

# Non-steroidal Anti-inflammatory Drugs Attenuate Hyperalgesia and Block Upregulation of Trigeminal Ganglionic Sodium Channel 1.7 after Induction of Temporomandibular Joint Inflammation in Rats

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**Objective:** To investigate the association between the analgesic effect of non-steroidal anti-inflammatory drugs (NSAIDs) and sodium channel 1.7 (Nav1.7) expression in the trigeminal ganglion (TG).

**Methods:** Temporomandibular joint (TMJ) inflammation was induced by complete Freund's adjuvant (CFA) in female rats. Ibuprofen, diclofenac sodium and meloxicam were given intragastrically before induction of TMJ inflammation. Histopathological evaluation and scoring of TMJ inflammation was used to evaluate the level of inflammation. The head withdrawal threshold and food intake were measured to evaluate TMJ nociceptive responses. The mRNA and protein expression of trigeminal ganglionic Nav1.7 was examined using real-time polymerase chain reaction and western blot.

**Results:** Twenty-four hours after the injection of CFA into the TMJs, NSAIDs attenuated hyperalgesia of inflamed TMJ and simultaneously blocked inflammation-induced upregulation of Nav1.7 mRNA and protein expression in the TG. However, ibuprofen and diclofenac sodium slightly attenuated TMJ inflammation and meloxicam did not affect TMJ inflammation.

**Conclusion:** Attenuation of hyperalgesia of inflamed TMJ by NSAIDs might be associated with their role in blocking upregulation of trigeminal ganglionic Nav1.7.

**Key words:** hyperalgesia, Nav1.7, NSAIDs, temporomandibular joint, trigeminal ganglion  
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Temporomandibular disorders (TMD) are characterised as pain in the temporomandibular joint (TMJ) or masticatory muscles or both, and joint inflammation

is thought to be a major cause of pain in patients with TMD<sup>1-3</sup>. The trigeminal ganglion (TG), as the location of the primary afferent neuron cell bodies for sensing and relaying the nociceptive sensations of the TMJ, has an important function in regulating TMJ nociception. TMJ inflammation increases the excitability of neurons in the TG<sup>4,5</sup> and also affects the expression of some pain-related genes in the TG<sup>6</sup>.

Sodium channel 1.7 (Nav1.7) has a prominent function in pain; its single disruption leads to a complete loss of pain<sup>7</sup>. Nav1.7 also plays an important role in inflammatory pain<sup>8,9</sup>. The increased tetrodotoxin-sensitive current amplitude following the inflammation of the hind paw is paralleled by an increase in Nav1.7 mRNA and protein in dorsal root ganglion<sup>10</sup>. Nociceptor-specific knockout of Nav1.7 abrogates inflammation-induced mechanical and thermal hyperalgesia<sup>8</sup>; the knock-down of Nav1.7 in primary afferents

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prevents inflammatory hyperalgesia<sup>9</sup>. Nav1.7 amplifies weak stimuli in the neurons and acts as the threshold channel for firing action potentials<sup>11,12</sup>. In particular, we have shown that complete Freund's adjuvant (CFA)-induced TMJ inflammation results in an increase in TG Nav1.7 in the neurons innervating the TMJ, and that inflammation-induced TMJ hyperalgesia is attenuated after blocking Nav1.7 function<sup>13</sup>.

It is widely known that the anti-inflammatory and analgesic effects of non-steroidal antiinflammatory drugs (NSAIDs) are mainly due to their inhibition of cyclooxygenase-1 and -2 (COX-1 and COX-2)<sup>14</sup>. COX-2 is a key enzyme involved in the synthesis of prostaglandins, amongst which prostaglandin E2 (PGE2) can particularly contribute to joint pain<sup>15</sup>. Considering that Nav1.7 plays an important role in inflammatory pain, we asked the question whether the analgesic effect of NSAIDs on inflamed TMJ was associated with their effect on trigeminal ganglionic Nav1.7 expression.

In this research, three kinds of NSAIDs were used to investigate the association between the analgesic effect of NSAIDs and Nav1.7 expression in the TG; ibuprofen, meloxicam and diclofenac sodium.

## Materials and methods

### Animals

Adult female Sprague-Dawley rats (200 to 220 g) were used in this study. The experimental protocols were approved by the Animal Use and Care Committee of Peking University and were consistent with the Ethical Guidelines of the International Association for the Study of Pain. The rats were housed under controlled temperature ( $22^{\circ}\text{C} \pm 1^{\circ}\text{C}$ ) on a 12 h light/dark cycle and had access to food and water. To examine the effects of NSAIDs on hyperalgesia in rats with TMJ inflammation, rats were divided into five groups (six rats per group): control, vehicle (TMJ inflammation without treatment with NSAIDs), ibuprofen, diclofenac sodium and meloxicam groups.

### NSAIDs administration

Tablets of ibuprofen (Smith, Kline & French, Tianjin, China), diclofenac sodium (Novartis Pharmaceuticals, Shanghai, China) and meloxicam (Yangtze River Pharmaceutical Group, Taizhou, China) were ground with a mortar into powder and dissolved with saline solution into suspension liquid for further use. The groups with NSAIDs were intragastrically given 1.5 ml suspensions

of ibuprofen (200 mg/kg body weight)<sup>16</sup>, diclofenac sodium (80 mg/kg body weight)<sup>17</sup> and meloxicam (3 mg/kg body weight)<sup>18</sup>, respectively, 24 h and 0.5 h before induction of TMJ inflammation, while the control and vehicle groups were intragastrically given 1.5 ml saline; the outline of the experimental design is presented in Fig 1a. The baseline of food intake was measured for 2 h at 28 h before induction of TMJ inflammation, while the baseline of the head withdrawal threshold was measured at 26 h before induction of TMJ inflammation. The influence of NSAIDs on the baseline of food intake was measured for 2 h at 4 h before induction of TMJ inflammation, while the influence of NSAIDs on the baseline of the head withdrawal threshold was measured at 2 h before induction of TMJ inflammation. Food intake was also measured at 20 h after induction of TMJ inflammation, while the head withdrawal threshold was measured at 22 h after induction of TMJ inflammation. The rats were sacrificed for TG dissection and RNA isolation at 24 h after induction of TMJ inflammation. Investigators were blinded to rat treatments.

### Induction of TMJ inflammation

All the groups were induced by TMJ inflammation with CFA, except the control group. Rats were anaesthetised with 1% pentobarbital sodium administered intraperitoneally, and were injected with 50  $\mu\text{l}$  of CFA (oil/saline at the ratio of 1:1, 0.025 mg Mycobacterium tuberculosis, Sigma, Missouri, USA) into the bilateral TMJs to induce TMJ inflammation as described in detail in our previous studies<sup>19,20</sup>; the control rats were injected with vehicle (50  $\mu\text{l}$  of saline).

### Measurement of the head withdrawal threshold

The head withdrawal threshold is usually measured to reflect the TMJ inflammation/pain of animals<sup>21</sup>. The head withdrawal threshold was measured from four rats in each group as described in detail previously<sup>20,21</sup>. Briefly, the von Frey filament (IITC Life Science, California, USA) was applied with progressive, increasing forces to the TMJ region until the head of a rat was withdrawn and the applied force was automatically recorded. The head withdrawal threshold was calculated as mean  $\pm$  standard deviation (SD), based on at least five measurements per joint. Investigators were blinded to rat treatment.

### Measurement of food intake

Food intake is negatively associated with TMJ inflammation/pain and is used as an indicator for TMJ inflam-

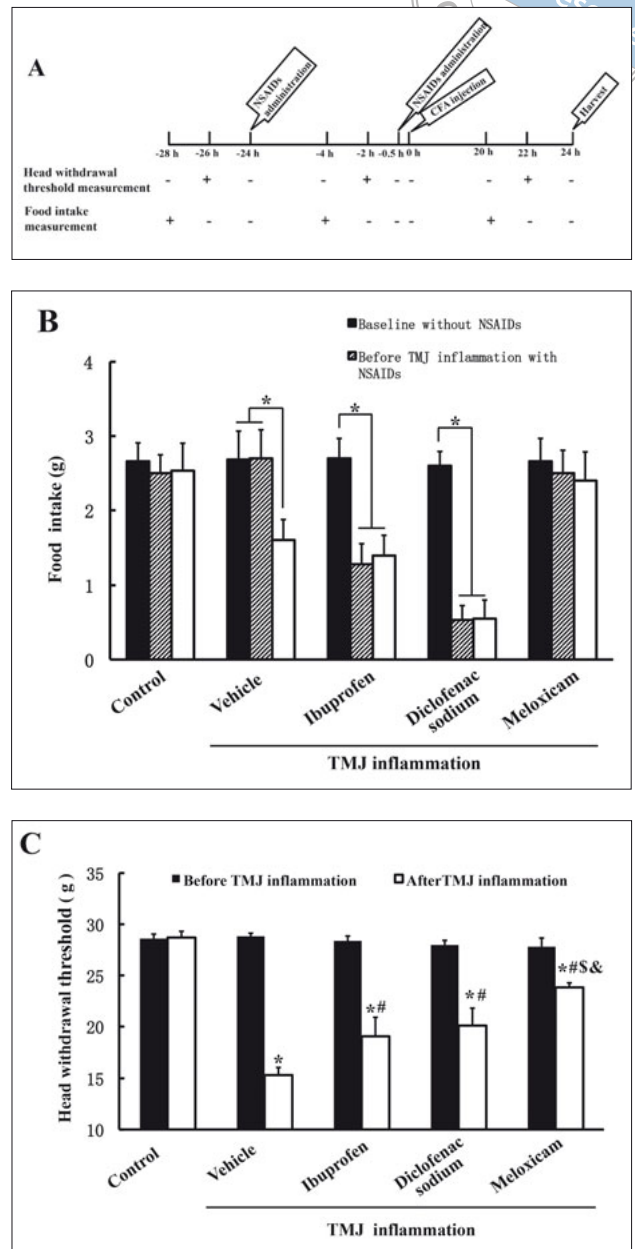
mation/pain<sup>20,22,23</sup>. Food intake was measured from four rats in each group as described in detail previously<sup>20</sup>. Briefly, each rat was kept in one cage supplied without food but with water for 15 h. The rat was then fed with food but without water for 2 h and the amount of food eaten by the rat during the 2 h period was recorded as food intake.

### Real-time quantitative Polymerase Chain Reaction

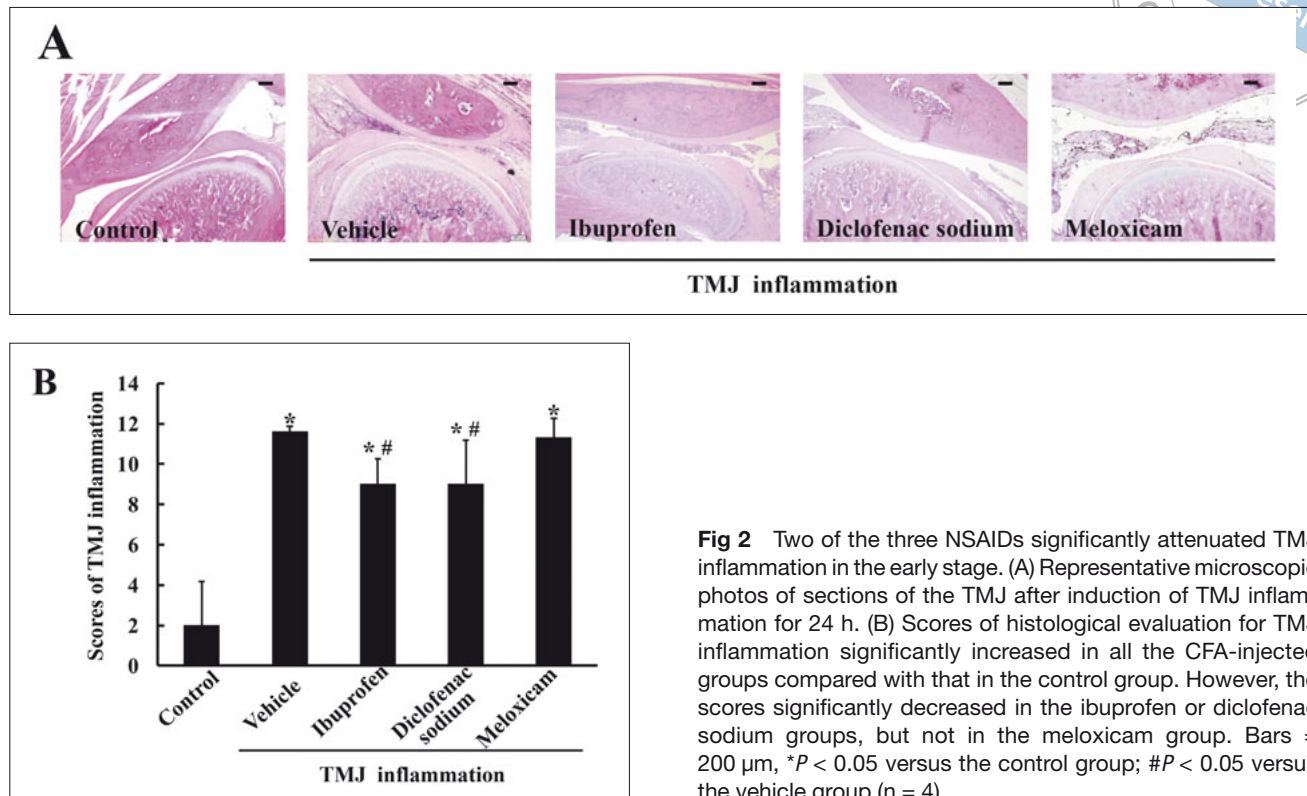
The TGs were dissected from four rats per group for total RNA isolation using TRIzol reagent (Invitrogen, California, USA). Reverse transcription and real-time Polymerase Chain Reaction (PCR) were performed, as described in detail previously<sup>20</sup>. Briefly, Reverse transcription was conducted with an iScript cDNA synthesis kit (Bio-Rad Laboratories, California, USA) in 20 µl reaction volume containing 1 µg of total RNA incubated at 25°C for 5 min, transcribed at 42°C for 30 min, and terminated by heating at 85°C for 5 min. The synthesised cDNA was stored at -20°C until use. Real-time PCR for Nav1.7 was performed with Power SYBR Green PCR Master Mix (Applied Biosystems, California, USA) using the 7500 Real-Time PCR System (Applied Biosystems). The reactions were run in duplicate with 1 µl of cDNA template in a 20 µl reaction volume; the program operated at 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 94°C for 15 s and 60°C for 1 min. The mRNA level of Nav1.7 was acquired from the value of the threshold cycle (Ct), as a relative level to that of β-actin through the formula  $2^{\Delta\Delta C_t}$  ( $\Delta C_t = \beta\text{-actin } C_t - \text{Nav1.7 } C_t$ ). Primers synthesised according to the sequence in previous reports<sup>13,19,20</sup> were as follows: rat β-actin sense/antisense, 5'-TGA CAG GAT GCA GAA GGA GA-3'/5'-TAG AGC CAC CAA TCC ACA CA-3', rat Nav1.7 sense/antisense and 5'-TCG TAC CCC ATA GAC CCC G -3'/ 5'-CTG ATT AGT CGT GCC GCT G -3'.

### Western blot analysis

The TGs were dissected from two rats per group and then homogenised by a homogeniser (Ultra-Turrax T10, IKA Laboratory Technology) in an ice-cold denaturing lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 1 µg/ml aprotinin, 1 µg/ml leupeptin) and centrifuged at 12,000 g for 30 min at 4°C. The supernatant was collected, and protein concentrations were determined via BCA assay (Pierce). Protein samples were subjected to the upper 6% and lower 10% SDS-PAGE and transferred to the PVDF membrane



**Fig 1** NSAIDs attenuated hyperalgesia of inflamed TMJ. (A) Outline of experimental design. (B) Food intake before and after TMJ inflammation. After TMJ inflammation, food intake decreased in the vehicle group, but not in the meloxicam group. Note that food intake even decreased in the ibuprofen and diclofenac sodium groups before TMJ inflammation, and did not decrease further after induction of TMJ inflammation. \* $P < 0.05$  ( $n = 6$ ). (C) The head withdrawal threshold before and after TMJ inflammation. After induction of TMJ inflammation, treatment with ibuprofen or diclofenac sodium or meloxicam could all partially block TMJ inflammation-induced decrease in the head withdrawal threshold, with meloxicam more effective than the other two drugs. \* $P < 0.05$  versus all groups before TMJ inflammation; # $P < 0.05$  versus the vehicle group after TMJ inflammation; \$ $P < 0.05$  versus the ibuprofen group after TMJ inflammation; & $P < 0.05$  versus the diclofenac sodium group after TMJ inflammation ( $n = 4$ ).



**Fig 2** Two of the three NSAIDs significantly attenuated TMJ inflammation in the early stage. (A) Representative microscopic photos of sections of the TMJ after induction of TMJ inflammation for 24 h. (B) Scores of histological evaluation for TMJ inflammation significantly increased in all the CFA-injected groups compared with that in the control group. However, the scores significantly decreased in the ibuprofen or diclofenac sodium groups, but not in the meloxicam group. Bars = 200  $\mu$ m, \* $P$  < 0.05 versus the control group; # $P$  < 0.05 versus the vehicle group (n = 4).

(Millipore, Massachusetts, USA). The membrane was blocked with 5% bovine serum albumin (BSA, Sigma, Missouri, USA) in TBS-T buffer (50 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 0.05% Tween 20) for 1 h and incubated with anti-Nav1.7 antibody (1:500, 20257-1-AP, Proteintech, Illinois, USA) and anti- $\beta$ -actin antibody (1:1000, sc-1616-R, California, USA) overnight at 4°C. After washing extensively with TBS-T, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. The membrane was visualised using the ECL kit (NC14109, Thermo, Illinois, USA) and Fusion system for Western blot and gel imaging (Fx, Vilber Lourmat, Marne-la-Vallée, France).

*Histopathological evaluation and scoring of TMJ inflammation*

TMJ inflammation was histopathologically evaluated and scored, as described in detail previously<sup>19</sup>. Briefly, two senior oral pathologists, who were blinded to information regarding the animal groups, evaluated and scored TMJ inflammation. The scoring of TMJ inflammation was performed according to the following histopathological criteria: 1) proliferation or erosion of the synovial lining,

scored on a scale of 0 to 3, where 0 =  $\leq$  3 layers of cells, 1 = 4 to 6 layers, 2 =  $\geq$  7 layers and 3 = erosion of the synovial layers or disorganised and broken synovial layers; 2) dilated vasculatures and tissue oedema, scored on a scale of 0 to 3, where 0 = absent, 1 = less than one-third of the synovial membrane length involved, 2 = one-third to two-thirds of the synovial membrane length involved, and 3 = more than two-thirds of the synovial membrane length involved; 3) fibrin-like exudate in the articular space, scored on a scale of 0 to 2, where 0 = absent, 1 = few and scattered and 2 = marked and cord-like; and 4) infiltration of mononuclear cells, scored on a scale of 0 to 4, where 0 = absent, 1 = mild infiltration in the sublining layer, 2 = moderate infiltration in the sublining layer and articular space, 3 = severe infiltration in the articular space and 4 = marked cellular infiltration full of the articular space. Higher scores represented more severe inflammation. For any group, the total scores were averaged by the number of examined joints and were presented as the mean  $\pm$  standard deviation (SD).

*Statistical analysis*

Statistical analysis was performed with SPSS 13 for Windows. All data were presented as mean  $\pm$  SD. Dif-

ferences between groups were examined by one-way analysis of variance (ANOVA). A value of  $P < 0.05$  was considered to be statistically significant.

## Results

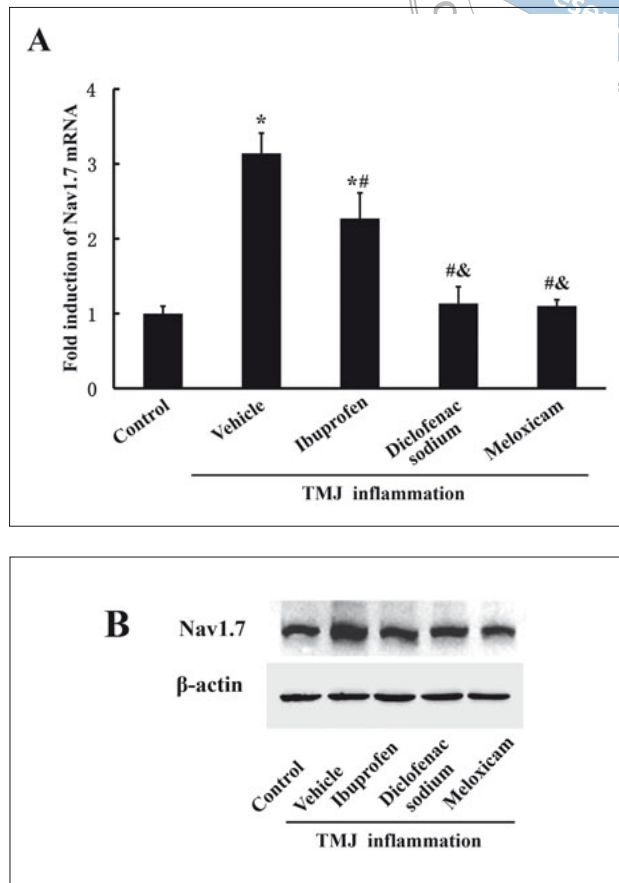
### NSAIDs attenuated hyperalgesia of inflamed TMJ

To examine whether NSAIDs could attenuate hyperalgesia of inflamed TMJ, we treated rats with three typical kinds of NSAIDs. Before induction of TMJ inflammation the food intake was significantly decreased in the rats with treatment of ibuprofen and diclofenac sodium compared with their respective baseline (Fig 1B), suggesting that ibuprofen and especially diclofenac sodium affected gastric intestinal functions. After induction of TMJ inflammation, food intake was significantly decreased in the vehicle group ( $P < 0.05$ ), but not in the meloxicam, and also there were no further changes in the ibuprofen and diclofenac sodium groups (Fig 1B). However, food intake as a parameter lost its ability to reflect TMJ inflammation/pain in ibuprofen and diclofenac sodium groups, since ibuprofen and diclofenac sodium affected food intake comparably or even more severely than TMJ inflammation in the vehicle group.

Before induction of TMJ inflammation, treatment with ibuprofen or diclofenac sodium or meloxicam did not affect the baseline of the head withdrawal threshold compared with the control and vehicle group ( $P > 0.05$ ); after induction of TMJ inflammation, treatment with ibuprofen or diclofenac sodium or meloxicam could all partially block a TMJ inflammation-induced decrease in the head withdrawal threshold ( $P < 0.05$ ), where meloxicam was more effective than the other two drugs ( $P < 0.05$ , Fig 1B).

### NSAIDs showed limited attenuation of TMJ inflammation

Twenty-four hours after the injection of CFA into the TMJs, histopathological examination showed the features of synovitis, including proliferation of synovial cells, the presence of a fibrin-like exudate in the superior joint space, and dilated vasculatures and infiltrated mononuclear cells under the synovial membrane in the CFA-injected TMJs; no such features were observed in control TMJ (Fig 2A). The inflammation scores significantly increased in all the CFA-injected groups compared with that in the control group ( $P < 0.05$ ). Although the inflammation scores for the ibuprofen and diclofenac



**Fig 3** NSAIDs blocked TMJ inflammation-induced upregulation of trigeminal ganglionic Nav1.7 mRNA expression. (A) Trigeminal ganglionic Nav1.7 mRNA expression was evaluated by real-time PCR after induction of TMJ inflammation for 24 h. \* $P < 0.05$  versus the control group; # $P < 0.05$  versus the vehicle group; & $P < 0.05$  versus the ibuprofen group ( $n = 4$ ). (B) Representative immunoblotting for Nav1.7 expression in the TG. The trigeminal ganglionic Nav1.7 protein expression corresponded to its mRNA expression pattern.  $\beta$ -actin served as an internal control for equal loading.

sodium groups were lower than that in the vehicle group ( $P < 0.05$ , Fig 2B), they were still much higher than that of the control group. Moreover, the inflammation score of the meloxicam group showed no difference with that of the vehicle group ( $P > 0.05$ , Fig 2).

### NSAIDs blocked TMJ inflammation-induced upregulation of Nav1.7 in the TG

After induction of TMJ inflammation, the mRNA expression of Nav1.7 in the TG was upregulated by about 3-fold, after induction of TMJ inflammation in the vehicle group, compared with that of the control group ( $P < 0.05$ ) (Fig 3), whereas the mRNA expression of

Nav1.7 in the TG was not affected in the diclofenac sodium and meloxicam groups, compared with that in the control group ( $P > 0.05$ , Fig 3). However, after induction of TMJ inflammation, the mRNA expression of Nav1.7 in the TG was still upregulated by about 2.3-fold in the ibuprofen group, compared with that in the control group ( $P < 0.05$ ). As shown in Figure 3B, the protein expression of Nav1.7 in the TG was induced in the vehicle group by TMJ inflammation compared with that in the control group, whereas ibuprofen, diclofenac sodium and meloxicam increasingly blocked TMJ inflammation-induced protein expression of Nav1.7 in the TG.

## Discussion

In the present study, we observed that ibuprofen, diclofenac sodium and meloxicam attenuated hyperalgesia of inflamed TMJ and simultaneously blocked TMJ inflammation-induced upregulation of Nav1.7 expression in the TG. These results suggested that Nav1.7 in the TG could be targeted by NSAIDs and the blocking of Nav1.7 upregulation in the TG might contribute to the analgesic effect of NSAIDs on TMJ inflammatory pain.

Attenuation of hyperalgesia of inflamed TMJ by NSAIDs might be associated with their role in blocking upregulation of Nav1.7 in the TG. It is well known that the anti-inflammatory and analgesic effects of NSAIDs are mainly due to their inhibition of COX-1 and COX-2<sup>14</sup>. Meloxicam is a selective inhibitor of COX-2, whereas diclofenac sodium and ibuprofen are inhibitors of both COX-1 and COX-2<sup>17,18,24</sup>. Therefore, through inhibiting COX-1 and mainly COX-2, NSAIDs reduce production of prostanoids, amongst which PGE2 has long been known as an important pro-inflammatory pain mediator. In the dorsal root ganglia (DRG), PGE2 release is dependent on microglia activation and ERK1/2 phosphorylation in the glial cells, and it contributes to hyperalgesia in a rat model of spinal cord injury<sup>25</sup>. PGE2 is also a mediator contributing to the signaling between the microglia and neurons<sup>25</sup>. Our previous study also found that TMJ inflammation-induced upregulation of trigeminal ganglionic Nav1.7 is dependent on ERK1/2 phosphorylation in the satellite glial cells in the TG. In the present study, we observed that NSAIDs attenuated hyperalgesia of inflamed TMJ and simultaneously blocked TMJ inflammation-induced upregulation of Nav1.7 expression in the TG. The change of Nav1.7 expression in the TG was associated with the presence or absence of hyperalgesia after induction of TMJ inflammation. Considering that upregulation of Nav1.7 is strongly

associated with hyperalgesia<sup>9,13,26</sup>, it is reasonable to believe that blocking upregulation of Nav1.7 in the TG by NSAIDs was also associated with their attenuation of hyperalgesia of inflamed TMJ. Our results were consistent with a previous study, in which ibuprofen and COX-2 selective inhibitor, NS-398, attenuated hyperalgesia of CFA-injected paw and also simultaneously blocked upregulation of Nav1.7 in the DRG<sup>16</sup>. However, the mechanism underlying NSAIDs role in blocking TMJ inflammation-induced upregulation of Nav1.7 expression in the TG remains to be fully understood. Given that treatment with NSAIDs will definitely lead to the reduction of PGE2, we speculated that PGE2 might affect Nav1.7 expression and the Na<sup>+</sup> current of the neurons. Therefore, reduction of PGE2 by NSAIDs could be a major reason for why NSAIDs block TMJ inflammation-induced upregulation of Nav1.7 in the TG. Future studies are needed to prove these theories and to provide a detailed mechanism underlying how PGE2 upregulates Nav1.7 expression.

No association between the anti-inflammatory effects of NSAIDs and pain reduction were observed in the early stage of CFA-induced TMJ inflammation. We observed that administration of NSAIDs 24 h and 0.5 h before induction of TMJ inflammation resulted in little improvement, in terms of the inflammation severity after induction of TMJ inflammation for 24 h, since the meloxicam group showed no improvement on the inflammation score and ibuprofen and diclofenac sodium groups showed only a limited improvement on their inflammation scores. However, all three drugs attenuated the hyperalgesia of inflamed TMJ, and meloxicam was more effective than the other two drugs. This data suggests that in the early stage of CFA-induced TMJ inflammation, NSAIDs' attenuation of the hyperalgesia of inflamed TMJ was not associated with its anti-inflammatory effect, but is more likely to be associated with its effect on the nerve system or neuron activity. In a previous study, the analgesic effect of ibuprofen and COX-2 selective inhibitor, NS-398, is also not dependent on the reduction of oedema in the early stage of CFA-induced paw inflammation<sup>16</sup>. Therefore, blocking upregulation of ganglionic Nav1.7 could be one of the mechanisms underlying NSAIDs' analgesic effects in the early stage of CFA-induced inflammation. This suggestion could also be supported by data that indicated meloxicam showed a better effect on blocking upregulation of Nav1.7 in the TG and a better analgesic effect than ibuprofen, although meloxicam did not improve TMJ inflammation, while ibuprofen slightly improved TMJ inflammation. However, it was difficult to explain that meloxicam also showed a

better analgesic effect than diclofenac sodium, while they both comparably blocked upregulation of Nav1.7 mRNA and protein.

Although Nav1.3, Nav1.8 and Nav1.9 also play an important role in the development of inflammatory pain<sup>10,27,28</sup>, we only evaluated Nav1.7 expression in the TG in the present study. The reason was mainly based on the results of our previous study, in which CFA-induced TMJ inflammation in the early period mainly upregulates trigeminal ganglionic Nav1.7 mRNA, slightly upregulates Nav1.8 and Nav1.9 mRNA expression and does not affect Nav1.3 mRNA expression<sup>13</sup>. In addition, Nav1.8 and Nav1.9 are mainly involved in the late period of inflammatory pain rather than in the early period<sup>10,16,29</sup>.

Since TMD is prevalent in women of childbearing age, by at least double that in men<sup>4</sup> and given that sex hormones may influence nociceptive sensitivity<sup>30</sup>, to mimic the clinical characteristics of TMD, only adult female rats were used in the present study. However, the effect of fluctuation of oestrogen in the oestrous cycle on nociceptive behaviours and trigeminal ganglionic Nav1.7 expression was not eliminated. Future studies are needed to explore whether fluctuation of oestrogen in the oestrous cycle could affect nociceptive behaviour and Nav1.7 expression in the TG.

Food intake is an important parameter for evaluating TMJ inflammatory pain in animals<sup>20,22,23</sup>. A combination of the head withdrawal threshold and the food intake parameter may help to better evaluate the nociceptive responses after TMJ inflammation than using one measure alone. However, ibuprofen and diclofenac sodium showed severe side effects on food intake. Therefore, food intake as a parameter for TMJ inflammatory pain, lost its ability to evaluate the nociceptive responses for TMJ inflammation or pain in the animals after treatment with ibuprofen and diclofenac sodium in the present study. Nevertheless, food intake as a parameter still corresponded well with the change in the head withdrawal threshold in the meloxicam group.

In conclusion, attenuation of hyperalgesia of inflamed TMJ by NSAIDs might be associated with their role in blocking upregulation of trigeminal ganglionic Nav1.7. Future studies are needed to explore whether PGE<sub>2</sub>, which are the products of COX, could activate ganglionic Nav1.7 expression.

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### Conflicts of interest

The authors reported no conflicts of interest related to this study.

### Author contribution

Dr Ruiyun Bi for carrying out the experiments and for writing the manuscript; Dr Yun Ding and Dr Yehua Gan for the design of the research and revision of the manuscript.

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