Cellular/Molecular

BoNT Injection into Temporomandibular Joint Alleviates TMJ Pain in Forced Mouth Opening Mouse Model

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Temporomandibular disorder (TMD) significantly impairs the quality of life of patients due to chronic pain and limited jaw function. Many treatment options have been used such as pharmacologic management, physical therapy, oral appliance therapy, and surgery. However, effective treatment options remain limited. In this study, we investigated the potential of botulinum toxin (BoNT) as a therapeutic approach for TMD using a forced mouth opening-induced TMD male mouse model. BoNT injection significantly alleviated mechanical hypersensitivity in the temporomandibular region over a 2 week period as demonstrated by von Frey behavioral tests. Additionally, the mouse grimace test confirmed that BoNT alleviated pain in mice. The open field test and pasta gnawing test showed that BoNT injection effectively alleviated mouth motor and food intake problems and did not cause impairments in general behavior. Moreover, direct observation of neural activity via in vivo Pirt-GCaMP3 calcium imaging of intact trigeminal ganglia (TG) revealed that BoNT suppressed both stimulus-evoked and spontaneous activity in TG neurons. Mechanistically, BoNT downregulated the expression of pain-promoting proteins (TRPV1, TRPA1, and TRPC1) and glutamate transporting protein (VGLUT2), thereby suppressing peripheral neural activity in the TG. In summary, our study identified a novel mechanism by which BoNT alleviates TMD pain. These new findings not only expand our understanding of the effects of BoNT on pain but also provide a new therapeutic approach to TMJ pain management.

Key words: **botulinum toxin; calcium imaging; temporomandibular disorder and pain; therapeutic approach**

Significance Statement

Temporomandibular disorders (TMDs) affect jaw function and cause chronic TMJ pain, significantly impacting quality of life. However, effective and long-term treatment options remain limited. In this study, we explored the potential of botulinum neurotoxin (BoNT) as a novel therapeutic option using a physiologically relevant animal model and real-time imaging of TG nerve activity. This approach allowed us to examine how peripheral sensory signals contribute to pain in TMD and how targeted modulation may offer TMJ pain relief. This study offers insight into TMJ pain mechanisms and supports BoNT as a potential long-lasting treatment option.

Introduction

Temporomandibular disorder (TMD) is characterized by severe pain and dysfunction in the temporomandibular joint (TMJ) and the muscles that control jaw movements. According to the National Institutes of Health (NIH), TMD affects 5–12% of the population (Sharma et al., 2011). Symptoms of TMD range from mild discomfort to severe pain and limited jaw function. Although chronic TMD is not a high-risk condition, the severity of pain can significantly affect quality of life. The World Health Organization (WHO) also emphasizes that the impact of TMD on quality of life is a serious health problem (1995). Although TMD is a common condition worldwide, the underlying mechanisms of TMD pain are still poorly understood, and effective treatments for pain relief are limited (Scrivani et al., 2008; Cairns, 2010; Manfredini et al., 2011).

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Botulinum neurotoxin (BoNT) is a well-known muscle relaxant that has been studied as a treatment for various pain disorders, including chronic migraine and myofascial pain syndrome (Aoki, 2005; Aurora et al., 2014). BoNT has recently been considered as a potential treatment for TMD due to its ability to reduce muscle hyperactivity and modulate pain transmission (Machado et al., 2020). Specifically, BoNT effectively suppressed bilateral trigeminal neuropathic pain and anxiety-like behavior in rats with chronic constriction injury of the distal inferior orbital nerve (ION-CCI; Chen et al., 2021). In addition, injection of BoNT into the TMJ relieved nocturnal grinding, relieved facial pain, improved jaw function, and improved TMD symptoms. Although BoNT is known to be an effective treatment for TMD, the underlying mechanism is not well understood. Consequently, BoNT has not been recommended for the treatment of TMD (Busse et al., 2023). One of the proposed mechanisms of BoNT is to reduce pain by inhibiting the release of pain-related neurotransmitters such as glutamate and by affecting pain sensors such as transient receptor potential vanilloid (TRPV1; Aoki, 2003; Kumar, 2018; Bagues et al., 2024). In fact, BoNT effectively reduced glutamate release, reducing the excitability of nociceptive neurons and relieving TMD-related pain (Bittencourt da Silva et al., 2014; Moga et al., 2018; Bagues et al., 2024). However, the fundamental mechanism by which BoNT alleviates TMD pain remains unclear, and elucidating the mechanism will expand the scope of BoNT as a therapeutic agent for TMD.

Various mouse models have been used to study TMD, the most common being the complete Freund's adjuvant (CFA) injection model and the forced mouth opening (FMO) model (Xiang et al., 2021; Hou et al., 2023). In our previous studies, the CFA and FMO mouse models were used to explore the most suitable animal models for in vivo GCaMP Ca^{2+} imaging of intact TG neurons (Alshanqiti et al., 2024; Son et al., 2024). The FMO model was found to be suitable for TMD studies because it allowed in vivo GCaMP3 Ca^{2+} imaging, facilitating direct observation of TG neuron activity in response to a variety of stimuli. Results obtained in this model captured the neurogenic components of pain and identified potential therapeutic targets, such as the calcitonin gene-related peptide (CGRP) pathway.

Here, we used the FMO-induced TMD mouse model to determine how BoNT reduces pain associated with TMD. We found that BoNT inhibited pain signaling pathways by reducing glutamate release and inhibiting nociception-related proteins. These findings not only provide a deeper understanding of the pain mechanism of TMD but also indicate that BoNT may provide a new therapeutic approach for treating TMD pain.

Materials and Methods

Animals. For Ca^{2+} imaging, 3–5-month-old male Pirt-GCaMP3 mice were prepared as described in our previous study (Kim et al., 2014, 2016). In addition, 8–12-week-old male C57BL/6 mice were used for behavioral testing. Mice were housed 4–5 per cage and maintained at 22°C ± 2°C and 50% ± 10% humidity with a 12 h light/dark cycle. Mice were given *ad libitum* access to tap water and commercially available chow. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Texas Health Science Center at San Antonio (UTHSA). All animal experiments complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Forced mouth opening. Mice were anesthetized by intraperitoneal injection of ketamine/xylazine (90/13.5 mg/kg; Zoetis, KET-00002R2; VetOne, 33197) and had their mouths mechanically opened using a Colibri retractor (Fine Science Tools, 17000-03) for 3 h daily for 5 consecutive days. Control mice were anesthetized for the same period without forced opening of their mouths.

TMJ injection of BoNT. The BoNT preparation used in this study was Nabota (Daewoong Pharmaceutical), which is a BoNT A derived from the same strain of *Clostridium botulinum* as onabotulinumtoxin A (BoNT). Anesthetized mice were injected bilaterally with 20 µl of BoNT (0.5 or 1 U) intra-articularly using a Hamilton syringe with a 30-gauge needle. For the control group, mice were similarly injected with 20 µl of saline.

TMJ von Frey test. Mice were acclimated to the experimenter's odor and touch for 2 d. The mice were then habituated to the experimental chamber containing a clear Plexiglas chamber equipped with a 4 oz paper cup for 2 h daily for 3–5 consecutive days. Fifty percent withdrawal thresholds were calculated using the up-down method (Chaplan et al., 1994). After acclimation, a baseline test was performed to measure skin sensitivity in the temporomandibular region using von Frey filaments for 5–7 d. When the baseline values of the von Frey test reached between 0.5 and 0.7 g, the baseline test was terminated (day –6), mice were subjected to FMO (days –5 to –1) and BoNT was administered (day 0). Thresholds were then assessed using the von Frey test for 2 weeks following the BoNT injection (days 1, 3, 7, and 14).

Mouse grimace scale (MGS). The mouse grimace scale (MGS) test was performed 1 h before the TMJ von Frey test. Mice were acclimated to the testing environment for 1 h before each experiment. Since the mice were already habituated to the cup for the von Frey test, the cup was placed horizontally, allowing the mice to enter before video recording began. A high-resolution camera (iPhone 13 Pro) was used to record the facial expressions of the mice. Recording was conducted for a total of 10 min. To evaluate facial expressions, screenshots were captured every 1–2 min, collecting a total of five images per mouse for assessment. Each image was independently assessed for four facial action units: orbital tightening, nose bulge, cheek bulge, and ear position. Each feature was scored on a scale from 0 (not present) to 2 (obviously present), in accordance with the original MGS manual (Langford et al., 2010). The MGS score for each image was calculated as the average of the four action unit scores. Then, the final MGS score for each mouse was obtained by averaging the five image-level scores.

Pasta gnawing test. The pasta gnawing test was conducted with modifications based on a previously established protocol (Rabl et al., 2016). Prior to the experiment, mice were individually housed in a cage identical in shape and structure to their home cage, and food was removed for a fasting period of 3 h. To record gnawing sounds, a microphone was positioned between the cage lid and the wire mesh where the food was typically placed. Each mouse was provided with four pieces of dry spaghetti (00000) measuring 5 cm in length, and gnawing sounds were recorded for 10 min. The recorded audio files were analyzed using Audacity software. Background noise was removed (the software has background noise removal function) to enhance the clarity of biting events, allowing for precise extraction of biting sounds. A continuous sequence of pasta consumption was defined as a single episode, and the number of gnawing events per episode was counted based on peak amplitudes in the waveform. An episode was defined as a continuous sequence of gnawing events and was considered to end when a pause of >1 s occurred between successive bites. Isolated biting events that were not part of a continuous sequence were not counted as episodes.

Open field test. Mice were acclimated to the testing room for 1 h prior to the experiment. Mice were tested individually in a 40 cm × 40 cm × 30 cm (length × width × height) square arena, and their movements were recorded for 10 min. Following the recording, the total distance traveled (cm) and movements of the mice were analyzed using ToxTrac software.

Trigeminal ganglion exposure surgery for in vivo Pirt-GCaMP3 Ca^{2+} imaging. Trigeminal ganglion (TG) exposure surgery was performed as described previously (Son et al., 2024). The trigeminal ganglion was

exposed under anesthesia, and a heating pad was provided to maintain the body temperature at 37°C ± 0.5°C. After removing the skin and muscle, a dental drill (Buffalo Dental Manufacturing) was used to remove an ~10 × 10 mm portion of the right dorsal skull (parietal bone between the right eye and ear). The cortical tissue was then aspirated to expose the TG. The TG was observed using a confocal microscope after hemostasis.

In vivo Pirt-GCaMP3 Ca^{2+} imaging of intact TG. In vivo Pirt-GCaMP3 Ca^{2+} imaging of intact TG was performed as described in previous studies (Son et al., 2024). In vivo Pirt-GCaMP3 Ca^{2+} imaging of intact TG in live mice was performed under the confocal microscope on a custom-designed platform for 2–3 h immediately after TG exposure surgery. The mice were maintained at 37°C ± 0.5°C on a heating pad during imaging sessions. Anesthesia was maintained by 1–2% isoflurane using a gas vaporizer with pure oxygen. Live images were acquired at 10 frames per cycle in frame-scan mode at ~4.5–8.79 s/frame, at a depth of 0–900 µm, using a 5 × 0.25 NA dry objective at 512 × 512 pixels or higher resolution with solid diode lasers tuned at 488 nm and emission at 500–550 nm. Von Frey filament (0.4 and 2 g) and noxious water (4 and 50°C) were applied around the jaw muscles as mechanical and thermal stimuli. Chemical stimulation was provided by intracutaneous injection of 10 µl capsaicin (50 mM). Raw image stacks were acquired, processed through deconvolution, and imported into ImageJ (NIH) software. The optical planes from consecutive time points were aligned and corrected using the stackreg plugin (Thévenaz et al., 1998), which utilizes a rigid-body cross-correlation method for image alignment. The calcium (Ca^{2+}) signal amplitudes were quantified as the ratio of Ft (the fluorescence intensity for each frame) to F0 (the average fluorescence intensity from the initial one to four frames). A thorough visual inspection of the raw imaging data was performed to confirm each cell that showed a response.

Immunohistochemistry. TGs were isolated from mice 3 d after BoNT injection or saline injection. The mice were perfused with cold PBS and 4% paraformaldehyde (PFA) solution after anesthesia. TGs were fixed with 4% PFA for 24 h at 4°C, washed with PBS, then embedded in OCT compound, and stored at −80°C. Tissues were sliced into 15 µm thicknesses using a cryostat. Tissues were attached to slides, heated at 50°C for 30 min, and washed with PBS. The slides were incubated with guinea pig polyclonal anti-VGLUT2 antibody (1:200, #135-404, Synaptic Systems), or rabbit polyclonal anti-transient receptor potential canonical 1 (TRPC1) antibody (1:200, #ACC-010, Alomone Labs), or rabbit polyclonal anti-transient receptor potential ankyrin 1 (TRPA1) antibody (1:200, #ACC-037, Alomone Labs), or rabbit polyclonal anti-TRPV1 antibody (1:200, #ACC-030, Alomone Labs), and with chicken polyclonal anti-Neuronal Nuclei (NeuN) antibody (1:400, #ABN91, MilliporeSigma) overnight at 4°C. Primary antibodies were washed out with PBST (0.3% Triton X-100 in PBS) and incubated with goat secondary antibody to guinea pig IgG-Alexa Fluor 568 (1:300, Invitrogen), goat secondary antibody to rabbit IgG-Alexa Fluor 488 (1:300, Invitrogen), and goat secondary antibody to chicken IgG-Alexa Fluor 488 (1:300, Invitrogen) for 2 h at room temperature. Secondary antibodies were washed out with PBST, and sections were stained with DAPI readymade solution (MBD0015, MilliporeSigma) for 5 min and then washed with PBS three times. Cover slides were applied with ProLong Diamond Antifade Mountant (Invitrogen) and dried. Immunofluorescence images were observed using a 10× dry or 40× water immersion objective lens by confocal microscopy.

Western blotting assay. TGs were lysed using the N-PER Neuronal Protein Extraction Reagent (Thermo Fisher Scientific) with the Halt Protease Inhibitor Cocktail (Thermo Fisher Scientific). The protein concentration of the tissue lysates was determined using the Bradford reagent (# B6916; Sigma). Thirty micrograms of protein from each sample were separated via 8–10% SDS-PAGE gel and transferred to a nitrocellulose membrane. The membrane was blocked with 5% skim milk in a 1× TBS-Tween-20 (TBST; containing 0.1% Tween-20) at room temperature for 1 h and incubated with primary antibodies against VGLUT2 (1:2,500), TRPC1 (1:2,500), TRPA1 (1:2,500), TRPV1 (1:2,500), and TRPM8 (1:2,500) diluted in 3% skim milk in a 1× TBST buffer at 4°C overnight. The blot was then washed three times with 1× TBST buffer and incubated with anti-guinea pig (goat anti-guinea pig IgG-HRP; #A18769; Invitrogen), rabbit (goat anti-rabbit IgG-HRP; #31466; Invitrogen), and mouse (sheep anti-mouse IgG-HRP, #NA931V; Cytiva) HRP-conjugated secondary antibodies diluted 1:5,000 in 3% skim milk in 1× TBST buffer at room temperature for 1 h. Next, the membrane was washed three times with 1× TBST buffer, and the protein bands were detected with ECL detection reagents (catalog # RPN2235; Cytiva) and semiquantified using a Bioanalytical Imaging System (Azure 280; Azure Biosystems). The protein expression level of β-actin was used as the loading control.

Statistical analysis. Statistics were performed using GraphPad Prism 8.0. All data were analyzed by one-way ANOVA followed by post hoc Tukey's test or post hoc Dunnett's test, as appropriate. The data are reported as the mean ± standard error of the mean.

Results

BoNT injection into TMJ reduces mechanical hyperalgesia of the temporomandibular region

In a mouse model of ION-CCI surgery, long-term effects of BoNT injection were observed, with pain relief for up to 53 d after subcutaneous injection of BoNT (Chen et al., 2021). However, adverse effects may occur if BoNT is injected into sites other than TMJ. Therefore, we used the FMO-induced TMD mouse model and injected BoNT directly into the TMJ. To determine the effect of BoNT injection on TMD-induced hypersensitivity in mice, the TMJ mechanical von Frey test was performed. Mice were acclimated to the investigator and chamber for 4–5 d. After acclimation, a baseline test was performed over 5–7 d to measure skin sensitivity in the temporomandibular region using von Frey filaments. The baseline established by this test was 0.5–0.6 g, which was used for the experiment (Fig. 1*A*). Then, the animal's mouth was forced open for 3 h daily for 5 consecutive days (days −5 to −1; Fig. 1*A*). On day 0, 20 µl of BoNT (0.5 or 1 U) or saline was injected into the TMJ, and the von Frey test was performed over the next 2 weeks (Fig. 1*A*). In the FMO + saline group, the TMJ withdrawal threshold (g) was significantly reduced from day 1 compared with the control group (Fig. 1*B*) and remained so over the full 2 weeks (Fig. 1*B*). These results indicate that mechanical TMJ pain was successfully induced in the FMO-induced TMD mouse model. In contrast, the TMJ withdrawal threshold (g) of the FMO + BoNT (0.5 U) and FMO + BoNT (1 U) groups were significantly reduced compared with the FMO + saline group (Fig. 1*B*), indicating that mechanical hypersensitivity was alleviated.

The FMO + BoNT (0.5 U) group exhibited a reduction in mechanical hypersensitivity at day 7, which gradually diminished by day 14, whereas the FMO + BoNT (1 U) group exhibited strong reduction up to day 14 (Fig. 1*B*). To further evaluate pain levels, we performed the MGS test to assess the degree of pain. Consistent with the previous results, the FMO + saline group exhibited significantly higher pain scores than the control group over the 2 weeks (Fig. 1*C*,*D*). The FMO group showed typical signs of severe pain, including ears pulled back, nasal muscles clenched, eyes more than half closed, and forward-extending whiskers (Fig. 1*C*). In contrast, mice in the FMO + BoNT (0.5 U) group showed significantly reduced facial pain expression, although their ears were still slightly pulled back (Fig. 1*C*). Mice in the FMO + BoNT (1 U) group appeared almost identical to the control group (Fig. 1*C*). This effect of BoNT injection persisted for the full 2 weeks (Fig. 1*C*). These results indicate that the effect of BoNT was maintained at least for 2 weeks after high-dose BoNT (1 U) injection into the TMJ. Taken together, these results suggest that if a surgeon can accurately administer

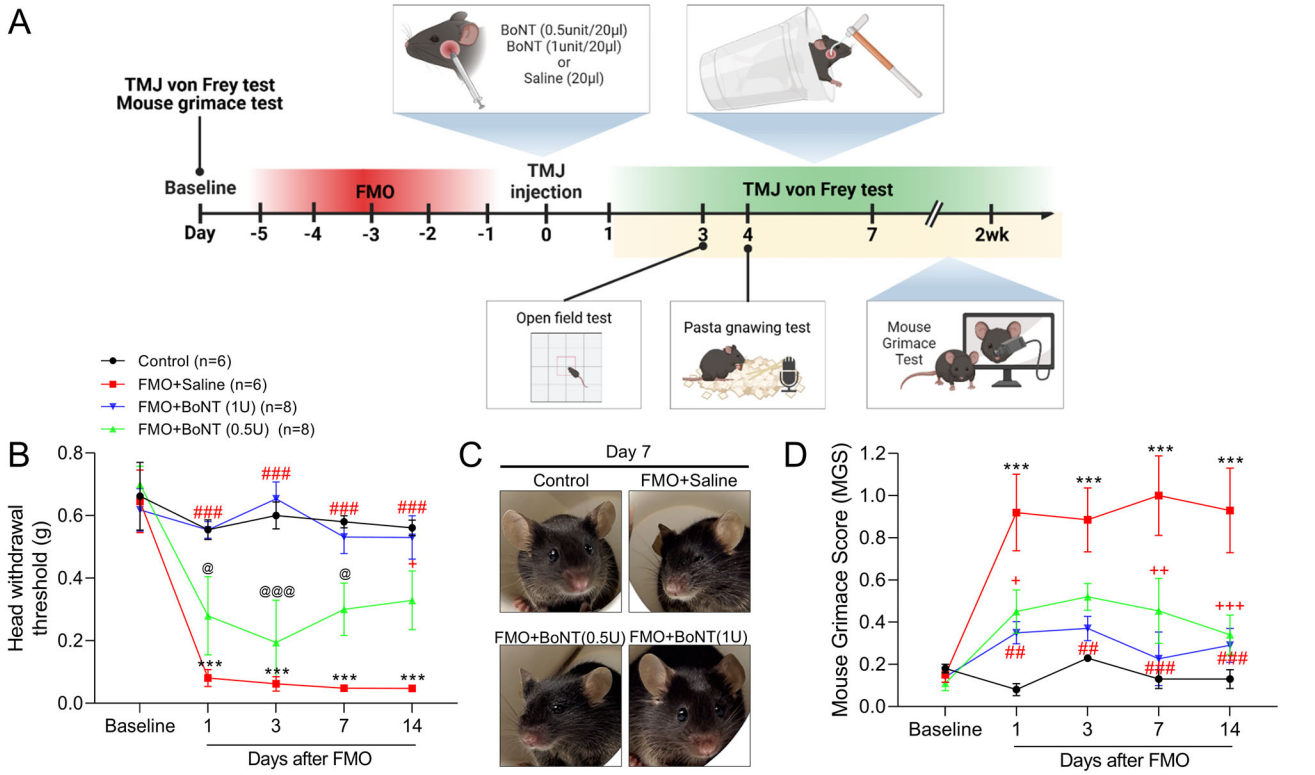


Figure 1. BoNT injection in TMJ reduces mechanical hyperalgesia of the temporomandibular region. ***A***, Experimental schedule. TMD induction using FMO was performed from day −5 to −1 after baseline tests. TMJ injection was done on day 0. TMJ mechanical pain sensitivity tests using von Frey filaments were performed on days 1, 3, 7, and 14. ***B***, Mechanical sensitivity is plotted as the 50% withdrawal threshold in grams. ***C***, ***D***, Mouse grimace scores (MGS) were measured at baseline and days 1, 3, 7, and 14. Comparisons of 50% withdrawal thresholds and MGS were performed with two-way ANOVA multiple comparisons followed by post hoc Tukey's test; Control versus FMO + saline: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$; Control versus FMO + BoNT (0.5 U): $^{@}p < 0.05$, $^{@@}p < 0.01$, $^{@@@}p < 0.005$; FMO versus FMO + BoNT (0.5 U): $^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.005$; FMO versus FMO + BoNT (1 U): $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.005$.

BoNT into the TMJ, a single BoNT injection can minimize possible side effects and achieve long-term pain relief.

BoNT injection relieves TMJ pain and facilitates food intake without impairing motor movement

The pasta gnawing test was conducted to evaluate the alleviation of TMJ pain and the related feeding challenges. The FMO + saline group showed a significant reduction in the amplitude peak and spectrum (biting episodes) of biting for food intake, showing that TMJ injury caused food intake problem and TMJ pain (Fig. 2*A*,*B*; Extended Data Audios 1–4). In contrast, the FMO + BoNT (1 U) group showed a significant increase in biting episodes compared with the FMO + saline group (Fig. 2*A*,*B*; Extended Data Audios 1–4). Localized facial and neck muscle weakness and chewing difficulties have been reported as side effects of BoNT injections (Mor et al., 2015). As a result, avoidance responses in tests like von Frey behavior test may not have been observed. To address this concern, we evaluated general motor behavior using the open field test (Fig. 2*C*,*D*) and pasta gnawing test for facial motor (Fig. 2*A*,*B*). The results showed that TMJ injury by FMO significantly reduced the total distance traveled (cm) in the FMO + saline group (Fig. 2*D*) and biting episodes were impaired in the FMO + Saline group (Fig. 2*A*,*B*). However, no significant differences were observed between control and BoNT-injected groups, indicating that BoNT did not impair general motor behavior (Fig. 2*B*,*D*). According to these results, eating issues are improved by TMJ injection of BoNT, which reduces TMJ pain without impairing overall motor behavior and movement. Given that the 1 U dose injection of BoNT showed higher efficacy in both the pasta gnawing and TMJ von Frey tests, we used 1 U as the final dose for further trials.

BoNT injection in TMJ reduces mechanical hypersensitivity in the temporomandibular region

To monitor spontaneously activated TG neurons in the absence of stimulation, in vivo Pirt-GCaMP3 Ca^{2+} imaging of intact TG was performed in the FMO-induced TMD mouse model. Representative heatmaps of spontaneously activated neurons are shown in Figure 3*A*. In the control group, a total of 26 ± 4.6 spontaneously activated neurons were observed in the TG including regions V1, V2, and V3 (Fig. 3*B*, Movies 1–3). In the FMO + saline group, the total number of spontaneously activated neurons (60 ± 7.6) was significantly increased compared with the control group (Fig. 3*C*), most prominently in V2 and V3 (Fig. 3*B*, Movies 1–3). In the control group, small-diameter TG neurons (<20 μm) accounted for the largest proportion of spontaneously activated TG neurons, followed by medium-diameter (20–25 μm) and large-diameter (>25 μm) neurons (Fig. 3*C*). In the FMO + saline group, the number of activated small- (42 ± 5.9) and medium-diameter neurons (17.2 ± 3.4) was significantly increased compared with the number of spontaneously activated small- (13.6 ± 3.0) and medium-diameter neurons (8.2 ± 1.6) in the control group (Fig. 3*C*). However, in the FMO + BoNT group, the number of spontaneously activated total (31.8 ± 3.9), small-diameter (16.6 ± 4.6), and medium-diameter (11.6 ± 3.2) TG neurons was significantly decreased compared with the FMO + saline group (total 60 ± 7.6, small 42 ± 5.9, medium 17.2 ± 3.4; Fig. 3*C*). Additionally, in of the FMO + saline group, the number of spontaneously activated neurons in the trigeminal ganglion mandibular branch (V3), which is directly linked to the TMJ, was significantly higher than the number of spontaneously activated neurons in other regions of the TG. On the other hand, in

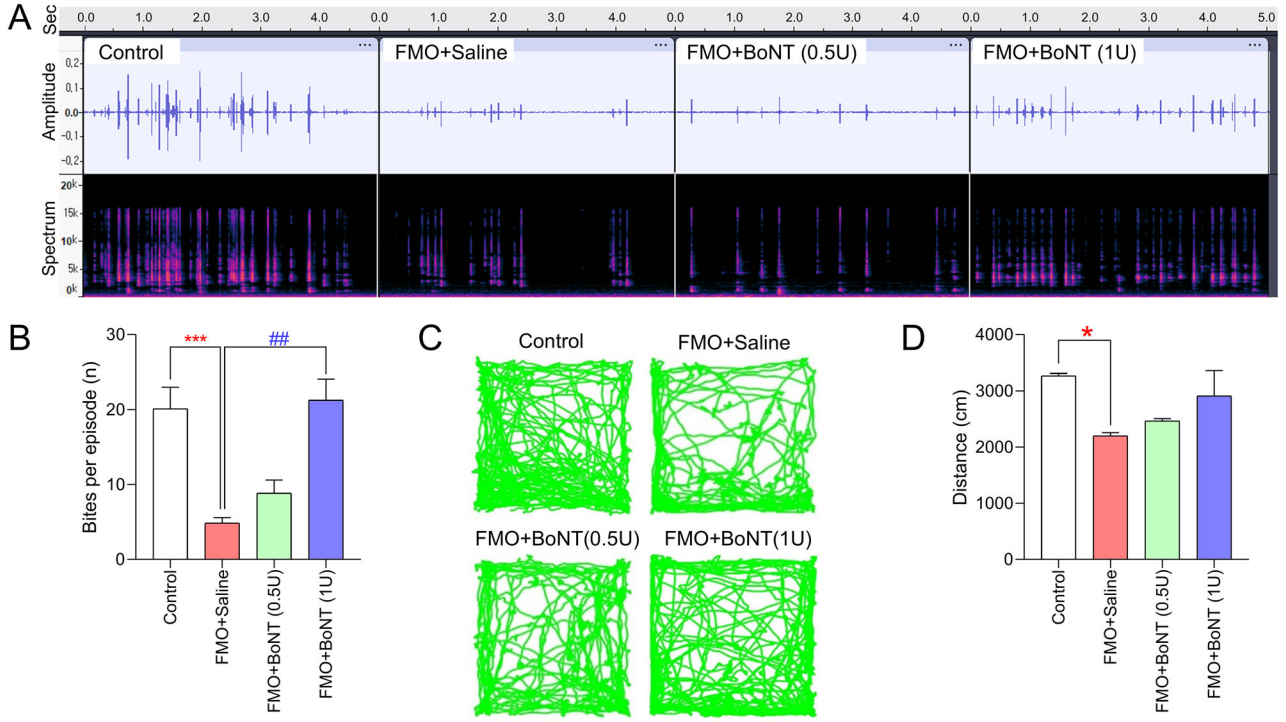


Figure 2. BoNT injection relieves TMJ pain and food intake problem without impairing movement. ***A***, Representative biting patterns for 5 s (amplitude and spectrum) of each condition. ***B***, Total number of bites per episode (*n*) for 10 min on day 4. ***C***, ***D***, Sample traces and total distance in the open field test. Comparisons of bites per episode (panel ***A***) and distance (panel ***C***) were performed with two-way ANOVA multiple comparisons with post hoc Dunnett's test compared to FMO + saline; Control versus FMO + saline: $*p < 0.05$, $**p < 0.01$, $***p < 0.005$; FMO + saline versus FMO + BoNT (1 U): $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.005$.

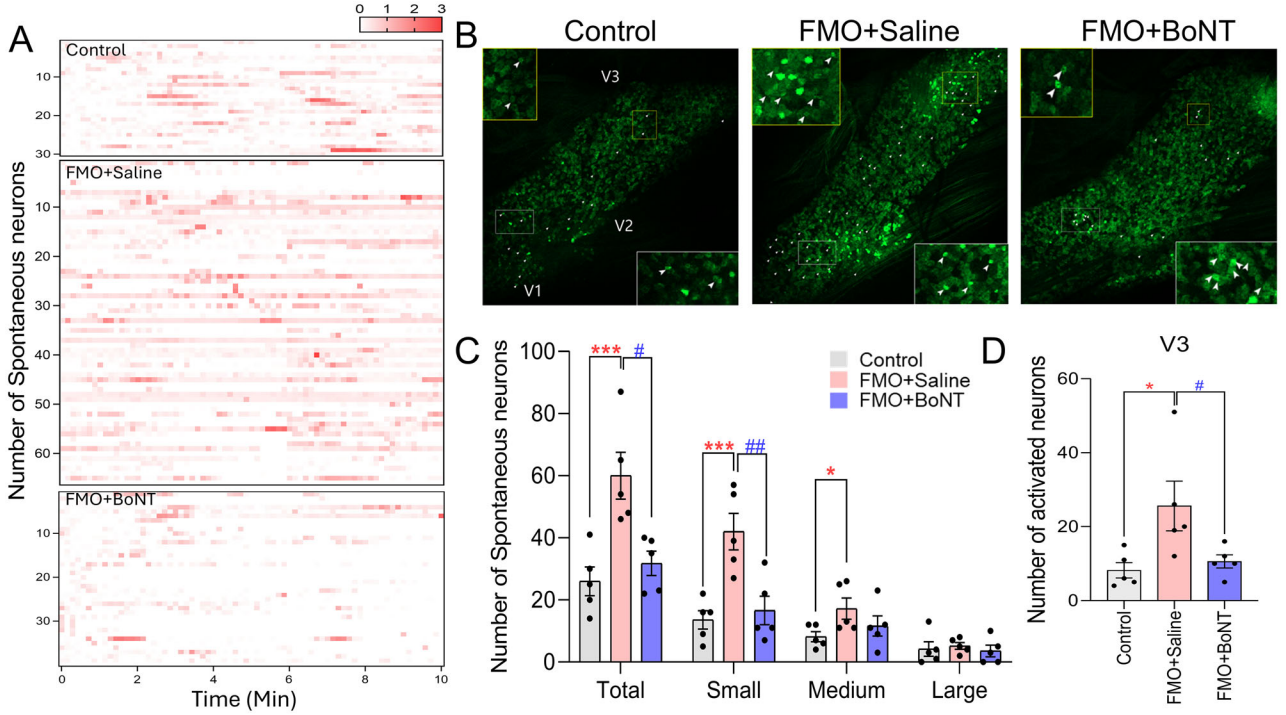
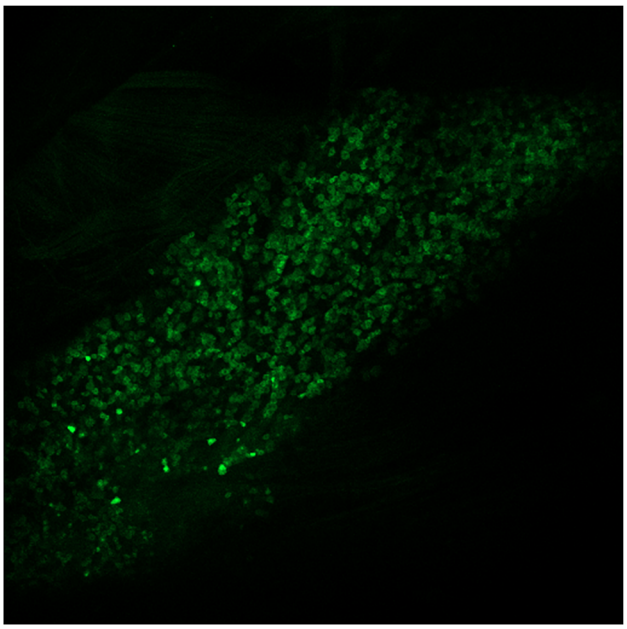
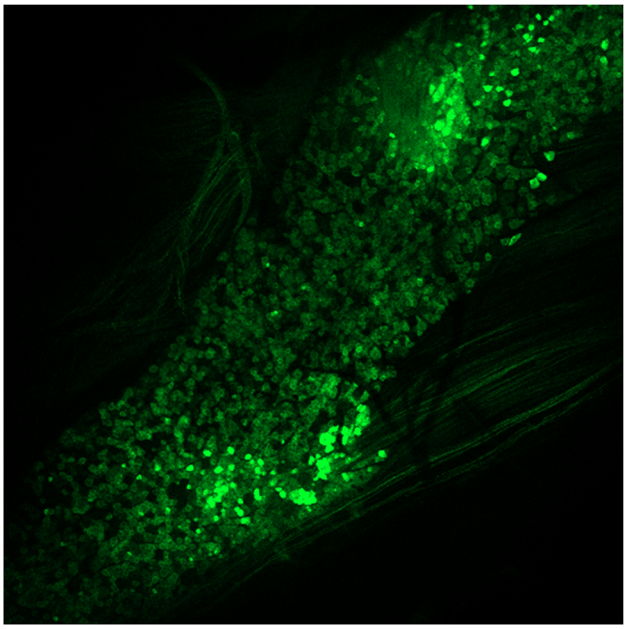


Figure 3. BoNT injection in TMJ reduces mechanical hypersensitivity in the temporomandibular region. ***A***, Representative heatmaps of spontaneously activated individual neurons. ***B***, Representative images of a single frame of spontaneous activities in in vivo Pirt-GCaMP3 Ca^{2+} imaging of intact TG in TMD-induced mice. V1, V2, and V3 indicate locations of TG regions and boxes are magnified areas from V2 and V3. ***C***, The number of total, small, medium, and large spontaneously activated neurons from each group ($n = 5$ per group). ***D***, Total number of activated neurons in V3 region. Comparisons of numbers of spontaneously activated neurons between control and experimental groups were performed with two-way ANOVA multiple comparisons followed by post hoc Dunnett's test compared to FMO + saline; Control versus FMO + saline: $*p < 0.05$, $**p < 0.01$, $***p < 0.005$; FMO + saline versus FMO + BoNT: $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.005$.



Movie 1. Related to Figure 3. Representative in vivo GCaMP imaging of an intact TG neuron from control naive mouse in spontaneous activity (no stimulus). [View online]

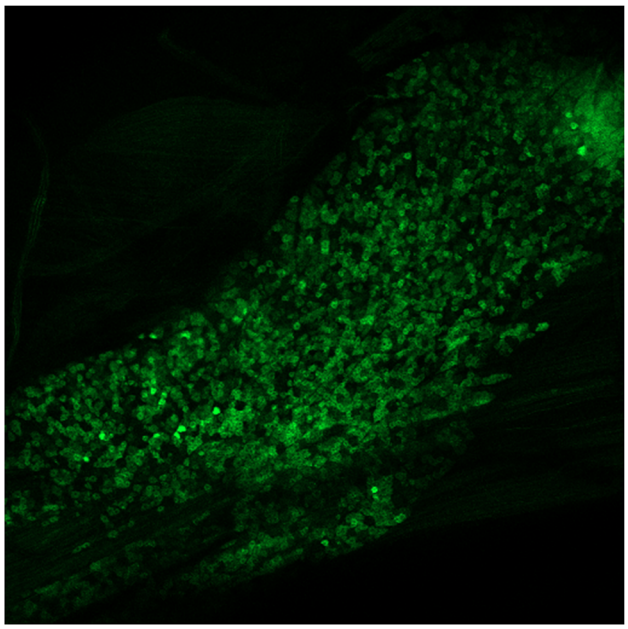


Movie 2. Related to Figure 3. Representative in vivo GCaMP imaging of an intact TG neuron from FMO mouse in spontaneous activity (no stimulus). [View online]

the FMO + BoNT group the number of spontaneously activated neurons was significantly reduced (Fig. 3*D*). These data suggest that TMJ injection of BoNT ameliorates TMJ hypersensitivity in mice.

BoNT injection into TMJ suppresses hypersensitivity of TG neurons

To monitor neurons activated by different stimuli, in vivo Pirt-GCaMP3 Ca^{2+} imaging of intact TG was performed in the FMO-induced TMD mouse model. First, von Frey filaments, ranging from weak (0.4 g) to powerful (2 g), were used to stimulate the TMJ mechanically. In the FMO + saline group, the



Movie 3. Related to Figure 3. Representative in vivo GCaMP imaging of an intact TG neuron from FMO + BoNT mouse in spontaneous activity (no stimulus). [View online]

number of activated neurons significantly increased compared with the control group regardless of strength of mechanical stimulus (Fig. 4*A*,*B*). However, in the FMO + BoNT group, following mechanical stimulation, significantly fewer activated neurons were observed compared with the control group (Fig. 4*A*,*B*). We then monitored neurons activated by stimulation with hot (50°C) or cold (4°C) water (Son et al., 2024). In the FMO + saline group, the number of neurons activated with hot or cold stimuli significantly increased compared with the control group (Fig. 4*C*,*D*). However, in the FMO + BoNT group, following hot or cold stimulation, significantly fewer activated neurons were observed compared with the control group (Fig. 4*C*,*D*). Finally, capsaicin, which causes pain and hyperalgesia by activating nonselective cation channels on small-diameter neurons that express TRPV1 receptors (Fattori et al., 2016), was used as a chemical trigger. In the FMO + saline group, the number of neurons activated with capsaicin stimulus significantly increased compared with the control group (Fig. 4*E*). However, in the FMO + BoNT group, the number of neurons activated with capsaicin stimulus significantly decreased compared with the control group (Fig. 4*E*). Overall, these data suggest that BoNT injection into TMJ after FMO suppresses the hypersensitivity of TG neurons to a variety of stimuli.

BoNT injection into TMJ suppresses the expression of pain sensing proteins in TG

To examine changes in pain sensing proteins in TG, immunohistochemical analyses of pain sensing proteins (TRPC1, TRPA1, and TRPV1) were performed. Transient receptor potential (TRP) channels are multimodal ion channels that function as sensors for stimuli that are chemically and physically harmful (Chen et al., 2020). TRPC1 monitors mechanical stimulation sensitivity (Garrison et al., 2012). TRPA1 is a sensor for a range of hazardous environmental stimuli, including reactive chemicals, cold, irritants, and mechanical stimulation (Naert et al., 2021). TRPV1 serves as a sensor for a range of pain stimuli, such as vanilloid and capsaicin, as well as heat, pressure, and

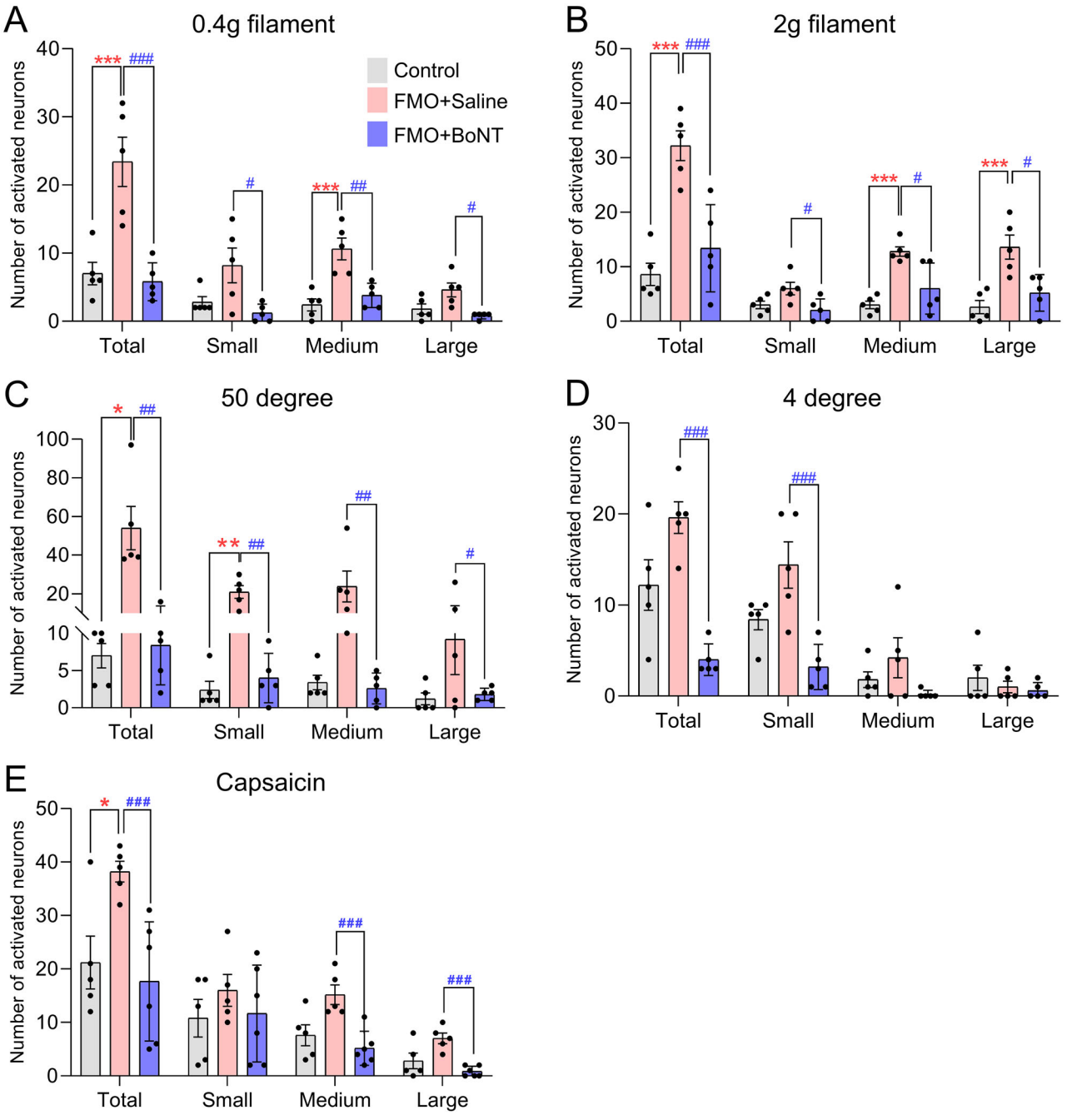


Figure 4. BoNT injection in TMJ suppresses hypersensitivity of TG neurons. Numbers of total, small, medium, and large neurons activated by each stimulus: 0.4 g von Frey filaments (***A***), 2 g von Frey filaments (***B***), 50°C water (***C***), 4°C water (***D***), and capsaicin (***E***). Comparisons of numbers of activated neurons in response to stimuli between control and experimental groups were performed with two-way ANOVA multiple comparisons followed by post hoc Dunnetts's test compared to FMO + saline; Control versus FMO + saline: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$; FMO versus FMO + BoNT: $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.005$.

pH (Tomohiro et al., 2013; Muller et al., 2018; Chen and Li, 2021; Seebohm and Schreiber, 2021). Since BoNT injection suppressed hypersensitivity to various stimuli in the TMD mouse model (Fig. 4), the expression of TRP channels that generate pain in response to chemical, physical, cold, and hot stimuli were examined using immunohistochemistry (IHC) and Western blot. IHC data showed that there was no difference in protein expression of TRPC1, TRPA1, or TRPV1 in the FMO + saline group compared with the control group (Fig. 5*A*,*B*). However, in the FMO + BoNT group, the expression of TRPC1 and TRPA1 was significantly reduced compared with the control group (Fig. 5*A*,*B*). To verify the expression of pain sensing proteins in TG, Western blot assays were performed. Western blot assay data showed that the expression of TRPA1 and TRPV1 proteins increased in the FMO + saline group compared with the control group and significantly decreased in the FMO + BoNT group (Fig. 5*C*,*D*) confirming the IHC findings. These results suggest that injection of BoNT into the TMJ inhibits the expression of pain sensing proteins TRPC1 and TRPA1.

BoNT injection into TMJ suppresses the expression of VGLUT2 in TG

To indirectly examine any changes in glutamate release from TG, immunohistochemical assays were performed on vesicular

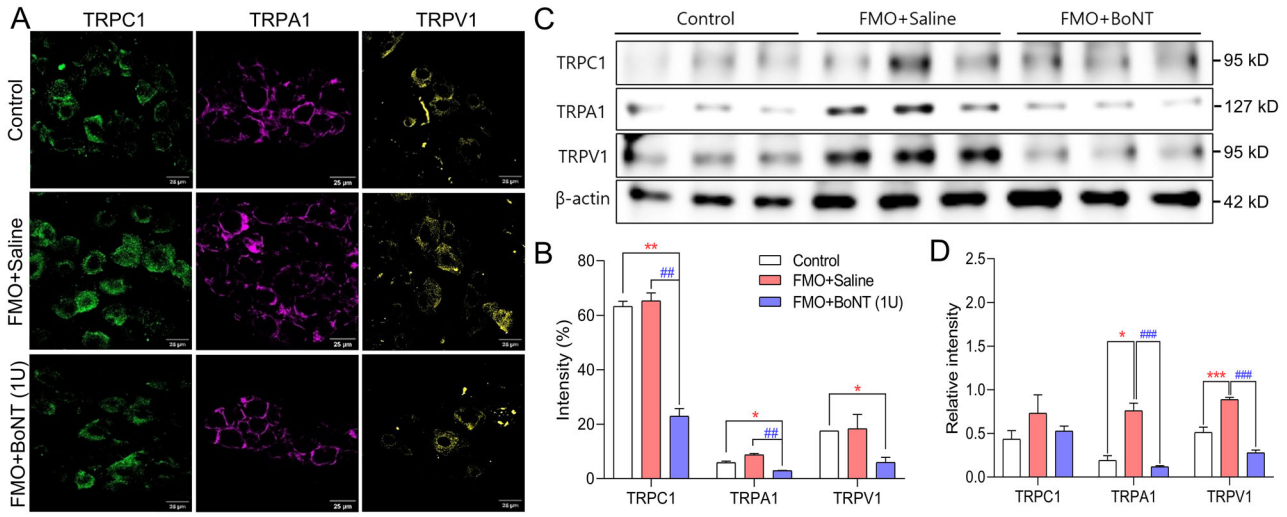


Figure 5. BoNT injection in TMJ suppresses the expression of pain receptor proteins. ***A***, Immunohistochemical analysis of TrpC1, TrpA1, and TrpV1 expression in TGs (scale bar: 25 µm, 400×). ***B***, Quantification of data from images in ***A***. ***C***, Western blot analysis of TRPC1, TRPA1, and TRPV1 in TGs. ***D***, Quantification of data shown in ***C***. Comparisons of intensity of staining of activated neurons in response to stimuli between control and experimental groups were performed with two-way ANOVA multiple comparisons followed by post hoc Tukey's test; Control versus FMO + saline: $*p < 0.05$, $**p < 0.01$, $***p < 0.005$; FMO versus FMO + BoNT: $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.005$.

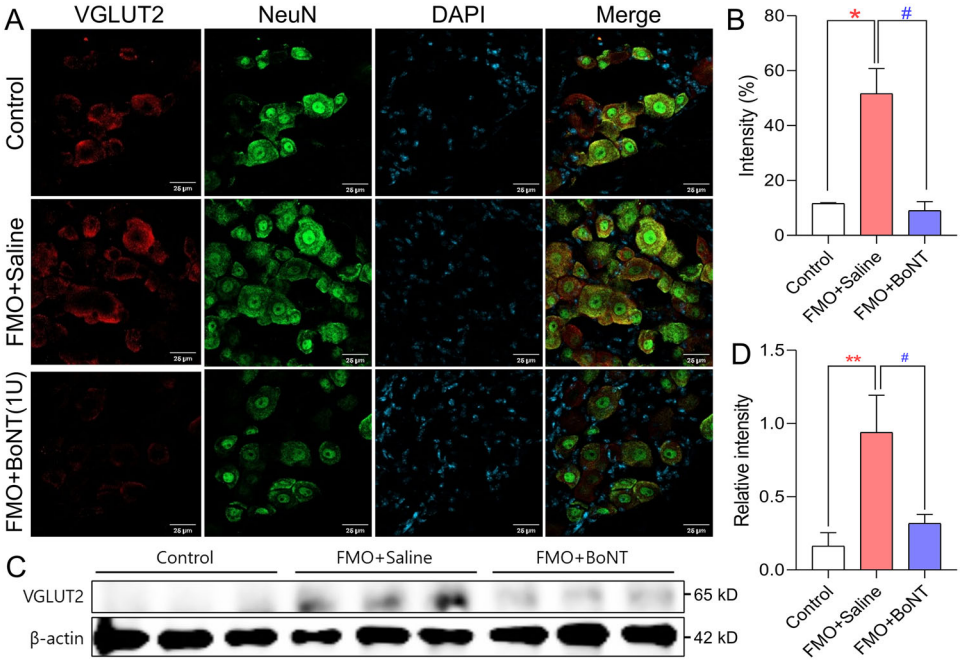


Figure 6. BoNT injection into TMJ suppresses the expression of VGLUT2. ***A***, Immunohistochemical analysis of VGLUT2, NeuN, and DAPI in TGs (scale bar: 25 µm, 400×). ***B***, Quantification of VGLUT2 expression data from images in ***A***. ***C***, Western blot analysis of VGLUT2 in TGs. ***D***, Quantification of data shown in ***C***. Comparisons of VGLUT2 staining intensity (***B***) or protein band intensity (***D***) of activated neurons between control and experimental groups were performed with two-way ANOVA multiple comparisons followed by post hoc Dunnett's test compared to FMO + saline; Control versus FMO + saline: $*p < 0.05$, $**p < 0.01$, $***p < 0.005$; FMO + saline versus FMO + BoNT: $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.005$.

glutamate transporter 2 (VGLUT2) protein. In the FMO + saline group, the expression of VGLUT2 protein was significantly increased compared with the control group (Fig. 6*A*, *B*). However, in the FMO + BoNT group, the expression of VGLUT2 protein was significantly decreased compared with the control group (Fig. 6*A*,*B*). To verify the expression of VGLUT2 in TG, Western blot assays were also performed. As shown in immunohistochemical assays, in the FMO + saline group, the expression of VGLUT2 protein was significantly increased compared with the control group (Fig. 6*C*,*D*). However, in the FMO + BoNT group, the expression of VGLUT2 protein was significantly decreased compared with the FMO + saline group (Fig. 6*C*,*D*). These results indirectly suggest that BoNT injection into TMJ reduces the expression of VGLUT2, which may be associated with a potential reduction in glutamate release.

Discussion

TMD causes difficulty chewing and is associated with jaw pain in the TMJ and surrounding muscles, significantly reducing

the quality of life of patients. TMD pain is initially caused by peripheral sensitization that activates pain sensing proteins resulting in the release of neurotransmitters and neuropeptides (List and Jensen, 2017; Ferrillo et al., 2022). Interactions with inflammatory mediators, such as prostaglandins, substance P, and CGRP, induce hypersensitivity in pain-sensitive nerve fibers in TMD (Iyengar et al., 2017; Shrivastava et al., 2021). Although the use of BoNT has been proposed and demonstrated to be effective in the treatment of TMD, the lack of understanding of the underlying pain modulation mechanisms by BoNT has limited its use. Here, we studied the effects of BoNT using in vivo Pirt-GCaMP3 Ca^{2+} imaging to observe changes in TG neurons in live mice in real time, which allowed us to understand the inhibitory effects of BoNT on neural activity. Specifically, after direct injection of BoNT into the TMJ, we found that the sensitivity of TMJ neurons was dramatically reduced. These data suggest that administration of BoNT significantly alleviates peripheral hypersensitivity of the TMD.

BoNT has been widely used in the treatment for chronic migraine, neuropathic pain, back pain, and pelvic pain (Pfau et al., 2009; Diener et al., 2010; Rivera Día et al., 2014). In addition, a single injection of BoNT was shown to be effective for 1 month in patients with nocturnal bruxism who present with TMD-like symptoms and facial discomfort (Shim et al., 2014, 2020). Our study using the von Frey behavioral test also confirmed that a single BoNT injection provides long-term pain relief lasting for at least 2 weeks. Clinical studies will be needed to determine whether BoNT injection into TMJ relieves facial pain in TMD lasting longer than 2 weeks. Our study using the von Frey behavioral test also confirmed that a single BoNT injection provides long-term pain relief lasting for at least 2 weeks. Although sex-based differences in TMJ pain sensitivity are widely known, our pilot study did not reveal any significant differences in the pain response between male and female mice under our experimental conditions. Therefore, to minimize hormonal fluctuations and ensure experimental consistency, male mice were used in the main study.

Many studies have used the subcutaneous injection into jaw muscles, whisker pad, or face rather than TMJ injection to confirm pain relief effects of BoNT (Wu et al., 2016; Chen et al., 2021). In our study, BoNT was injected directly into the TMJ to confirm antinociceptive effects of BoNT in TMJ pain. TMJ injection of BoNT has been reported in several studies to be effective in relieving TMD symptoms (Béret et al., 2023); however, there can be adverse effects if given incorrectly or excessively. When BoNT is injected into incorrect areas or in inappropriate doses, several side effects may occur, including muscle weakness, facial asymmetry, and difficulty swallowing or speaking (Mor et al., 2015). Therefore, the key to avoiding side effects lies in the precision of the injection, which relies on the provider's expertise. We injected BoNT into the TMJ intra-articular area, which is anatomically situated beneath the zygomatic arch and anterior to the external auditory meatus (Morel et al., 2019).

To assess whether our direct TMJ injection method resulted in similar adverse effects, we conducted behavioral tests, including the open field test, pasta gnawing test, and mouse grimace test. Our results showed that BoNT-injected mice exhibited scores similar to the control group, with no observable muscle abnormalities, facial dysfunction, or ocular issues. We confirmed that carefully injecting BoNT into the TMJ produced pain relief while minimizing adverse side effects.

Furthermore, our findings suggest that BoNT effectively reduces pain in the FMO model by modulating trigeminal ganglion activity. However, the precise mechanism by which BoNT exerts its effects remains to be fully elucidated. One possibility is that BoNT directly acts on TMJ pathology. Our previous study demonstrated a significant increase in the level of CD45+ immune cells in the TMJ area (Alshanqiti et al., 2024). Given this, BoNT may mitigate pain by directly modulating inflammatory pathways within the TMJ rather than merely serving as a site of entry. Alternatively, it is possible that BoNT primarily acts within the trigeminal ganglion after being transported from the TMJ. Further studies will be necessary to distinguish between these mechanisms and to determine whether the primary site of BoNT action is within the TMJ itself or via trigeminal ganglion.

The fact that the exact mechanism of action of BoNT has not yet been determined is another crucial factor contributing to its incomplete consideration as a therapy for TMD. The pain relief mechanism of botulinum toxin (BoNT) is still not fully understood, although it is believed to involve both central and peripheral pathways (Kumar, 2018). According to Beatrice Oehler et al., when BoNT was injected into the hindpaw, no changes in signal transmission were observed in dorsal root ganglion (DRG) neurons that had been hyperactivated by CFA (Oehler et al., 2022). The research team suggested that the analgesic effect of BoNT may be mediated through a central rather than a peripheral neural mechanism. In contrast, we found that direct injection of BoNT into the temporomandibular joint (TMJ) alleviated the hyperactivity of peripheral neurons in the trigeminal ganglion (TG), thereby reducing pain. This was confirmed through in vivo Pirt-GCaMP3 Ca^{2+} imaging of intact TG neurons. Some studies have hypothesized that BoNT may influence peripheral nociceptive pathways by preventing the synthesis of neuropeptides such as substance P, CGRP, and glutamate, which are known to be involved in inflammation and pain transmission (Matak and Lacković, 2014). This suggests that peripheral sensitization may play a significant role in the analgesic action of BoNT, especially in conditions affecting the muscles and skeleton including temporomandibular disorders (TMD). Thus, it may be that the effects of BoNT involve both central and peripheral pathways. Although peripheral effects are usually transient, central mechanisms, such as brainstem or spinal cord modulation, may promote longer-lasting pain reduction. It is likely that complete understanding of the BoNT pain-relieving mechanism will enable more effective therapeutic usage in illnesses such as TMD, especially in the treatment of TMJ pain.

Sensitization of peripheral neurons leads to the activation of pain sensing ion channels such as TRPV1 and TRPA1, which induce hypersensitivity and hyperalgesia in the TMJ or vice versa (Wang et al., 2023). Using a live TMD mouse model induced by FMO, we observed spontaneous activity and responses of TG neurons to stimulation and showed that these neurons were more sensitive than control neurons, consistent with other reports (Khalil et al., 2018; Zhang et al., 2023). In our study, the protein expression levels of TRPC1, TRPA1, and TRPV1 were decreased after BoNT injection into the TG. These results showed a reduction in neurons activated in response to various stimuli (mechanical, hot, cold, and capsaicin) suggesting that BoNT reduced the expression of pain receptors such as TRPC1, TRPA1, and TRPV1, thereby reducing neuronal responsiveness to these stimuli. Unexpectedly, in the FMO mouse model, the expression of TRPV1, TRPC1, and TRPA1 in the TG was not increased, which was not consistent with the increased sensitivity to heat, mechanical, and capsaicin

stimulation of TG neurons following FMO. However, the expression of all of these ion channels decreased after BoNT injection in the FMO-induced TMD mouse model. Nonetheless, we cannot definitively conclude that pain relief was entirely due to the decreased expression of nociceptors. Our results suggest that hypersensitivity to capsaicin, cold, heat, or mechanical stimulation is likely due to posttranslational modifications such as phosphorylation of TRPV1, TRPC1, and TRPA1 rather than to ectopic expression of nociceptors in the TG (Khalil et al., 2018). To fully understand the effects of BoNT, it will be important to delineate the neurochemical and gene expression changes in the TG following BoNT injection.

High levels of glutamate are usually associated with high pain levels. Primary afferent synapses and neurons use glutamate as their primary excitatory neurotransmitter (Wozniak et al., 2012). VGLUT2, an isoform of the vesicular glutamate transporter that regulates glutamate release, has been identified as a key player in neuropathic pain (Moechars et al., 2006; Weston et al., 2011). Some studies on the peripheral nervous system showed a reduction in TMJ pain by inhibiting neurotransmitter and glutamate release (List and Jensen, 2017; Bagues et al., 2024; Hosseindoost et al., 2024). Studies have reported that BoNT primarily alleviates pain in the central and peripheral nervous systems by inhibiting glutamate release (Tsutsuki et al., 2007; Bagues et al., 2024). In addition, intraplantar injection of BoNT in the hindpaw downregulated SNAP-25 and consequently decreased VGLUT2 expression in a mouse model of neuropathic pain induced in the chronic constriction injury model (Wang et al., 2019). In our study, VGLUT2 protein expression in the FMO group was significantly increased, but BoNT injection decreased the VGLUT2 level in the TG. These results indicate that BoNT injection into TMJ effectively suppressed VGLUT2 expression in the TG, which may be linked to a reduction in glutamate release.

In summary, we observed that BoNT suppresses TMJ hypersensitivity in the distal nerve of the TG, and this analgesic effect lasts for at least 2 weeks. These data suggest that TMJ injection of BoNT may be an option for effective, long-lasting treatment of TMJ pain.

Data Availability

Data sharing is applicable to this article as new data were created or analyzed in this study.

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