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# Sensory neuron-TRPV4 modulates temporomandibular disorder pain via CGRP in mice

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## Abstract

Temporomandibular disorder (TMD) pain that involves inflammation and injury in the temporomandibular joint (TMJ) and/or masticatory muscle is the most common form of orofacial pain. We recently found that transient receptor potential vanilloid-4 (TRPV4) in trigeminal ganglion (TG) neurons is upregulated after TMJ inflammation, and TRPV4 co-expresses with calcitonin gene-related peptide (CGRP) in TMJ-innervating TG neurons. Here, we extended these findings to determine the specific contribution of TRPV4 in TG neurons to TMD pain, and examine whether sensory neuron-TRPV4 modulates TMD pain via CGRP. In mouse models of TMJ inflammation or masseter muscle injury, sensory neuron-*Trpv4* conditional knockout (cKO) mice displayed reduced pain. Co-expression of TRPV4 and CGRP in TMJ- or masseter muscle-innervating TG neurons was increased after TMJ inflammation and masseter muscle injury, respectively. Activation of TRPV4-expressing TG neurons triggered secretion of CGRP, which was associated with increased levels of CGRP in peri-TMJ tissues, masseter muscle, spinal trigeminal nucleus, and plasma in both models. Local injection of CGRP into the TMJ or masseter muscle evoked acute pain in naïve mice, while blockade of CGRP receptor attenuated pain in mouse models of TMD. These results suggest that TRPV4 in TG neurons contributes to TMD pain by potentiating CGRP secretion.

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TRPV4; CGRP; Pain; Temporomandibular joint; Masseter muscle

## Introduction

Temporomandibular disorders (TMD) pain refers to a family of symptoms characterized chiefly by jaw function-associated pain in the TMJ and/or masticatory muscles[56, 71, 76, 91, 92]. Mastication is fundamentally relevant for vertebrate nourishment, including humans. In cases of tissue inflammation or injury to the TMJ and masseter muscle, mastication becomes painful[9, 28, 41, 56, 91, 92, 98]. Current pharmacotherapies for TMD pain have limited efficacy and serious side effects[6, 16, 24, 34, 69, 101]. For instance, non-steroidal anti-inflammatory drugs (NSAIDs) effective for acute inflammation cause gastrointestinal morbidity with long term use. Muscle relaxants are frequently used for acute pain of TMD but have not been proven efficacious in chronic conditions. Repeated injections of corticosteroids can lead to chondrocyte apoptosis and condylar degeneration. To better understand TMD pain mechanisms is needed for developing more effective treatments.

TMD pain critically depends on trigeminal ganglion (TG) sensory neurons that transmit nociceptive signals from the periphery to the central nervous system. TRPV4, a multi-modally activated ion channel, is abundantly expressed in TG neurons[18, 20, 55]. We previously found that the TMJ-innervating TG neurons express TRPV4 and that TRPV4 expression in these neurons is increased after joint inflammation[20]. Using bite force measurement, we further demonstrated that global knockout (KO) or systemic inhibition of TRPV4 significantly attenuated TMJ inflammation-induced masticatory pain[20]. However, it remains unclear whether TG neurons are a crucial cellular site where TRPV4 drives TMD pain. Furthermore, the mechanism whereby sensory neuron-TRPV4 mediates TMD pain is unknown.

Calcitonin gene-related peptide (CGRP) is an important pain mediator for migraine[27, 88], visceral pain[29], neuropathic pain[43], knee and ankle joint arthritis pain[54], fibromyalgia[57], and complex regional pain syndrome[57]. Of note and with relevance to TMD, CGRP-containing nerve fibers have been detected in the joint capsule, articular disc, and synovial membrane of both rodent and human TMJs[33, 40, 46, 94]. Elevated levels of CGRP in synovial fluid have been found in human arthritic TMJs[39, 59, 89] and correlated with the impairment of mandibular mobility and pain of patients[7, 8]. While these reports suggest that increased CGRP in TMJ-associated tissues might be involved in the pathophysiology of TMD, the mechanisms underlying the elevation of CGRP and the functional contribution of CGRP to TMD pain remain largely elusive.

We previously found that CGRP co-expresses with TRPV4 in a subset of TG neurons which innervate the TMJ[20], suggesting that TRPV4 in TG neurons might drive TMD pain via secretion of CGRP. Using two mouse models of chronic TMD pain, TMJ inflammation and masseter muscle injury, we sought to investigate whether: 1) conditional KO of sensory neuron-*Trpv4* attenuates TMD pain; 2) activation of TRPV4 in TG neurons triggers CGRP release and leads to increased levels of CGRP in TMD-associated tissues; 3) blockade of

CGRP receptor ameliorates TMD pain. To enhance the translational relevance, we also examined whether TRPV4 co-expresses with CGRP in human TG neurons.

## Methods

### Animals

Mice with conditional KO (cKO) of *Trpv4* in primary sensory neurons were generated via Cre-loxP-mediated recombination by mating mice carrying *Trpv4* (*Trpv4*$^{fl/fl}$) with a mouse line expressing Cre recombinase under control of the *Nav1.8* promoter (*Nav1.8*-Cre)[19]. The Cre mice enable gene recombination commencing at birth selectively in nociceptive sensory neurons, without affecting gene expression in the spinal cord, brain, or other tissues in the body. Efficiency of targeting was verified with immunohistochemistry and functional $Ca^{2+}$ imaging studies showing that the number of TRPV4-expressing DRG/TG sensory neurons were reduced by ~80%[19] and TRPV4-responding neurons in response to the TRPV4 selective agonist 4α-PDD was reduced by ~83%[113]. Male WT (background: C57bl/6j) and sensory neuron-*Trpv4* cKO mice (background: C57bl/6j) were used at 2.5–3 months of age (weight 24–26g). Animals were housed in climate-controlled rooms on a 12/12h light/dark cycle with water and standardized rodent diet available *ad libitum*. Animal protocol was approved by the Duke University-IACUC in compliance with NIH guidelines.

WT and sensory neuron-Trpv4 cKO mice were randomly assigned to experimental groups receiving complete Freund's adjuvant (CFA), incomplete Freund's adjuvant (IFA), ligation of the tendon of the anterior superficial part of masseter muscle (TASM) or sham-TASM. Additional WT mice were also randomly assigned to receive injections of CGRP or CGRP receptor inhibitor or vehicle. Experimenters performing pain assessments, immunostaining analysis, and enzyme-linked immunosorbent assay (ELISA) were blinded to animal genotypes and drug treatment conditions. Sample 'N' for each group or condition are indicated in figure legends.

### Induction of TMJ inflammation and masseter muscle injury

For TMJ inflammation, mice were briefly anesthetized with 2% isoflurane and injected with 10μL of complete Freund's adjuvant (CFA, 5mg/mL; Chondrex, Woodinville, WA) into the joint using a 30G needle on a Hamilton syringe[20]. Control animals were injected with the same volume of incomplete Freund's adjuvant (IFA). For masseter muscle injury, ligation of the tendon of the anterior superficial part of masseter muscle (TASM) model was conducted as previously described[31]. Mice were anesthetized with ketamine/xylazine (i.p. 80mg/8mg/kg, Sigma-Aldrich, St. Louis, MO) during the procedure. A retractor was used to open the animal's mouth, and a 3 mm long incision was made posterior-anteriorly along but just lateral to the gingivobuccal margin in the buccal mucosa, beginning immediately next to the first molar. The TASM was freed gently from surrounding connective tissues and clearly visualized. The tendon was tied with two chromic gut (6.0) ligatures, 1.5 mm apart. The incision was closed with two 5.0-silk sutures. Sham-operated control mice received the same procedure, except that the masseter muscle's tendon was not ligated. Since the bite performed by the mice was an incisor bite, both CFA/IFA and TASM/sham-

TASM procedures were performed bilaterally to reduce the sided variability in bite force measurements (see bite force test below).

## TMD pain behavioral tests

The bite force test, as we and others described[32, 110 20], was used to measure masticatory pain of TMD. The bite force transducer consists of two aluminum beams, each instrumented with two single-element strain gauges. The four strain gauges are connected in a Wheatstone bridge. Deformation of parallel-mounted beams results in a proportional change in resistance and subsequently in the voltage output to the Wheatstone bridge. One end of each beam served as bite plate and was covered with an acrylic coating (Micro-Measurements, Wendell, NC) to protect the animals' teeth. Mice were acclimated to the testing facility and handling prior to behavioral testing. Mice were placed in a cylindrical tube with an opening at one end for accommodation of the mouse's head. When the bite transducer was slowly moved towards the mouse, a bite was invariably elicited. The voltage output during each bite was recorded as a continuous wave at 500Hz using Labview 8.0 (National Instruments, Austin, TX). The bite force transducer was calibrated and checked for linearity by suspending a series of calibration weights ranging from 0.1 to 0.5 kg from the bite plates. The voltage output from each weight was regressed against force exerted by calibration weights. Output for calibrations was both linear and reproducible, with correlation coefficients ($R^2$) ranging from 0.98–0.99. The peak voltage of each bite was determined and converted into force (newton) based on the regression equation derived from calibration. Each animal was tested 3–5 times per time point and the values were averaged. The interval between two trials was >1 min. Mice were randomly assigned to treatment groups. The experimenter was blinded to the treatment conditions and animal genotypes. Although the baseline values for bite force were not statistically different between groups or genotypes, there were variations (from 12.63 to 16.34 newtons). To statistically analyze the data in a more objective way, bite force values after treatment were normalized to the baseline values for each group and % of bite force changes were compared.

## Chemical injections

To determine the effect of systemic inhibition of CGRP receptor on CFA- or TASM-induced TMD pain, mice received a single intraperitoneal (i.p.) administration of the CGRP receptor inhibitor olcegepant (Sigma-Aldrich)[72] at 3 mg/kg and 10 mg/kg on day 3 after CFA or day 7 after TASM, respectively, when the TMD pain was the most prominent (see Fig. 1). To test the local inhibitory effect, olcegepant at 30μg/10μL and 100μg/10μL was bilaterally, intraarticularly (i.a.) injected into the TMJs on day 3 after CFA or intramuscularly (i.m.) injected into the masseter muscle on day 7 after TASM. Control animals received the same volume of 2% of DMSO. TMD pain was measured at 1h, 3h, and 5h following injections. To examine whether local injection of CGRP (Sigma-Aldrich) induces pain, CGRP at 10μg/10μL and 30μg/10μL was bilaterally i.a. or i.m. injected into TMJs and masseter muscle, respectively. Control animals received the same volume of normal saline. Mice were measured at 0.5, 1, 3, and 5h following injections.

To track TMJ or masseter muscle innervation by the TG neurons, mice were injected with 2 μL of neural tracer fast blue (FB, 2% aqueous solution; Polysciences, Warrington, PA) into

the TMJ or masseter muscle 15 minutes before administration of CFA or IFA and ligation of TASM or sham-TASM.

### Immunohistochemistry and quantitative analysis

Mice were perfused transcardially with ice-cold phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (PFA, Sigma-Aldrich) at the experimental time points under studies. Mouse TGs were dissected and post-fixed in 4% PFA for 4–5h then cryo-protected in 20% sucrose overnight. Non-diseased human TGs from donors were obtained through NIH-NeuroBioBank with an exemption of Duke University-IRB. Postmortem TGs were dissected from 4 male donors aged 21–55 years. Mouse TG sections at 12 μm and human TG sections at 8 μm were blocked with 5% normal donkey serum (Jackson ImmunoResearch, West Grove, PA), and incubated overnight with primary antibodies: rabbit anti-TRPV4 (1:2500, Novus Biologicals, Littleton, CO) and goat anti-CGRP (1:3000, Abcam, Boston, MA). Immunodetection was accomplished with secondary antibodies (AlexaFluor594 or AlexaFluor488; 1:600; Invitrogen, Waltham, MA) for 2h, and cover-slipped with Vectashield (Vector, Burlingame, CA). Digital micrographs were acquired using a BX61 Olympus upright microscope equipped with high-resolution CCD (Olympus, Tokyo, Japan). For every mouse or human TG, 4–6 sections were analyzed. TG neurons were identified by morphology. Using ImageJ software, the cutoff density threshold was determined by averaging the density of three neurons per section that were judged to be minimally positive. All neurons for which the mean density exceeded the threshold of 25% were counted as positive.

### TG sensory neurons culture

Following our studies[18, 19], TGs from WT and sensory neuron-*Trpv4* cKO mice were dissected and digested with 1 mg/mL collagenase (Worthington Biochemical Co., Lakewood, NJ) and 5 mg/mL dispase (Invitrogen) for 1 h, then triturated. The resulting cell suspension was filtered through a 70 μm cell strainer (BD Falcon, Franklin Lakes, NJ) to remove debris. Neurons were cultured in DH10 medium (1:1 DMEM:Ham F12, Invitrogen) with 10% fetal bovine serum (Sigma), 100 U/mL penicillin, and 100μg/mL streptomycin (Gibco, Waltham, MA), and 50 ng/mL nerve growth factor (USBiological, Salem, MA) on coverslips coated with poly-D-lysine and laminin (Invitrogen), and incubated with 5% $CO_2$ at 37°C. To stimulate the secretion of CGRP, sensory neurons were incubated with the TRPV4 selective agonist GSK1016790A[99] (GSK101, Sigma-Aldrich) next day after culture. Supernatant was collected 15 min after GSK101 stimulation. To test the effect of inhibition of TRPV4 on CGRP secretion, cultured neurons were pretreated with the TRPV4 selective inhibitor GSK205[79] (Sigma-Aldrich) for 15min.

### Mouse tissue dissection and processing for ELISA assay

Blood was collected via cardiac puncture and centrifuged for 15 min. Plasma was quickly frozen and stored at −80°C until use. After blood collection, mice were perfused transcardially with ice-cold PBS at the experimental time points under study. Dissected TG and spinal trigeminal nucleus tissues were homogenized and incubated on ice in radio-immunoprecipitation assay (RIPA, Sigma-Aldrich) buffer for 30 min. RIPA was added according to tissue weights. During incubation, each sample was homogenized 3 times

(20 seconds/time). Samples were then centrifuged at 4°C for 15 min at 13000 rpm. The supernatant was collected and stored at −80°C until use. Following previous study[52], the peri-TMJ tissues, which include the synovial membrane, joint capsule, retrodiscal tissue, and articular disc were excised. In addition, the anterior superficial part of the masseter muscle (3–4mm long) and the mandibular condyle (3–4mm long, including the head of condyle) were also dissected. The peri-TMJ tissues, masseter muscle, and mandibular condyle were weighed and sonicated in 2N acetic acid 3 times (15 seconds/time). Homogenates were heated at 90°C for 10 min then centrifuged at 4°C for 15 min at 13,000 rpm. The supernatant was collected and vacuumed until lyophilized. Samples were re-suspended in enzyme immunoassay (EIA) buffer according to tissue weights and stored at −80°C until use.

### ELISA measurement of CGRP

CGRP levels in collected supernatant from cultured TG neurons, plasma, TG, spinal trigeminal nucleus, peri-TMJ tissues, masseter muscle, and mandibular condyle were measured by CGRP EIA kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's instructions. CGRP content was determined photometrically by measuring absorbance at 405 nm on an automated plate reader (Molecular Devices, San Jose, CA). Absorbance values were corrected by blank subtraction, averaged, and converted to CGRP concentrations using a CGRP standard curve.

### Statistical analysis

All data are expressed as mean ± SEM. Two-tail *t* test, one-way ANOVA followed by Tukey post-hoc test, or two-way repeated measures ANOVA followed by Bonferroni post-hoc test was used for groups comparison. Experimental 'N' as used was based on a power analysis of our previous relevant studies involving bite force test, immunohistochemistry, and ELISA[18, 20, 61]. $P<0.05$ was considered statistically significant.

## Results

### Conditional KO of Trpv4 in TG sensory neurons attenuates TMD pain-induced by TMJ inflammation or masseter muscle injury in mice

Whereas TMD has multifactorial etiologies[30, 63, 71, 91], a significant subgroup of patients suffer joint inflammation and/or masseter muscle injury[11, 38, 64, 91, 97]. To mimic these conditions in mice, we induced TMJ inflammation by injecting CFA into the joint and masseter muscle injury by ligating the TASM in mice. Following our previous method[20], bite force measurement, as a clinically relevant read-out, was used to assess masticatory pain of TMD. Using this method, we have found that global knockout or systemic inhibition of TRPV4 attenuated the reduction of bite force after TMJ inflammation-induced by CFA[20]. Here, we extended this finding by determining whether TG sensory neurons are the critical cellular site where TRPV4 drives masticatory pain in TMJ inflammation model, as well as in another model of masseter muscle injury. Both TMJ inflammation and masseter muscle injury resulted in long-lasting masticatory pain, as indicated by a substantial reduction of bite force from day 1 to 11 after CFA (Fig. 1A) and from day 7 to 21 after TASM (Fig. 1B). We took advantage of these two chronic pain models to study the specific role of sensory neuron-TRPV4 in TMD pain. Sensory neuron-Trpv4 cKO mice displayed normal

general appearance and body weight during development (data now shown). Behavioral tests demonstrated that cKO of *Trpv4* in TG neurons significantly suppressed the reduction of bite force in both models (Fig. 1A–C), suggesting an essential role of sensory neuron-TRPV4 in masticatory pain of TMD.

## Colocalization of TRPV4 and CGRP in TMJ- and masseter muscle-innervating TG neurons is increased after TMJ inflammation or masseter muscle injury in mice, and TRPV4 colocalizes with CGRP in human TG neurons.

We previously found that TRPV4-containing TG neurons co-express CGRP[20]. To examine whether these neurons innervate the TMJ and masseter muscle and whether the innervations are upregulated after CFA or TASM, we micro-injected a neural tracer fast blue (FB) into the TMJ or masseter muscle for CFA and TASM models, respectively. First, we found that the percentage of TG neurons-expressing TRPV4 (Fig. 2A) and CGRP (Fig. 2B) are increased, but FB-labeled neurons remain unchanged (Fig. 2C), after CFA or TASM. Second, neural tracing analysis with FB-labeled neurons showed that the percentage of TRPV4 and CGRP co-expression in TMJ- or masseter muscle-innervating TG neurons was doubled in mice treated with CFA compared with IFA and in mice received TASM compared with sham-TASM (Fig. 2D), respectively. Third, we found that % of TRPV4 expression in CGRP-containing TG neurons (TRPV4+CGRP/CGRP) was not significantly different between CFA vs. IFA or TASM vs. Sham groups (Fig. 2E), and *vice versa* (CGRP+TRPV4/TRPV4) (Fig. 2F). Typical images showing expression of TRPV4, CGRP and FB and their colocalizations in TG neurons are displayed in Fig. 2G–V.

We also examined whether TRPV4 and CGRP are colocalized in human TG neurons. Indeed, immunohistochemical analysis demonstrated that TRPV4 was also co-expressed with CGRP, with 41.4% of TG neurons immuno-reactive for TRPV4, 40.8% for CGRP, and 27.6% for both TRPV4 and CGRP (Fig. 3A–C).

## Specific activation of TRPV4 in mouse TG sensory neurons triggers the release of CGRP

We next examined whether activation of TRPV4 in TG neurons triggers secretion of CGRP. Incubation of cultured mouse TG neurons with TRPV4 selective agonist GSK101 at 10 nM for 15 min significantly elevated the secretion of CGRP, which was abolished by conditional KO of *Trpv4* or pretreatment with TRPV4 selective inhibitor GSK205 at 10 μM (Fig. 4).

## Increased systemic and local levels of CGRP after TMJ inflammation or masseter muscle injury are dependent on sensory neuron-TRPV4 in mice

Based on the data that activation of TRPV4 triggers the release of CGRP from cultured TG sensory neurons (Fig. 4) and colocalization of TRPV4 and CGRP is increased in TMJ- and masseter muscle-innervating TG neurons after TMJ inflammation or masseter muscle injury (Fig. 2D), we next asked whether CGRP levels are elevated in mice after CFA or TASM and its dependence on sensory neuron-TRPV4. ELISA assays demonstrated that the relative levels of CGRP in TG, peri-TMJ tissues, masseter muscle, spinal trigeminal nucleus, and plasma, but not mandibular condyle, are increased in CFA and TASM models (Fig. 5A–L). Interestingly, the increase of CGRP was drastically suppressed by conditional KO of sensory neuron-*Trpv4* (Fig. 5M–R), suggesting its critical dependence on TRPV4 in TG neurons.

### CGRP administration into the TMJ or masseter muscle evokes pain in mice

Increased CGRP levels in peri-TMJ tissues and masseter muscle after TMJ inflammation or masseter muscle injury suggest that CGRP may play a pro-nociceptive role in TMD pain. We then tested if CGRP evokes pain in naïve mice. I.a. or i.m. micro-injection of CGRP at doses of 10μg/10μL and 30 μg/10μL into the TMJ or masseter muscle significantly lowered the bite force (Fig. 6A–B), indicating CGRP contributes to TMD pain.

### Pharmacological inhibition of CGRP receptor attenuates TMD pain in mice

Receptor activity modifying protein 1 (RAMP1) forms a complex with calcitonin receptor-like receptor (CLR) and receptor component protein (RCP) to constitute the receptor for CGRP[13]. Systemic blockade of CGRP receptor by i.p. injection of olcegepant (3mg/kg and 10 mg/kg), a CGRP receptor inhibitor via binding to RAMP1/CLR[25, 68], significantly blunted the reduction of bite force (Fig. 7A–B). Considering an increase of CGRP levels in peri-TMJ tissues and masseter muscle in CFA and TASM models and local injection of CGRP into TMJ and masseter muscle evoked pain, we next tested the effect of local inhibition of CGRP receptor on TMD pain. Interestingly, we observed that i.a. or i.m. injection of olcegepant (30μg/10μL and 100μg/10μL) into the TMJ or masseter muscle also attenuated the reduction of bite force (Fig. 7C–D). In addition, the effects of olcegepant on TMD pain were comparable between two doses for i.p., i.a., or i.m., or i.p injections. Nevertheless, these data further support that CGRP contributes to TMD pain.

## Discussion

Activation of TG neurons innervating the TMJ and masticatory muscles provides a critical nociceptive pathway for TMD pain[93]. Although TRPV4, a pro-nociceptive ion channel, is abundantly expressed in TG neurons, direct evidence regarding whether and how sensory neuron-TRPV4 drives TMD pain remains unknown. Here, we found that specific deletion of *Trpv4* in TG neurons blunted TMD pain after TMJ inflammation or masseter muscle injury. We further demonstrated that activation of TRPV4-expressing TG neurons triggered secretion of CGRP, which might contribute to increased local levels of CGRP in TG, masseter muscle, peri-TMJ tissues, and spinal trigeminal nucleus and elevated systemic levels CGRP in plasma in mouse models of TMD pain. Local injection of CGRP into the TMJ or masseter muscle evoked acute pain, while systemic or local blockade of CGRP receptor attenuated TMD pain. Together, these data suggest that sensory neuron-TRPV4 contributes to TMD pain *via* CGRP.

TMD is known for its mastication-associated pain. Clinical research has shown that TMD patients have significantly reduced bite force compared to healthy controls, and this reduction correlates with pain severity[17, 26, 36, 50, 77, 78, 80, 111]. Based on this background, we and others developed and validated a method of measuring bite force in rodents as a clinically-relevant read-out of TMD masticatory pain[20, 32, 83, 110]. Using this method, we previously demonstrated that global knockout or systemic inhibition of TRPV4 significantly attenuated the reduction of bite force in a mouse model of TMJ inflammation[20]. Further, wild-type mice exhibited increased TRPV4 expression in TMJ-innervating TG neurons after TMJ inflammation[20]. These data raised an important question as to whether TG neurons

are a crucial site where TRPV4 drives TMD pain. To address this question, here, we examined the specific contribution of sensory neuron-TRPV4 to TMD pain with two mouse models: TMJ inflammation and masseter muscle injury. In line with the model of TMJ inflammation, we found an increase of TRPV4 expression in TG neurons after masseter muscle injury. Importantly, bite force reduction was significantly attenuated by cKO of sensory neuron-*Trpv4* in both models, providing the first demonstration that TRPV4 in TG neurons is essential for TMD pain.

Although studies have demonstrated that activation of TRPV4 in DRG neurons can increase CGRP levels in urinary bladder, airways, and esophagus[65, 106], it is unknown whether TRPV4 in TG neurons promotes CGRP secretion under TMD conditions. Delineation of the dependence of CGRP levels on TRPV4 in TG neurons and the functional roles of CGRP in TMD pain is important to deconstruct the mechanisms underlying sensory neuron-TRPV4 in TMD pain because 1) the trigeminal sensory system has distinct characteristics from the spinal sensory system under pathophysiological conditions[14, 37, 60, 81, 85, 104] and 2) TMD pain involves unique target tissues and has its own distinct etiologies[30, 37, 63]. Studies have shown that CGRP is increased in TG neurons and spinal trigeminal nucleus after TMJ inflammation or masseter muscle injury[22, 44, 51, 95, 96]. Further, CGRP has been detected in the synovial membrane, articular disc, periosteum, and joint capsule of the TMJ[46, 103]. To gain greater insight into the role CGRP in TMD pain, we conducted a thorough investigation of temporal change of local levels of CGRP in peri-TMJ tissues, masseter muscle and mandibular condyle, as well as TG and spinal trigeminal nucleus and circulating plasma. We found that TMJ inflammation and masseter muscle injury resulted in increased levels of CGRP in all tissues examined except for the mandibular condyle and masseter muscle for TMJ inflammation model. Importantly, elevation of CGRP levels was completely suppressed in sensory neuron-*Trpv4* cKO mice. This is surprising because TRPV4 expression in TG/DRG neurons was not abolished in *Trpv4*-cKO mice (Nav1.8-cre::*Trpv4*$^{fl/fl}$, ~80% reduction)[19, 113]. Although further studies are warranted to investigate whether the residual TRPV4 in sensory neurons (~20%), most likely outside of Nav1.8-cre$^{+}$ neuronal population, plays a functional role in CGRP release, it is plausible that deletion of *Trpv4* from Nav1.8-cre$^{+}$ neurons can have a dramatic effect on secretion of CGRP because CGRP is exclusively expressed within a subset of Nav1.8-cre$^{+}$ neurons[75].

It is yet unclear how TRPV4 in TG neurons causes elevated levels of CGRP after TMJ inflammation or masseter muscle injury. At the site of tissue inflammation or injury, pro-inflammatory mediators can be released from peripheral non-neuronal cells. Many of these mediators activate or sensitize ion channels and receptors in sensory neurons which can subsequently release neuropeptides, notably CGRP and substance P (SP), from their terminals[82, 90]. Previous studies have demonstrated that elevated levels of pro-inflammatory mediators, such as TNF-α, IL-1α, IL-1β, IL-6, IL-17, and PGE2, are present in TMJ-associated tissues of TMD patients[3–5, 35, 45, 100, 107]. Since many of these mediators can sensitize TRPV4[2, 49, 58, 90], an enhanced activation of TRPV4 in TG neurons may trigger secretion of CGRP, possibly via an exocytotic process[23, 87], which leads to increased levels of CGRP in local tissues and blood after TMJ inflammation or masseter muscle injury. In support of this putative mechanism, we found that activation of TRPV4-expressing TG neurons with TRPV4 selective agonist GSK101 triggered secretion of CGRP, and we also

observed an increased co-expression of TRPV4 and CGRP in TMJ- and masseter muscle-innervating TG neurons in CFA or TASM models. We have previously shown that activation of TRPV4 can induce an increase of phosphorylated extracellular signal-regulated kinase (p-ERK) in TG neurons[20]. Interestingly, p-ERK can upregulate CGRP expression[53] and CGRP can cause phosphorylation of ERK in TG neurons[105]. Thus, it is plausible that p-ERK and CGRP, as downstream targets of TRPV4, form a positive feedback loop in TMD pain regulation.

Given a known pro-nociceptive role of CGRP[42, 112] and increased levels of CGRP after TMJ inflammation or masseter muscle injury, we next evaluated the functional contribution of CGRP to TMD pain. Systemic or local TMJ/masseter muscle injection of the CGRP receptor inhibitor olcegepant significantly attenuated TMD pain. Meanwhile, local administration of CGRP into the TMJ or masseter muscle evoked acute pain. Based on the data presented and in view of CGRP's role in peripheral and central sensitization[15, 42, 86], it is likely that CGRP in the peri-TMJ tissues, masseter muscle, and TG may cause local release of pro-inflammatory mediators from neighboring non-neural cells or vascular tissue, which sensitize or activate trigeminal nociceptive neurons to drive pain. Supporting this hypothesis, CGPR has been shown to rapidly stimulate IL-1β, IL-6 and TNF-α secretion from the synovial mesenchymal stem cells of TMJ[59]. Secretion of CGRP from soma of TG neurons can also induce release of a variety of cytokines from satellite glial cells[1], which can signal back to sensory neurons[67]. Moreover, CGRP, released from TG neurons, can activate the second order neurons and glial cells in spinal trigeminal nucleus[15, 66]. Interestingly, elevated levels of CGRP in the spinal trigeminal nucleus can reversely promote sensitization of TG nociceptive neurons[21]. Further studies are needed to test if these possibilities may account for TMD pain driven by CGRP. Nevertheless, our gain- and loss-of-function results strongly suggest that elevated CGRP regulated by sensory neuron-TRPV4 promotes TMD pain.

We found that TRPV4 co-expresses with CGRP in human TG neurons, corroborating the findings in mice. Interestingly, CGRP-containing nerves were detected in joint capsule, articular disc, and synovial membrane of human TMJs[33, 40, 46, 94] and elevated levels of CGRP in synovial fluid were found in human arthritic TMJs[39, 59, 89]. Further experiments are needed to elucidate whether co-expression of TRPV4 and CGRP is increased in TG neurons of TMD patients and whether elevated levels of CGRP are dependent on TRPV4 in TG neurons, as found in mice.

There remain some limitations of this study. First, only male mice and human subjects were included in this study. Considering there is a sexual dimorphism of TMD pain[12, 73] and CGRP plays sexually differential roles in pain modulation (e.g., migraine and osteoarthritis)[74, 102], our dedicated future work will include females. Second, other pain-related ion channels, such as TRPV1 and TRPA1, have also been implicated in TMD pain[10, 62, 84, 108–110], and activation of these channels in TG neurons triggers CGRP release[66, 70]. Further investigations are needed to explore if TRPV4 has a functional interaction with these channels in secretion of CGRP under TMD condition. In support of this possibility, a recent study demonstrated that TRPV4 can interact with TRPV1 in sensory neurons for itch transmission[47]. Third, it is yet unknown why CGRP levels in mandibular

condyle are not altered in mouse models of TMD. We speculate this might be attributed to the minimal CGRP expression in nerve fibers located in condyle[48].

In summary, our study demonstrates that TRPV4 in TG neurons contributes to TMD pain via a mechanism of action involving the pain mediator CGRP. Considering CGRP/CGRP receptor-blocking biologics have been recently approved for treating migraine, for which TMD shares significant co-morbidity, it would be important to examine whether TMD patients can benefit from these biologics in pain relief.

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## Highlights

- Conditional knockout of Trpv4 in TG neurons attenuates pain in mouse models of TMD.
- Activation of TRPV4-expressing TG neurons triggers secretion of CGRP.
- Increased CGRP in TMJ-associated tissues are dependent on sensory neuron-TRPV4.
- Blockade of CGRP receptor reduces pain in mouse models of TMD.

**Perspective:**

This study demonstrates that activation of TRPV4 in TG sensory neurons drives pain by potentiating the release of pain mediator CGRP in mouse models of TMJ inflammation and masseter muscle injury. Targeting TRPV4 and CGRP may be of clinical potential in alleviating TMD pain.

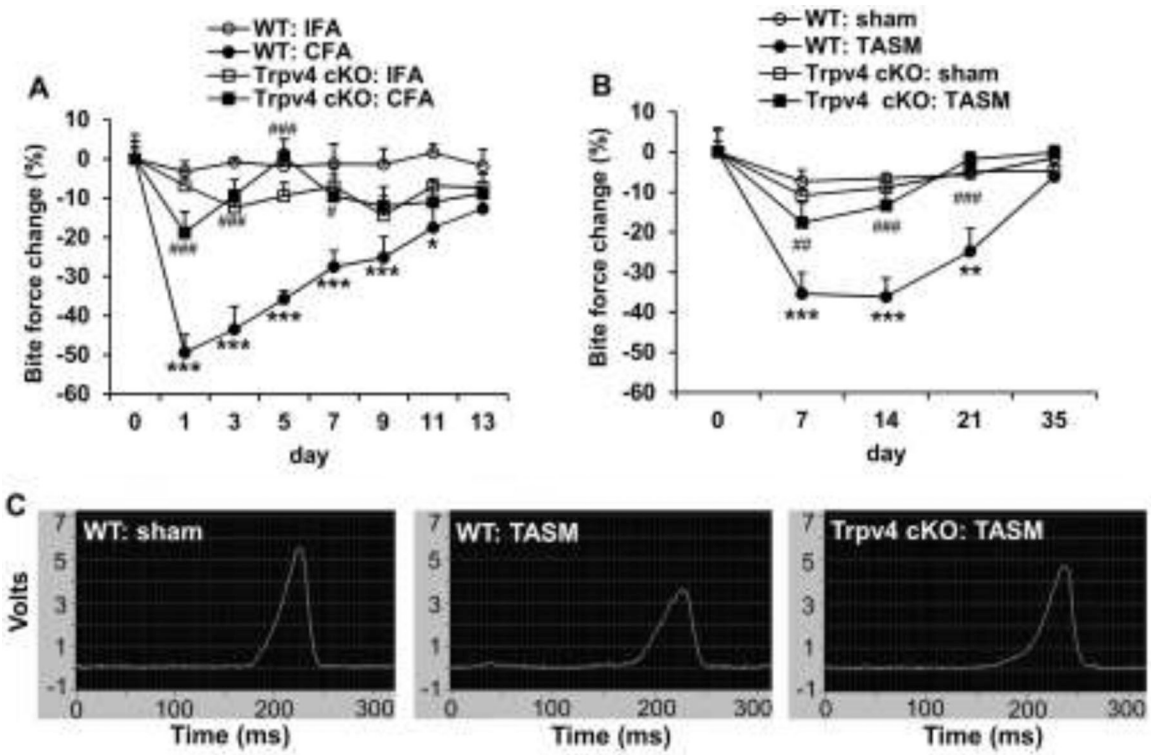


**Figure 1.**

Conditional KO of *Trpv4* in TG sensory neurons attenuates TMD pain after TMJ inflammation or masseter muscle injury. The reduction of bite force was significantly suppressed in sensory neuron-*Trpv4* cKO (Nav1.8-cre::*Trpv4*$^{fl/fl}$) mice from day 1 to 7 after CFA (A) and from day 7 to 21 after TASM (B). (C) Exemplary changes of bite force signal for WT: sham, WT: TASM, and *Trpv4* cKO: TASM on day 7. *p<0.05, **p<0.01, and ***p<0.001 vs. WT: IFA or WT: sham; $^{\#}$p<0.05, $^{\#\#}$p<0.01, and $^{\#\#\#}$p<0.001 vs. WT: CFA or WT: TASM, two-way repeated measures ANOVA followed by Bonferroni's post hoc test. N=6–9 male mice/group.

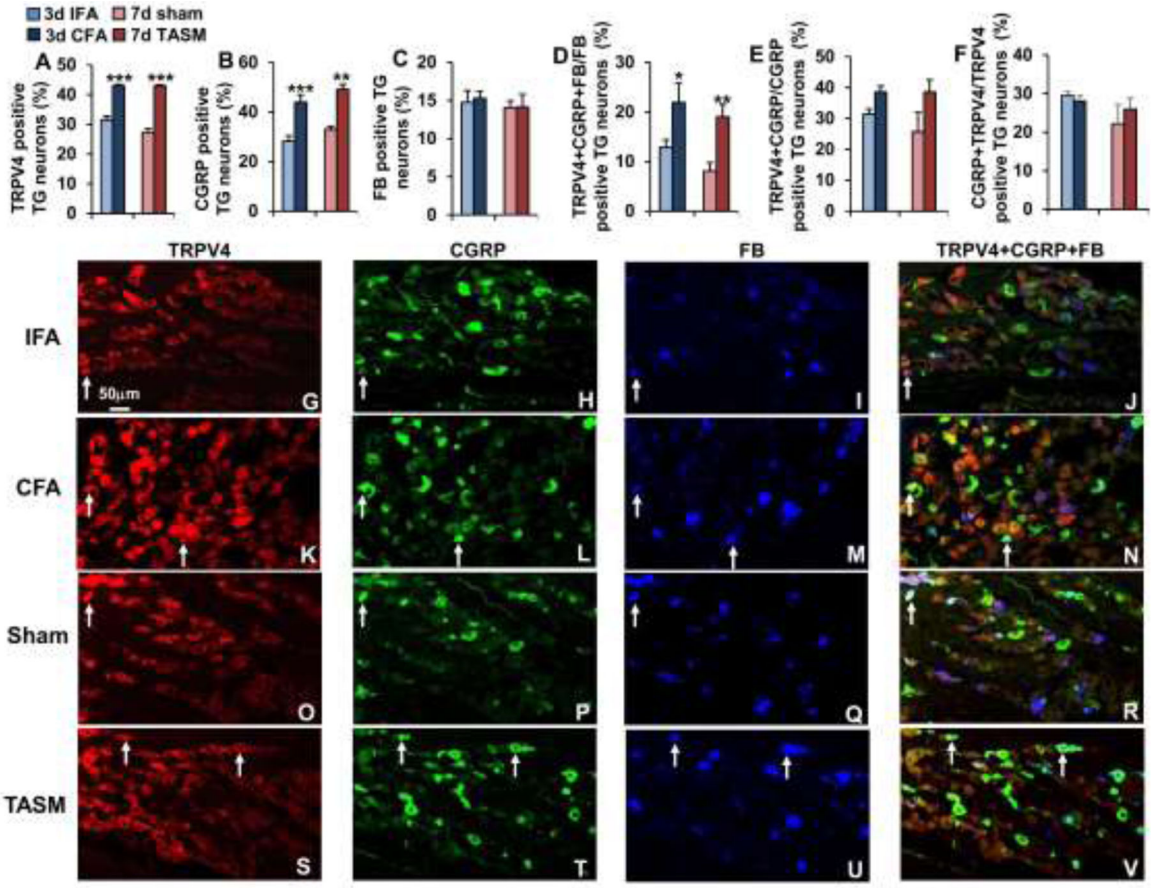


**Figure 2.**

Co-expression of TRPV4 and CGRP in TMJ- or masseter muscle-innervating TG neurons is increased after TMJ inflammation and masseter muscle injury, respectively. (A-C) image quantitative analysis shows an increased percentage of TRPV4 and CGRP, but an unchanged percentage of FB, positive TG neurons, relative to the total of TG neurons, after TMJ inflammation and masseter muscle injury; (D) shows an increased percentage of colocalization of TRPV4, CGRP, and FB, relative to the total of FB positive TG neurons, after TMJ inflammation and masseter muscle injury; (E-F) show unchanged colocalization of TRPV4 and CGRP relative to the total of CGRP or TRPV4 positive TG neurons after TMJ inflammation and masseter muscle injury. (G-V) show colocalization of TRPV4, CGRP, and FB in TMJ (G-N)- or masseter muscle (O-V)-innervating TG neurons after TMJ inflammation and masseter muscle injury. Arrows indicate co-expressed neurons. *p<0.05, **p<0.01, ***p<0.001 vs. IFA or sham, two-tail *t* test. N=4–5 male mice/group, 4–6 sections/TG/mouse.

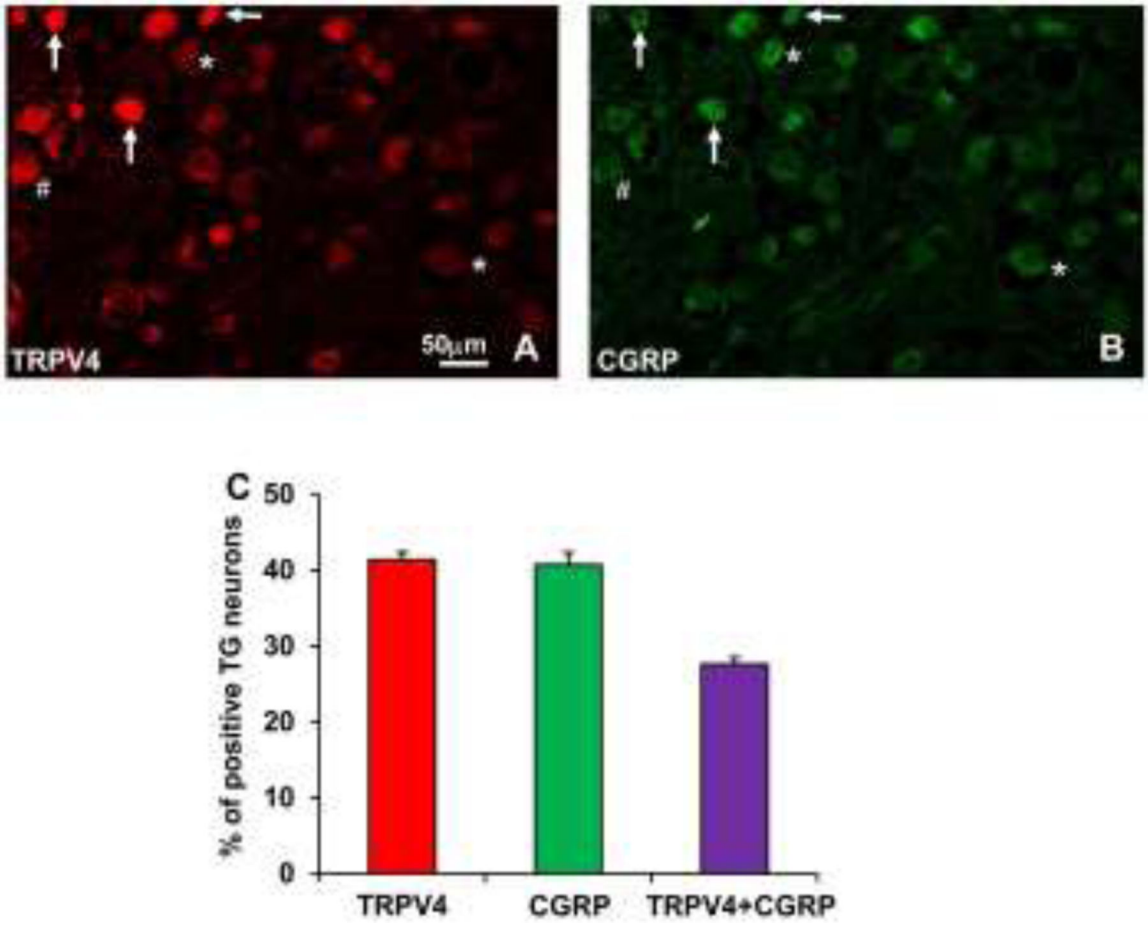


**Figure 3.**

TRPV4 is colocalized with CGRP in human TG neurons. (A-B) Immunostaining images show co-expression of TRPV4 and CGRP. Arrow=co-expressed neuron, #=TRPV4 positive but CGRP negative neuron, and *= TRPV4 negative but CGRP positive neuron. (C) shows quantitative analysis relative to the total of TG neurons. N=4 human male TGs, 4–6 sections/TG.

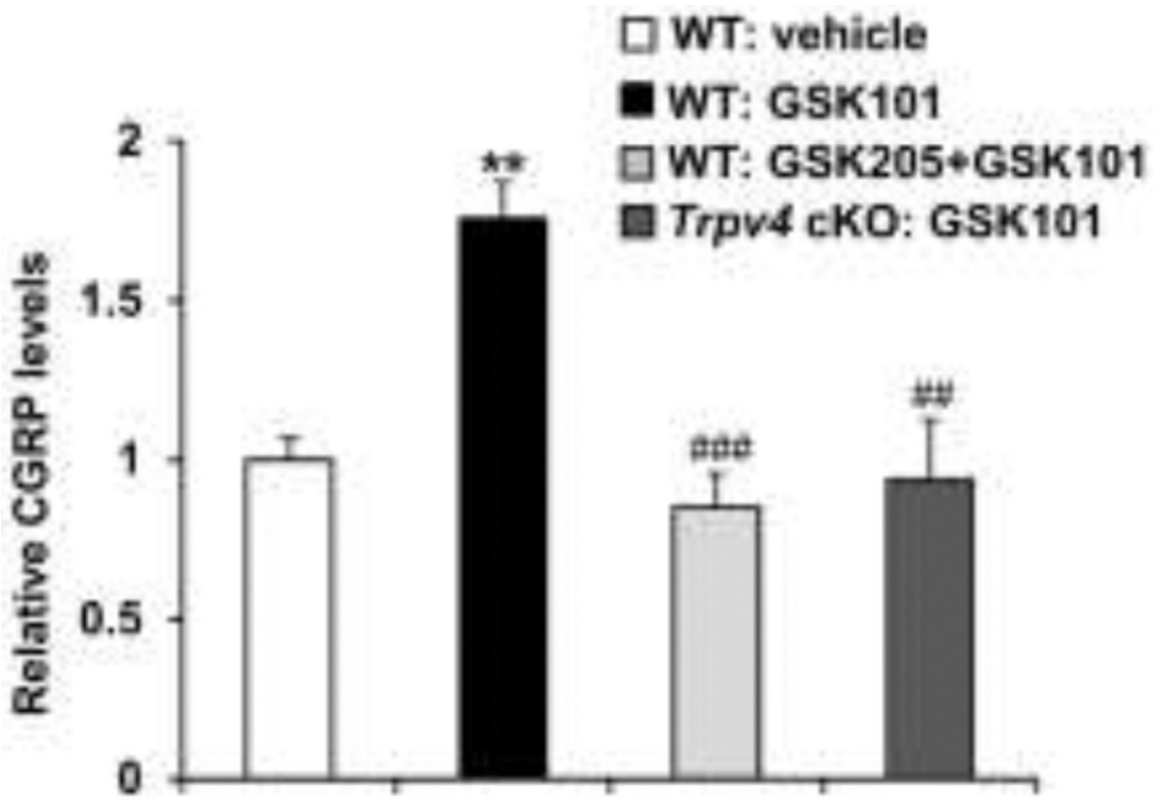


**Figure 4.**

Specific activation of TRPV4 in cultured TG sensory neurons triggers CGRP secretion. Acute incubation of GSK101 (10nM), the selective TRPV4 agonist, significantly increased the level of CGRP in supernatant of cultured TG neurons. The increase of the CGRP secretion was abolished by pretreatment with TRPV4 selective inhibitor GSK205 (10μM) or in TG neurons isolated from *Trpv4*-cKO mice. **p<0.01 vs. WT: vehicle (0.5% DMSO), $^{\#\#}$p<0.01 and $^{\#\#\#}$p<0.001 vs. WT: GSK101, one-way ANOVA followed by Tukey's post hoc test. N=4–5 cultures, one culture per male mouse.

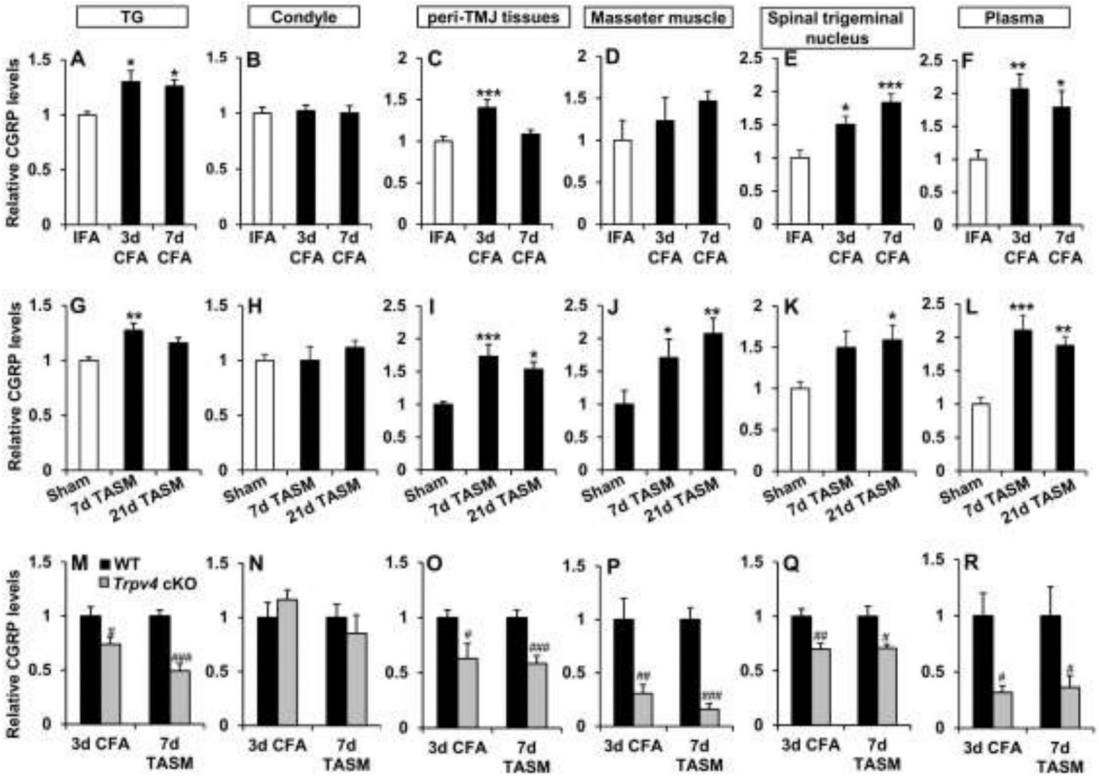


**Figure 5.**

TMJ inflammation or masseter muscle injury induces an increase of local and systemic levels of CGRP, which is dependent on sensory neuron-TRPV4. (A-L) TMJ inflammation elevated CGRP levels in TG (A), peri-TMJ tissues (C), spinal trigeminal nucleus (E), and plasma (F), but not in condyle (B). The trend for increase of CGRP levels in masseter muscle did not achieve significance (D). (G-L) Masseter muscle injury elevated CGRP levels in TG (G), peri-TMJ tissues (I), masseter muscle (J), spinal trigeminal nucleus (K), and plasma (L), but not in condyle (H). (M-R) cKO of *Trpv4* in TG sensory neurons dramatically lowered the levels of CGRP. *p<0.05, **p<0.01, ***p<0.001 vs. IFA or sham, one-way ANOVA followed by Tukey's post hoc test (A-L). $^{\#}$p<0.05, $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001 vs. WT, two-tail *t* test (M-R). N=8–10 male mice/group.

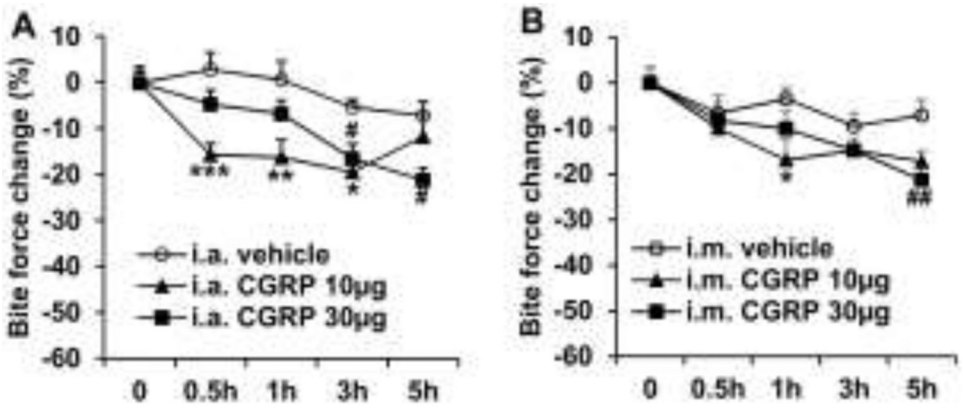


**Figure 6.**

Local injection of CGRP evokes pain in naïve mice. (A) intraarticular (i.a.) or (B) intramuscular (i.m.) injection of CGRP, at 10µg/10 µL and 30µg/10 µL, reduced bite force. *p<0.05, **p<0.01, ***p<0.001, $^{\#}$p<0.05, and $^{\#\#}$p<0.01, vs. vehicle (normal saline), two-way repeated measures ANOVA followed by Bonferroni's post hoc test. N=4–8 male mice/group.

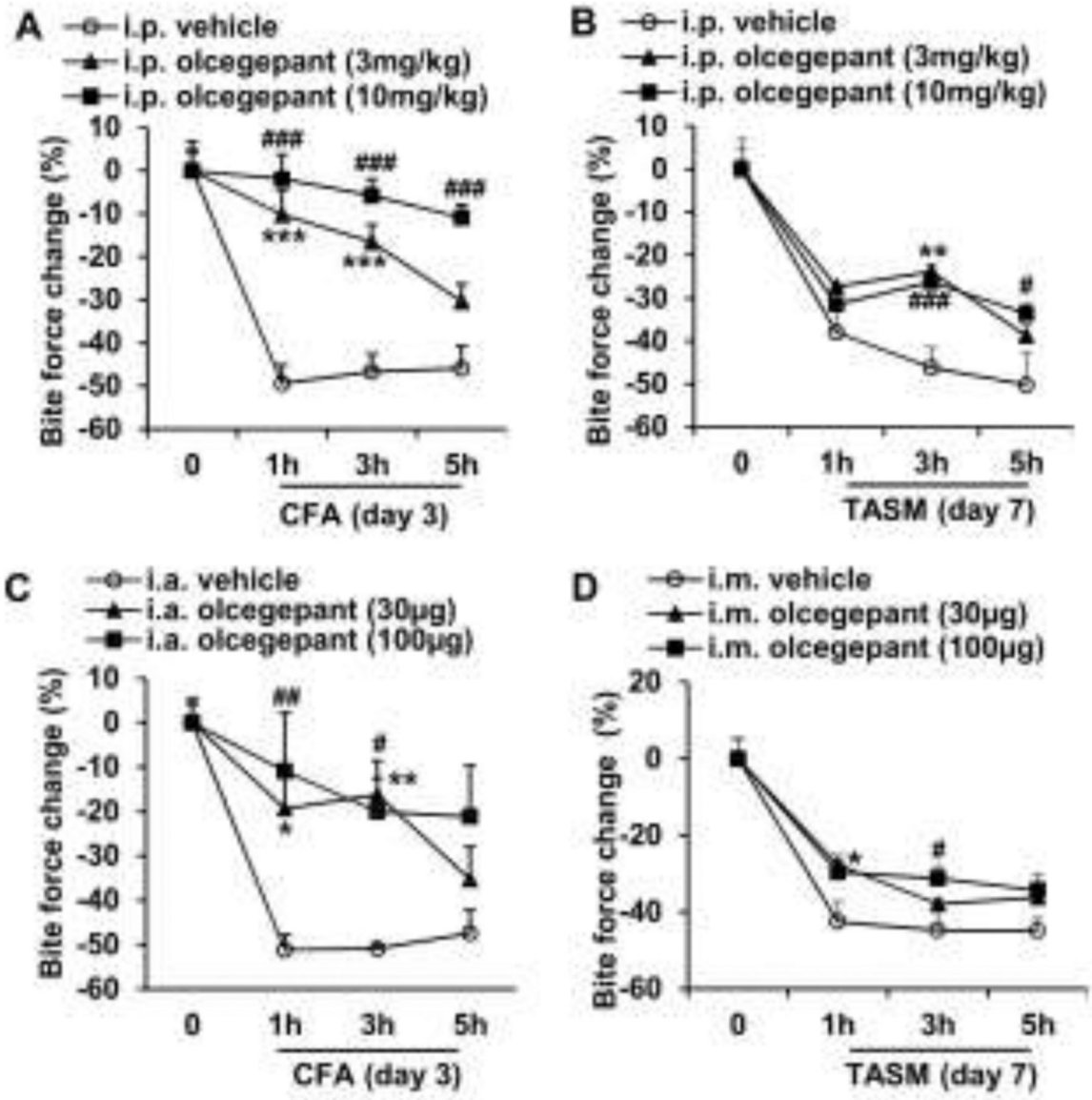


**Figure 7.**

Inhibition of CGRP receptor attenuates TMD pain. (A-B) Systemic inhibition of CGRP receptor with its selective inhibitor olcegepant (i.p., 3mg/kg and 10mg/kg) significantly blunted the reduction of bite force after TMJ inflammation (A) or masseter muscle injury (B). (C-D) local injection of olcegepant into TMJ (i.a.) or masseter muscle (i.m.) (30μg/10μL and 100μg/10μL) also attenuated the reduction of bite force after TMJ inflammation (C) or masseter muscle injury (D). *p<0.05, **p<0.01, ***p<0.001, $^{\#}$p<0.05, $^{\#}$p<0.01, and $^{\#\#\#}$p<0.001 vs. vehicle (2% DMSO). Two-way ANOVA followed by Bonferroni's post hoc test. N=5–7 male mice/group. Note: '0' on x-axis represents baseline (before CFA or TASM).