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ORIGINAL ARTICLE

Prostaglandin E_2 Upregulated Trigeminal Ganglionic Sodium Channel 1.7 Involving Temporomandibular Joint Inflammatory Pain in Rats

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Abstract—Prostaglandin E_2 (PGE_2) is a key proinflammatory mediator that contributes to inflammatory hyperalgesia. Voltage-gated sodium channel 1.7 ($Na_v1.7$) plays an important role in inflammatory pain. However, the modulation of $Na_v1.7$ in inflammatory pain remains poorly understood. We hypothesized that PGE_2 might regulate $Na_v1.7$ expression in inflammatory pain. We here showed that treatment of rat trigeminal ganglion (TG) explants with PGE_2 significantly upregulated the mRNA and protein expressions of $Na_v1.7$ through PGE_2 receptor EP2. This finding was confirmed by studies on EP2-selective antagonist PF-04418948. We also demonstrated that $Na_v1.7$ and COX-2 expressions, as well as PGE_2 levels, were upregulated in the TG after induction of rats' temporomandibular joint (TMJ) inflammation. Correspondingly, hyperalgesia, as indicated by head withdrawal threshold, was observed. Moreover, TMJ inflammation-induced upregulation of $Na_v1.7$ expression and PGE_2 levels in the TG could be reversed by COX-2-selective inhibitor meloxicam given by oral gavage, and meanwhile, the hyperalgesia of inflamed TMJ was also mitigated. So we concluded that PGE_2 upregulated trigeminal ganglionic $Na_v1.7$ expression to contribute to TMJ inflammatory pain in rats. Our finding suggests that PGE_2 was an important regulator of $Na_v1.7$ in TMJ inflammatory pain, which may help increase understanding on the hyperalgesia of peripheral inflammation and develop a new strategy to address inflammatory pain.

KEY WORDS: prostaglandin E_2; sodium channel 1.7; inflammatory pain; trigeminal ganglion; temporomandibular joint.

INTRODUCTION

Voltage-gated sodium channels are essential for electrogenesis in excitable cells. Tetrodotoxin-sensitive (TTX-S) voltage-gated sodium channel 1.7 ($Na_v1.7$) is highly expressed in the dorsal root ganglia (DRG), trigeminal ganglia (TG), sympathetic ganglia, and pain-sensing free nerve endings (nociceptors) close to areas where stimuli are initiated [1]. $Na_v1.7$ plays a key role in pain perception. It amplifies weak stimuli in the neurons and acts as a threshold channel for firing action potentials [2, 3]. Mutations in this gene contribute to three human pain syndromes including primary erythromelalgia [4], paroxysmal extreme pain disorder [5], and congenital inability to experience pain [6]. Accompanied by increased TTX-S current amplitude, the messenger RNA (mRNA) and protein expressions of $Na_v1.7$ are elevated in the DRG of a rat hindpaw inflammation model [7]. Nociceptor-specific knockout of $Na_v1.7$ abolishes inflammation-induced mechanical and thermal hyperalgesia [8]. Meanwhile, when

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knocking down Na$_v$1.7 in primary afferents, inflammatory hyperalgesia was prevented [9]. Recently, our group also showed that Na$_v$1.7 in TG is involved in temporomandibular joint (TMJ) inflammatory pain [9]. However, the regulation of Na$_v$1.7 expression remains poorly understood, except only two studies showing that Na$_v$1.7 is potentially regulated by nerve growth factor (NGF) [10] and tumor necrosis factor-α (TNF-α) [11].

Tissue inflammation caused by infection or injury is associated with a number of abundantly increased proinflammatory mediators, including interleukin-1 (IL-1), TNF-α, NGF, serotonin (5-HT), and prostaglandins, especially prostaglandin E$_2$ (PGE$_2$) [12]. PGE$_2$ is synthesized by constitutive cyclooxygenase-1 (COX-1) and, to a greater extent, by its inducible isoform COX-2 [13]. PGE$_2$ as an inflammatory mediator sensitizes peripheral nociceptors through receptors for E prostanoid (EP) with designated subtypes EP1, EP2, EP3, and EP4 [14]. Among these subtypes, EP2 plays a key role in spinal inflammatory hyperalgesia [15]. Nonsteroidal anti-inflammatory drugs (NSAIDs), the most commonly used analgesics, reduce the production of prostanoids, mainly PGE$_2$, by inhibiting COX-1 and, mainly, COX-2 action, to suppress inflammation and inflammatory pain [16]. PGE$_2$ can increase both tetrodotoxin-resistant (TTX-R) (including increasing Na$_v$1.9 sodium current and promoting Na$_v$1.8 trafficking in the DRG) and TTX-S sodium currents [17–19]. However, it is not clear whether PGE$_2$ could modulate Na$_v$1.7. So we hypothesized that PGE$_2$ could upregulate Na$_v$1.7 expression to contribute to inflammatory pain.

In this study, we tested the hypothesis and demonstrated evidence that PGE$_2$ is an important regulator of Na$_v$1.7 in TMJ inflammatory pain.

METHODS

Animals

Adult male Sprague-Dawley rats (230–250 g, Vital River Experimental Animal Technique Company, Beijing, China) were housed in a pathogen-free area with *ad libitum* access to water and food and under a 12-h light/dark cycle. The experimental protocols utilized in the study were approved by the Animal Use and Care Committee of Peking University. The employed procedures were also consistent with the Ethical Guidelines of the International Association for the Study of Pain.

TG Explant Culture

After the rats were decapitated, the TGs were dissected. After rinsing with Hank's balanced saline twice, the TGs were incubated in 2 mL of Dulbecco's modified Eagle's medium (DMEM, Gibco, Invitrogen, USA) provided with 10% heat-inactivated fetal bovine serum (FBS) and penicillin/streptomycin (1:100) in the presence of the following treatments: PGE$_2$ (10^{-6}–10^{-4} M, Sigma, USA), PF-04418948 (10 μM, Sigma, USA), or both PGE$_2$ and PF-04418948 for 24 h. The samples were incubated in a humid incubator at 37 °C with 5% CO$_2$ and 95% air.

Induction of TMJ Inflammation

Under anesthesia with 1% sodium pentobarbital (40 mg/kg, i.p.), rats received 50 μL injections of complete Freund's adjuvant (CFA; Sigma, USA) (1:1 oil/saline emulsion) into each of the TMJs to induce bilateral TMJ inflammation for 24 h as described in the previous studies [20–22]. Rats in the control group received 50 μL injections of sterile saline into each of the TMJs.

Behavioral Testing

Behavioral testing was performed by blinded observers. Head withdrawal threshold was measured as an indicator for hyperalgesia of the facial region or TMJ inflammation as previous studies [20–22]. Head withdrawal threshold was measured immediately before and 24 h after administration of COX-2 inhibitor and induction of TMJ inflammation, respectively. The electronic von Frey filament (IITC Life Science, Woodland Hills, CA, USA) was applied with the gradual increasing forces to the skin of the TMJ region of each rat until the head withdrew. The applied force was then automatically recorded. Head withdrawal threshold was calculated on the basis of at least five measurements per joint and showed as mean ± standard deviation (SD).

Administration of COX-2 Inhibitor

Meloxicam, a selective COX-2 inhibitor, is 13-fold more active against COX-2 than against its isoform COX-1 [23]. Rats were randomized to the control or inflammation group. Meloxicam (Yangtze River Pharmaceutical Group, Jiangsu, China) was suspended in 0.5% methyl cellulose and was administered to rats by oral gavage at 10 mg/kg 30 min before the induction of TMJ inflammation as modified from a previous study [24]. Control animals were given 0.5% methyl cellulose alone by oral gavage.

Real-Time Quantitative PCR

Total RNA was isolated with TRIzol reagent (Invitrogen, USA) following the manufacturer's instructions. Reverse transcription was performed as described in detail previously [21]. The primers were customized according to the sequence from previous reports [22, 25] as follows: for rats' $Na_v1.7$, sense 5′-TCG TAC CCC ATA GAC CCC G-3′, anti-sense 5′-CTG ATT AGT CGT GCC GCT G-3′; for rats' COX-2, sense 5′-TAC AAG CAG TGG CAA AGG CC-3′, anti-sense 5′-CAG TAT TGA GGA GAA CAG ATG GG-3′; and for rats' β-actin, sense 5′-TGA CAG GAT GCA GAA GGA GA-3′, anti-sense 5′-TAG AGC CAC CAA TCC ACA CA-3′.

Western Blot Analysis

TG explants were homogenized by a homogenizer (Tissue Lyser II, Qiagen, Germany) in RIPA buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 mg/mL aprotinin, and 1 mg/mL leupeptin) containing protease inhibitor cocktail (Sigma, USA). The supernatant was collected, and the protein concentrations were determined using the bicinchoninic acid assay (Pierce, USA). Samples with equal amounts of protein (50 μg) were loaded and then separated by 6–10% gradient sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to the nitrocellulose membrane (Millipore, USA). The membranes were incubated with 5% nonfat milk and then incubated with anti-$Na_v1.7$ antibody (1:1000, 20257-1-AP, Proteintech, USA) or anti-COX-2 antibody (1:1000, 12282s, Cell Signaling Technology, USA) and anti-β-actin antibody (1:1000, sc-1616-R, Santa Cruz, USA) overnight at 4 °C. After washing extensively with TBS-T (50 mmol/L Tris–HCl, pH 7.5, 150 mmol/L NaCl, and 0.05% Tween 20), the membrane was incubated with horseradish peroxidase-conjugated secondary antibodies (1:10,000, ZB2301, ZSGB-BIO, China) for 1 h at room temperature. After extensive washing with TBS-T, the membranes were visualized using the eECL kit (CW0049, Cwbiotech, China) and Fusion FX5 imaging system (Vilber Lourmat, Marne-la-Vallée, France).

Tissue PGE₂ Determination

PGE_2 in the TG was assayed using enzyme immunoassay as reported previously [26]. Animals were anesthetized with an overdose of pentobarbital sodium (50 mg/kg, i.p.) and decapitated. The TGs were then excised within 30–60 s after decapitation, weighed, and flash frozen in liquid nitrogen for storage at −80 °C. The TGs were homogenized by a homogenizer (Tissue Lyser II, Qiagen, Germany) in ice-cold lysis buffer (0.1 M phosphate, pH 7.4, 1 mM EDTA, and 10 μM indomethacin). Then the samples were centrifuged at $12,000 \times g$ for 30 min, after which, the supernatants were collected. PGE_2 in the sample was measured in triplicate with a PGE_2 enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI, USA) in accordance with the manufacturer's instructions. The kit exhibits little cross-reactivity between structurally related PGE_3 and PGE_1.

Statistical Analysis

Experimental data were analyzed with SPSS 17 for Windows (SPSS Inc., Chicago, IL, USA). All data were expressed as mean ± SD. Differences between two groups were examined using an independent samples t test, whereas differences between groups were examined by one-way analysis of variance. P value <0.05 was considered statistically significant.

RESULTS

PGE₂ Upregulated Na_v1.7 Expression in TG Explants

We first tested whether PGE_2 could upregulate $Na_v1.7$ expression in cultured TG explants. As shown in Fig. 1, both the mRNA and protein expressions of $Na_v1.7$ in TG explants were upregulated in dose- and time-dependent manners by PGE_2 treatment.

PGE₂-Induced Upregulation of Na_v1.7 Expression Was Dependent on Its Receptor EP2 in TG Explants

The PGE_2 receptor EP2 was shown to mediate inflammatory pain [15]. We also treated TG explants with PF-04418948, a selective antagonist for EP2 [27]. As shown in Fig. 2a, b, treatment with PF-04418948 totally blocked PGE_2-induced upregulation of $Na_v1.7$ mRNA and protein expressions.

Trigeminal Ganglionic Na_v1.7 and COX-2 Expressions, As Well As PGE₂ Levels, Were Concurrently Increased with Hyperalgesia After Induction of TMJ Inflammation

We previously showed that the trigeminal ganglionic $Na_v1.7$ is upregulated by induction of TMJ inflammation [25]. However, it remains unknown whether TMJ
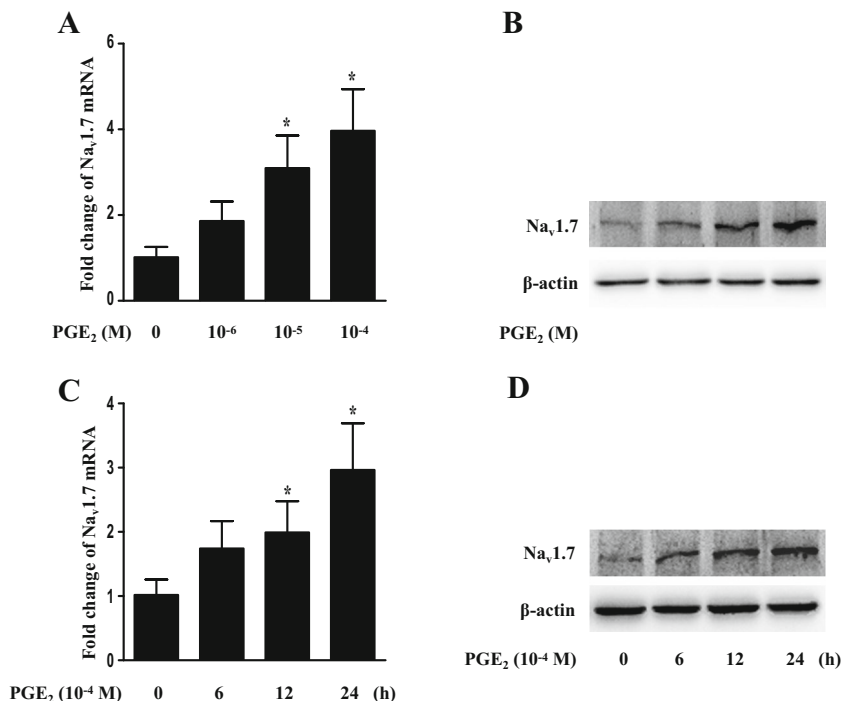
Fig. 1. Upregulation of Na$_v$1.7 expression in TG explants after treatment with PGE$_2$. **a** Dose course of Na$_v$1.7 mRNA expression in TG explants after PGE$_2$ treatment for 24 h. One-way ANOVA, *$P < 0.05$ *versus* control group, $n = 3$. **b** Dose course of Na$_v$1.7 protein expression in TG explants after PGE$_2$ treatment for 24 h. **c** Time course of Na$_v$1.7 mRNA expression in TG explants after treatment with PGE$_2$. One-way ANOVA, *$P < 0.05$ *versus* control group, $n = 3$. **d** Time course of Na$_v$1.7 protein expression in TG explants after treatment with PGE$_2$.

inflammation-induced upregulation of Na$_v$1.7 could be dependent on COX-2/PGE$_2$ signal pathway in TG. We first examined whether TMJ inflammation could concurrently upregulate trigeminal ganglionic Na$_v$1.7 and COX-2 expressions. As shown in Fig. 3a, b, the mRNA and protein expressions of both Na$_v$1.7 and COX-2 were significantly upregulated after induction of TMJ inflammation for 24 h compared with that in the control group ($P < 0.05$).

Correspondingly, the PGE$_2$ levels in the TG were also significantly upregulated after induction of TMJ inflammation for 24 h compared with those of the control group (180.4 ± 31.4 pg/mg tissue *versus* 58.9 ± 26.9 pg/mg tissue; $P < 0.05$) (Fig. 3c). Conversely, the head withdrawal threshold significantly decreased after induction of TMJ inflammation for 24 h (Fig. 3d), suggesting that hyperalgesia ensued after TMJ inflammation.
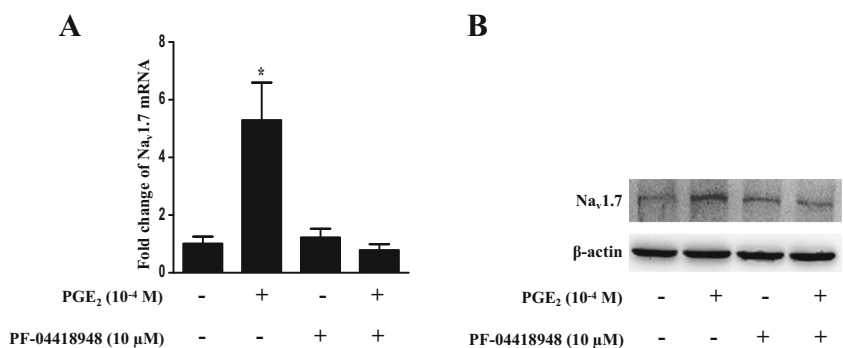

Fig. 2. PGE$_2$-induced upregulation of Na$_v$1.7 expression was dependent on its receptor EP2 in TG explants. **a** PGE$_2$ receptor subtype EP2-selective antagonist PF-04418948 blocked the upregulation of Na$_v$1.7 mRNA expression in TG explants after PGE$_2$ treatment for 24 h. **b** PF-04418948 blocked the upregulation of Na$_v$1.7 protein expression in TG explants after PGE$_2$ treatment for 24 h. One-way ANOVA, *$P < 0.05$ *versus* control group, $n = 3$.
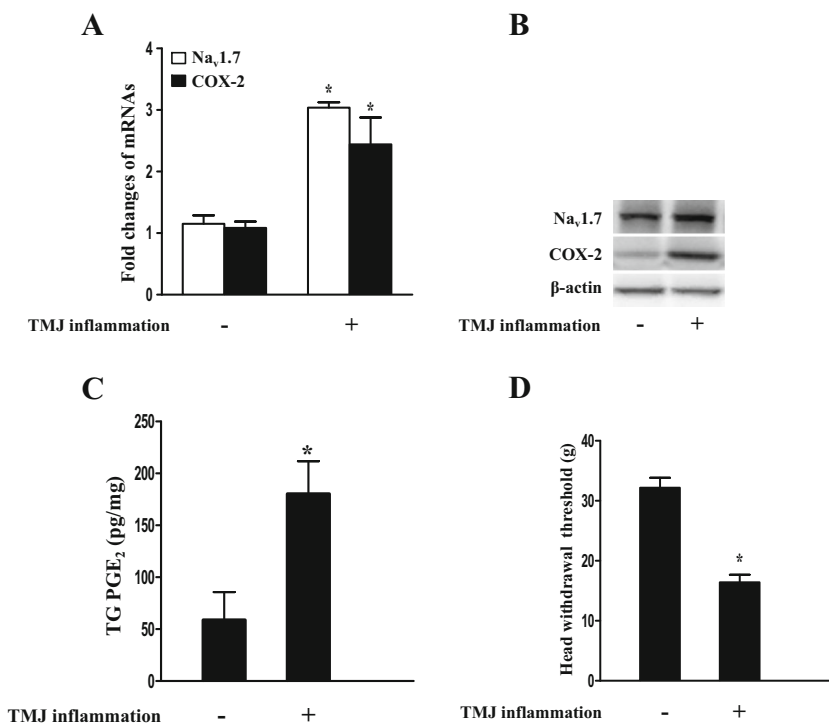
Fig. 3. Induction of $Na_v1.7$ and COX-2 expressions, as well as PGE_2 level increase and hyperalgesia, by TMJ inflammation. **a** Upregulation of the mRNA expression of both $Na_v1.7$ and COX-2 in TG after induction of TMJ inflammation for 24 h. Independent Student's t test, two-tailed, $*P < 0.05$ *versus* control group, $n = 3$. **b** Upregulation of protein expression of both $Na_v1.7$ and COX-2 in TG after induction of TMJ inflammation for 24 h. **c** Increase in PGE_2 level in the TG after induction of TMJ inflammation for 24 h. Independent Student's t test, two-tailed, $*P < 0.05$ *versus* control group, $n = 3$. **d** Decrease in head withdrawal threshold after induction of TMJ inflammation for 24 h. Independent Student's t test, two-tailed, $*P < 0.05$ *versus* control group, $n = 5$.

Pretreatment with COX-2-Selective Inhibitor Blocked TMJ Inflammation-Induced Upregulation of $Na_v1.7$ and PGE_2 in TG and Decreased the Hyperalgesia of Inflamed TMJ

To examine whether the COX-2/PGE_2 signal pathway was involved in TMJ inflammation-induced upregulation of $Na_v1.7$ in TG, we then pretreated rats with meloxicam by oral gavage before the induction of TMJ inflammation. As shown in Fig. 4a, b, pretreatment with meloxicam totally blocked TMJ inflammation-induced upregulation of mRNA and protein expressions of $Na_v1.7$, but not COX-2, in the TG. Pretreatment with meloxicam also totally blocked the rise in PGE_2 levels induced by TMJ inflammation in the TGs (Fig. 4c) and also partially blocked the decrease in head withdrawal threshold ($P < 0.05$) (Fig. 4d).

DISCUSSION

In this study, we showed that PGE_2 could upregulate trigeminal ganglionic $Na_v1.7$ expression in the explant culture and that involved in TMJ inflammatory pain. First, we showed that PGE_2 upregulated $Na_v1.7$ expression in TG explants. This upregulation could be blocked by the PGE_2 receptor EP2-selective antagonist. Second, TMJ inflammation-induced upregulation of $Na_v1.7$ expression and PGE_2 levels were confirmed to be dependent on activity of COX-2, which is the key synthase of PGE_2. For the first time, these results revealed that PGE_2 is a key regulator for the trigeminal ganglionic $Na_v1.7$ involved in TMJ inflammatory pain. These observations might help us further understand the mechanisms underlying inflammatory pain and the regulation of trigeminal ganglionic $Na_v1.7$ expression in inflammatory pain.

The involvement of trigeminal ganglionic $Na_v1.7$ in TMJ inflammatory pain was regulated by COX-2/PGE_2 signaling. PGE_2 has been known as an important proinflammatory pain mediator. Many studies showed that PGE_2 can regulate the excitability of murine DRG neurons [17, 18, 28] or sensitize the peripheral terminals of sensory fibers [29, 30]. However, the detailed targets of PGE_2 remain to be fully understood. In the present study, we showed that PGE_2 could upregulate trigeminal ganglionic
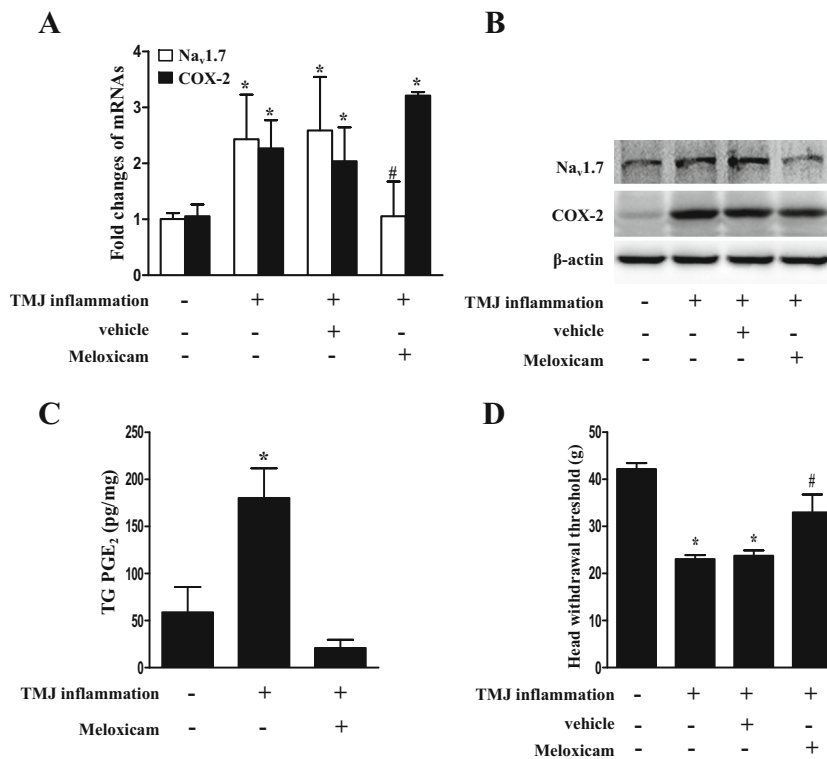
Fig. 4. Blocking of the upregulation of trigeminal ganglionic Na$_v$1.7 expression induced by TMJ inflammation, as well as the PGE$_2$ increase and hyperalgesia, by COX-2-selective inhibitor meloxicam. **a** Pretreatment with meloxicam abolished the upregulation of the mRNA expression of Na$_v$1.7, but not COX-2, in TG after induction of TMJ inflammation for 24 h. One-way ANOVA, *$P < 0.05$ *versus* control group, $n = 3$. **b** Pretreatment with meloxicam abolished the upregulation of protein expression of Na$_v$1.7, but not COX-2, in TG after induction of TMJ inflammation for 24 h. **c** Pretreatment with meloxicam abolished the increase in PGE$_2$ levels in TG after induction of TMJ inflammation for 24 h. One-way ANOVA: *$P < 0.05$ *versus* control group, $n = 3$. **d** Pretreatment with meloxicam partially reversed the decrease in head withdrawal threshold after induction of TMJ inflammation for 24 h. One-way ANOVA, *$P < 0.05$ *versus* control group, $n = 5$.

Na$_v$1.7 expression. More importantly, we observed that TMJ inflammation-induced upregulation of Na$_v$1.7 expression and PGE$_2$ levels in TG and the hyperalgesia were dependent on COX-2, which is the key synthase of PGE$_2$. The biophysical property of Na$_v$1.7 is to amplify weak stimuli and act as a threshold channel for firing action potentials in neurons [3]. Moreover, upregulation of Na$_v$1.7 expression accompanies the increase in TTX-S current amplitude in the neurons [7]. Hence, our results suggest that targeting trigeminal ganglionic Na$_v$1.7 by PGE$_2$ could be an important cause for hyperalgesia in inflamed TMJ, especially in the early period of inflammation (within 24 h). This targeting may also underlie the function of PGE$_2$ as a proinflammatory pain mediator. Furthermore, PGE2 production may also, to some extent, explain why COX-2 knockout mice failed to develop thermal hyperalgesia and mechanical allodynia of the inflamed tibiotarsal joint after induction of arthritis by CFA in a

previous study [31]. The COX-2 downstream production of PGE2 in the DRG of the COX-2 knockout mice can be reasonably believed to not be induced by the tibiotarsal arthritis. Consequently, Na$_v$1.7 expression may not have increased in the DRG, and hence, hyperalgesia did not develop. Na$_v$1.7 (coded by SCN9A) is a unique pain-related gene, in which loss-of-function mutations result in a congenital inability to experience pain. Our findings would expand our knowledge on PGE$_2$ targets and the Na$_v$1.7 regulatory mechanism. Na$_v$1.7 could be an important target for anti-inflammatory pain. However, further studies are needed to test whether PGE$_2$ could upregulate Na$_v$1.7 expression *in vivo*.

The PGE$_2$-induced upregulation of Na$_v$1.7 expression was dependent on EP2 receptor. PGE$_2$ performs its function through acting on a group of G protein-coupled receptors designated as EP1 to EP4 [14]. EP2 was shown to play an important role in PGE$_2$-mediated spinal inflammatory

hyperalgesia [15]. Our results also demonstrated that the regulation of trigeminal ganglionic $Na_v1.7$ expression by PGE_2 could be mediated through the EP2 subtype, since EP2-selective antagonist PF-04418948 totally blocked the effects of PGE_2 on $Na_v1.7$ expression. However, we did not test whether the specific antagonists of the other PGE_2 receptor subtypes could also block PGE_2 effects on trigeminal ganglionic $Na_v1.7$ expression. Hence, we might not have fully excluded the involvement of the other PGE_2 receptor subtypes in the effect of PGE_2 on trigeminal ganglionic $Na_v1.7$ expression. For example, the administration of both an EP4 antagonist (AH23848) and EP4 knockdown through intrathecally used short hairpin RNA decreases inflammation-induced thermal and mechanical hyperalgesia [32]. AH23848 also decreases the PGE_2-mediated sensitization of capsaicin-evoked currents in DRG neurons *in vitro*, suggesting that EP4 plays an important role in inflammatory pain [32]. PGE_2 failed to induce mechanical allodynia in $EP1^{(-/-)}$ mice [33]. Meanwhile, the acid-induced writhing response in $EP3^{(-/-)}$ mice pretreated with lipopolysaccharide exhibited a significantly less enhanced number of writhings [34]. These results indicate that both EP1 and EP3 play significant roles in inflammatory nociception. Additional studies are needed to elucidate the signaling pathway downstream of EP2 that is involved in the PGE_2-induced upregulation of $Na_v1.7$ expression. Meanwhile, the cis-element in the promoter of $Na_v1.7$ responding to PGE_2 must also be investigated.

Upregulation of $Na_v1.7$ expression by PGE_2 might contribute to PGE_2-induced TTX-S sodium currents. Previous studies showed that PGE_2 can increase both TTX-R and TTX-S sodium currents [17–19]. Our results showed that PGE_2 could upregulate trigeminal ganglionic $Na_v1.7$ expression, by which the increased TTX-S current amplitude is usually accompanied [7], contributing to inflammatory pain. Therefore, PGE_2-induced TTX-S sodium currents could possibly be in part mediated by the upregulation of $Na_v1.7$ expression. Nevertheless, future studies are certainly needed to confirm whether PGE_2-induced $Na_v1.7$ expression contributes to PGE_2-induced TTX-S sodium currents.

In conclusion, our results showed that PGE_2 could upregulate trigeminal ganglionic $Na_v1.7$ expression through its receptor EP2; the TMJ inflammation-induced upregulation of trigeminal ganglionic $Na_v1.7$ was dependent on the COX-2/PGE_2 signal pathway in TG and therefore contributed to TMJ inflammatory pain. Our results may help increase understanding on the hyperalgesia of peripheral inflammation and develop a new strategy to address inflammatory pain.

COMPLIANCE WITH ETHICAL STANDARDS

The experimental protocols utilized in the study were approved by the Animal Use and Care Committee of Peking University. The employed procedures were also consistent with the Ethical Guidelines of the International Association for the Study of Pain.

Conflict of Interest. The authors declare that they have no competing financial interests.

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