNF-α, GFAP, CCL2, p-ERK1/2, and p-p38. The findings above suggest that the $P2Y_{14}$ receptor in the TG may play an important role in the initiation and maintenance of inflammatory pain.

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Declaration of competing interest

None of the authors has any conflicts of interest to declare. All generated and analyzed data in our study are contained in this article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuint.2019.104567.

Author contributions

Jiu Lin and Jie-fei Shen were involved in designing the study. Jiu Lin, Yan-yan Zhang, Xin-yi Fang, Meng-ke Liu, Chao-lan Huang, and Da-qing Liao conducted experiments and analyzed the data. Jiu Lin, Jie-fei Shen, Fei Liu, Cheng Zhou, and Hang Wang were involved in the writing and editing. All authors critically reviewed the manuscript.

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