

Botulinum Neurotoxin A Prevents the Development of Nitroglycerin-Induced Chronic Migraine via Inhibition of CGRP and NLRP3 Inflammasomes in Mice

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Abstract

Background This study was designed to examine the therapeutic effects of Botulinum neurotoxin A (BoNT/A) on chronic migraine and to explore the potential mechanisms by using a mouse model of chronic migraine, which was established by repeated intraperitoneal (i.p.) injection with nitroglycerin (NTG).

Methods NTG-induced basal and evoked mechanical hypersensitivity were evaluated using the von Frey filament test. Before the first injection of NTG, a single facial injection of BoNT/A was administered in the supraorbital region to explore its preventive effects on the development of chronic migraine in mice. The expression of calcitonin gene-related peptide (CGRP) and synaptosomal-associated protein25 (SNAP25) in the trigeminal ganglia (TG) and the trigeminal nucleus caudalis (TNC) were detected by Western blotting and immunostaining.

Results Repeated administration of NTG resulted in basal and evoked mechanical hypersensitivity in mice. Single facial BoNT/A injection prevented the development of NTG-induced mechanical hypersensitivity in mice. Western blotting results revealed that peripheral BoNT/A injection decreased the NTG-induced upregulation of expression of CGRP and SNAP25 in the TGs and NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3) inflammasomes in the TNC. Immunostaining results revealed peripheral BoNT/A injection also decreased the NTG-induced upregulation of CGRP expression in the TNC.

Conclusions Thus, these results indicate that single facial injection of BoNT/A may be a preventive treatment of chronic migraine, possible due to the inhibitory effect of BoNT/A on the expression of CGRP and SNAP25 in the TGs and NLRP3 inflammasomes in the TNC.

Background

Chronic migraine is a very common neurological disease worldwide, and the global prevalence is typically range from 1.4–2.2%[1]. Chronic migraine has great negative effects on the quality of life, psychological health, interpersonal relationships, and financial stability of family[2]. Studies suggest that chronic migraine usually develops from episodic migraine that gradually increases in attack frequency, with an annual progression rate of about 3%[3]. The most significant factors that raised the risk of conversion from episodic to chronic migraine are the overuse of acute migraine medication, often ineffective acute treatment[4]. The development of novel preventive treatment of chronic migraine is urgently required to improve the current management of chronic migraine.

To date, the mechanisms underlying chronic migraine are still poorly understood [5]. Generally, the activation of the trigeminovascular system is considered as a common pathway involved in many primary headache disorders. The trigeminovascular system plays a critical role in the connection between peripheral events and central sensitization. The promising results from recent clinical trials provided a strong indication that calcitonin gene-related peptide (CGRP) plays an important role in the initiation and

development of chronic migraine[6]. CGRP can be released from the trigeminal ganglion neurons, and it can interact with adjacent neurons and satellite glial cells to perpetuate peripheral sensitization. CGRP can also be transported to and released from the central terminals of TG neurons that located in the trigeminal nucleus caudalis (TNC), which can drive the central sensitization of the second-order neurons in the TNC[7]. A shift from activity-dependent to activity-independent central sensitization may be a crucial mechanism driving the progression of episodic migraine to chronic migraine[7]. Recently, neuroinflammation is appreciated as an important mechanism of central sensitization induced by chronic migraine[8]. Glia (especially microglia) surrounding TNC neurons can directly or indirectly influence the establishment of central sensitization related to chronic migraine[9]. Interestingly, it was reported that microglia NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3) inflammasomes were activated in the TNC in NTG-induced chronic migraine mouse model [8]. Thus, targeting neuroinflammation (especially NLRP3 inflammasomes) may be a novel strategy to develop novel preventive treatments of chronic migraine.

Botulinum neurotoxin A (BoNT/A) is a protein neurotoxin produced by the bacterium *Clostridium botulinum*. At the neuromuscular junction, BoNT/A prevents the release of the neurotransmitter acetylcholine from axon endings and thus causes flaccid paralysis. Nowadays, BoNT/A is clinical applied to treat many types of chronic pain, including chronic migraine. Intriguingly, the clinical use of BoNT/A in chronic migraine is now continuously expanding because of its outstandingly high potency and long-lasting duration of action[10]. However, the molecular mechanisms underlying the therapeutic effects of BoNT/A for chronic migraine are largely unknown. Traditionally, the blockade of neurotransmitters release by BoNT/A were thought to be a major contributor to its analgesic effects. In a recent study, appearance of cleaved SNAP25 in rat spinal cord following peripheral administration of BoNT/A suggests that BoNT/A is retrogradely transported through the peripheral nervous system (PNS) to central nervous system (CNS). Thus, we postulated that peripheral administration of BoNT/A may be a powerful method to modulate neuron–glia interactions in the CNS, especial under chronic pain conditions[11].

Our present research was aim to investigate the possible role of CGRP, SNAP25, and NLRP3 inflammasomes in therapeutic effects of BoNT/A on chronic migraine, which was modeled by NTG-induced chronic migraine in mice. Herein, chronic migraine-related behavioral and neurochemical changes were investigated in the TGs and TNC of the NTG-treated mice. Our results demonstrated for the first time that the preventive effects of BoNT/A on chronic migraine may be attributed to its inhibitory effects on the upregulation of CGRP and NLRP3 inflammasomes in mice. Taken together, our results suggest BoNT/A therapy is a preventive treatment of chronic migraine, and indicate CGRP and NLRP3 inflammasomes may be possible targets for BoNT/A.

Methods

Animals

Male adult C57BL/6 mice, weighing 20–25 g were purchased from Shanghai Laboratory Animal Center (Shanghai, China). All animals were housed in separated cages under standard laboratory conditions with a 12-h light/dark cycle and the room was kept at 22 ± 2 °C and 40–60% humidity. Food and water were offered ad libitum. The protocols in this study followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All animal care and experimental procedures were approved by the Institutional Animal Care and Use Committee of Soochow University. Animal experiments were conducted between 9:00 a.m. and 17:00 p.m.

Drug administration

NTG(Beijing Yimin Pharmaceutical Co., Ltd,China) was prepared from a stock solution of 5.0 mg/mL NTG in 30% alcohol,30% propylene glycol, and water. NTG was freshly diluted in 0.9% saline to a dose of 10 mg/kg. β -hydroxybutyric acid (BHB) was diluted in 0.9% saline to 100 mg/kg. All injections were administered as a 10 mL/kg volume[12]. BoNT/A was bought from the Lanzhou Institute of Biological Products, Lanzhou, China. Each vial contains 100 U of purified *C. botulinum* type A neurotoxin complex. The toxin was frozen in liquid nitrogen and stored at – 80 °C. In our experiment BoNT/A was reconstituted in an adequate volume of 0.9% NaCl to a dose of 0.18U/ 100 μ L. Each mouse was injected 100 μ L.

Establishment of chronic migraine mouse model

To simulate the progression of chronic migraine, we administered a dose of 10 mg/kg of NTG every second day for 9 days, resulting in a total of 5 NTG injection/test days. Baseline responses were tested immediately before intraperitoneal (i.p.) injection with NTG. 2 h after NTG administration, mechanical responses were tested again[12]. For the prevention group, mice were administered with a single injection of BoNT/A 1 h before the first NTG injection. BoNT/A was injected at three points in bilateral supraorbital (SO) region. For the treatment group,mice were injected with same dose of BoNT/A 1 h after last NTG injection.

Behavioral tests

To determine mechanical sensitivity, the threshold for responses to punctate mechanical stimuli (mechanical hyperalgesia) was tested according to the up-and-down method. In brief, the plantar surface of the animal hindpaw was stimulated with a series of eight von Frey filaments (bending force ranging from 0.008 to 1.0 g). The first filament tested was 0.4 g. Each filament was tested for ten times. Less than five positive responses a heavier filament (up) was tried, otherwise a lighter filament (down) was tested. A positive response was defined as withdrawal, shaking, or licking of the paw in response to stimulation. In this experiment all mice were tested with the left hindpaw.

Rota-rod test

We used Rota-Rod equipment to assess the effect of BoNT/A on the motor function in mice. Each mouse was pretrained for 3–5 consecutive days with the rod rotating at a speed of 20 rpm until they could stay on it for 5 min without falling. Every test day, mice were tested at the speed of the rotor (20 rpm) for three times and the average duration of running time was recorded.

Weight

Body weight was measured by electronic balance and recorded before drug administration every test day.

Western blotting

Two hours after the last NTG i.p.injection, mice were terminally anesthetized with 4% chloral hydrate (intraperitoneally 10 ml/kg) and transcardially perfused with saline; the TGs and TNC were rapidly removed and homogenized in a lysis buffer containing phosphatase inhibitors and a cocktail of protease inhibitors for total protein extraction and assay. The protein concentrations were determined using the BCA Protein Assay (Pierce, Rockford, Illinois, USA). SDS-PAGE was performed on 10% polyacrylamide gels at 80 V for 30 min and at 120 V for 2 h. After transfer onto a PVDF membrane, the blots were blocked with 5% nonfat milk in Tris-buffered saline Tween 20 (TBST) and incubated overnight at 4 °C with primary antibodies including anti-TRPV1 (rabbit, 1 : 1000; Novus), anti-TRPA1 (rabbit, 1 : 1000; Novus), anti-TRPV4(rabbit,1:500;Abcam,UK), anti-CGRP (mouse,1:500;Santa Cruz, USA), anti-SNAP25(rabbit,1:2000;Abcam,UK), anti-GFAP(mouse,1:1000;Cell Signaling,USA), anti-NLRP3(rabbit,1:1000; Cell Signaling, USA),and anti-GAPDH (mouse, 1 : 2000; Mesgen). The membranes were washed with TBST three times and incubated with a secondary antibody conjugated to horseradish peroxidase. (1 : 2000; Mesgen). Protein bands were visualized using an enhanced chemiluminescence detection kit (Pierce) and the band densities were detected and analyzed using Tanon 5200 Multi (Tanon, Shanghai).

Quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR)

Mice were anesthetized and decapitated 2 h after the last NTG or saline injection, and the TNC tissues were obtained and immediately stored in liquid nitrogen until analysis. We used TRIzol reagent (Invitrogen, Carlsbad, CA) to extract total RNA according to the protocol supplied by the manufacturer, and yield and purity were assessed with a NanoDrop 2000 spectrophotometer (Thermo, Waltham, MA, USA) with absorbance at 260 and 280 nm. cDNAs were synthesized using a Revert Aid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA). Q-PCR was performed with SYBR GREEN PCR Master Mix (Roche, Balse, Switzerland) using the ABI 7500 Real-Time PCR system. Relative gene

expression was normalized to the internal reference glyceraldehyde 3-phosphate dehydrogenase (GAPDH) using the $2 - \Delta\Delta CT$ method. The primers were as follows: GAPDH: forward: GAAGGTGGTGTGAACGGAT, reverse: AATCTCCACTTGCCACTGC; IL-1 β : forward: AGAGCATCCAGCTCAAATCTC, reverse: CAGTTGTCTAATGGGA ACGTCA; IL-18: forward: GACTCTTGCCTCAACTCAAGG, reverse: CAGGCT GTCTTTGTCAACGA.

Immunofluorescence

Two hours after the final drug injection, the mice were anaesthetized and transcardially perfused with 0.9% saline, followed by 4% paraformaldehyde (PFA) in PBS (0.01 M, pH 7.4). Thereafter, TNCs originating from the regions between the medulla oblongata and the first cervical spine were separated immediately and post-fixed with 4% PFA overnight at 4 °C, transferred to 10% sucrose in PBS overnight then to 20% sucrose overnight and serially cross-sectioned 1–5 mm to the obex (20 μ m thick) with a cryostat (Leica). For immunostaining, sections were washed in 0.01 M PBS (10 min \times 3), permeabilized with 0.2% Triton X-100 (Sinopharm Chemical Reagent Co.,Ltd, China) and incubated with 5% normal goat serum (Solarbio, China) for 60 min at 37 °C. Afterward, the sections were incubated with primary antibodies overnight at 4 °C. The following antibodies were used: mouse anti-CGRP (1:200, Abcam). After the sections were washed in PBS (10 min \times 3), they were incubated with secondary antibodies that were conjugated with Alexa Fluor 488 and Alexa Fluor 555 (goat anti-rabbit IgG, goat anti-mouse IgG; 1:200; CST) at 37 °C for 1 h. Finally, the sections were mounted with DAPI and imaged using a confocal microscope (Leica).

Statistical analysis

Statistical analysis of the data sets was carried out using Graphpad Prism, version 6.02 (Graphpad Software, La Jolla, California, USA). All data were expressed as mean \pm SEM. An unpaired t-test was used for two-group comparisons. One-way analysis of variance with the Bonferroni post-test was used for multiple comparisons. Two-way repeated-measures analysis of variance was also used to analyze the data at multiple time points. $P < 0.05$ was considered statistically significant.

Results

Repeated NTG injection induced basal and evoked mechanical hypersensitivity in mice

We first examined whether repeated NTG injection would induce basal and evoked mechanical hypersensitivity in mice as previous reported [12]. The results showed that repeated NTG injection evoked mechanical hypersensitivity, and these pain behavioral responses returned to the basal levels 7 days after the last NTG injection (Fig. 1A; $F_{time}(8, 176) = 19.83, P < 0.0001$; $F_{NTG}(1, 22) = 69.65, P < 0.0001$; $F_{NTG \times time}(8, 176) = 8.190, P < 0.0001$). In addition, the mechanical pain sensitivity of the NTG group were significantly higher than those of the VEH group 2 h after NTG injection (Fig. 1B; $F_{time}(4, 88) = 3.165, P = 0.0176$; $F_{NTG}(1, 22) = 68.89, P < 0.0001$, $F_{NTG \times time}(4, 88) = 1.151, P = 0.3379$).

Clinically, BoNT/A therapy may be effective in the management of chronic migraine[13]. We subsequently assessed whether BoNT/A would attenuate chronic migraine in this mouse model. The results showed that single supraorbital injection of BoNT/A before the first NTG treatment prevented NTG-induced basal and evoked mechanical hypersensitivity and the therapeutic effects of BoNT/A could be long-lasting in NTG-treated mice (Fig. 1C; Ftime (8, 112) = 9.441, P < 0.0001, FNTG (1, 14) = 58.07, P < 0.0001, FNTG × time (8, 112) = 6.708, P < 0.0001; Ftime (8, 112) = 5.884, P < 0.0001, Fprevent (1, 14) = 24.66, P = 0.0002, Fprevent × time (8, 112) = 4.878, P < 0.0001; Fig. 1D; Ftime (4, 56) = 4.442, P = 0.0035, FNTG (1, 14) = 38.98, P < 0.0001, FNTG × time (4, 56) = 1.316, P = 0.2753; Ftime (4, 56) = 1.607, P = 0.1852, Fprevent (1, 14) = 11.68, P = 0.0042, Fprevent × time (4, 56) = 1.221, P = 0.3123). In addition, by using the Rotarod test, we found that motor function was not altered following pretreatment with BoNT/A (Fig. 1E; Ftime (1.917, 26.84) = 1.511, P = 0.2389, FNTG (1, 14) = 0.02203, P = 0.8841, FNTG × time (4, 56) = 0.4002, P = 0.8077; Ftime (2.471, 34.59) = 4.290, P = 0.0157, Fprevent (1, 14) = 0.03311, P = 0.8582, Fprevent × time (4, 56) = 2.546, P = 0.0493). Moreover, BoNT/A injection also has little effect on the body weight in mice (Fig. 1F; Ftime (2.686, 34.92) = 11.89, P < 0.0001, FNTG (1, 13) = 5.825, P = 0.0313, FNTG × time (8, 104) = 3.114, P = 0.0034; Ftime (2.299, 29.89) = 5.382, P = 0.0077, Fprevent (1, 13) = 1.512, P = 0.2406, Fprevent × time (8, 104) = 1.285, P = 0.2596).

Subsequently, we investigated whether BoNT/A would reverse NTG induced chronic migraine pain or not in mice. For this purpose, single facial injection of BoNT/A was performed 1 h after the last NTG injection. In the VEH group, mechanical threshold returned to the basal level 7 days after the last NTG injection. Unexpected, we found that BoNT/A treatment did not reverse the mechanical hypersensitivity in NTG-treated mice (Fig. 1G; Ftime (3.495, 48.93) = 27.63, P < 0.0001, Ftreatment (1, 14) = 0.2683, P = 0.6125, Ftreatment × time (5, 70) = 1.298, P = 0.2746).

In addition, we administered a single facial injection of BoNT/A to examine the possible effects of BoNT/A on the mechanical sensitivity in naïve mice. The results showed that facial injection of BoNT/A treatment did not affect the mechanical sensitivity in naïve mice (Fig. 1H; Ftime (8, 112) = 1.512, P = 0.1608, Fdrug (1, 14) = 0.2621, P = 0.6167, Fdrug × time (8, 112) = 0.4313, P < 0.9001).

Single facial BoNT/A injection attenuated NTG-induced upregulation of CGRP in the TGs and TNC in mice

We further investigated whether NTG treatment induced increases in the protein expression levels of the CGRP and SNAP25 in the TGs or not. The results showed that protein expression of CGRP was increased in both TGs and TNC of CM mice compared with the VEH group. Pretreatment of BoNT/A attenuated the upregulation of CGRP expression in the TGs and TNC in NTG-treated mice (Fig. 2C; for TG: F (2, 9) = 17.16, P = 0.0008; for TNC: F (2, 8) = 22.30, P = 0.0005). We further evaluated the changes of SNAP25 expression in the TGs and TNC by western blotting. The results indicated that pretreatment of BoNT/A abolished the upregulation of SNAP25 expression in the TGs in NTG-treated mice (Fig. 2C; for TG: F (2, 9) = 13.11, P = 0.0022). The expression of SNAP25 in the TNC did not show any difference among all three groups (Fig. 2C; for TNC: F (2, 9) = 0.9611, P = 0.4185). In addition, GFAP expression both in the TGs and

TNC did not show any significant change in all three groups (Fig. 2C; for TG: $F(2, 9) = 1.547$, $P = 0.2647$; for TNC: $F(2, 9) = 1.285$, $P = 0.3229$). Immunofluorescence analysis confirmed that pretreatment with BoNT/A abolished the NTG-induced up-regulation of CGRP immunoreactivity in the TNC (Fig. 2D-E; $F(2, 11) = 16.38$, $P = 0.0005$). Thus, these results indicated that peripheral application of BoNT/A inhibited the up-regulation of CGRP expression in the TGs and TNC.

The roles of TRPV1, TRPV4, and TRPA1 in preventive effects of BoNT/A on chronic migraine in mice

TRP channels (such as TRPV1, TRPV4, and TRPA1) play important roles in the pathogenesis of chronic pain, including chronic migraine[5]. We postulated the therapeutic effects of BoNT/A on chronic migraine may be possibly involved in these TRP channels. Thus, we assessed whether pretreatment with BoNT/A 1 h before the first NTG injection would alter the expression of TRPV1, TRPV4, and TRPA1 in the TGs. Unexpected, the expression of TRPV4, TRPV1 and TRPA1 in both the TGs (Fig. 3A-B; for TRPV4: $F(2, 8) = 0.06325$, $P = 0.9392$; for TRPV1: $F(2, 8) = 1.794$, $P = 0.2271$; for TRPA1: $F(2, 9) = 0.005035$, $P = 0.9950$) and TNC (Fig. 3C-D; for TRPV4: $F(2, 9) = 1.299$, $P = 0.3194$; for TRPV1: $F(2, 9) = 1.143$, $P = 0.3611$; for TRPA1: $F(2, 8) = 0.07510$, $P = 0.9283$) did not show any differences among all tested groups.

The roles of TNC NLRP3 inflammasome activation in preventive effects of BoNT/A on chronic migraine in mice in the TNC

To determine the role of microglia NLRP3 inflammasome pathways in the pathogenesis of chronic migraine and the therapeutic effects of BoNT/A in mice, we examined the expression of NLRP3 and IL-1 β in the TNC between VEH group and repeated NTG administration group. Compared with VEH group, the protein expression level of NLRP3 (Fig. 4A; $F(2, 8) = 19.22$, $P = 0.0009$) and the mRNA expression level of IL-1 β (Fig. 4B; $F(2, 7) = 9.261$, $P = 0.0108$) was significantly decreased in the BoNT/A treatment group. However, the mRNA expression level of NLRP3 (Fig. 4B; $F(2, 6) = 0.001269$, $P = 0.9987$) and IL-18 (Fig. 4B, $F(2, 6) = 0.5473$, $P = 0.6049$) in the TNC did not change among all three groups. We subsequently investigated whether NLRP3 inhibitor could treat NTG induced chronic migraine pain or not in mice. NLRP3 inhibitor BHB (100 mg/kg; intraperitoneally) was injected 30 minutes before the NTG injection daily, then the mechanical sensitivity was tested before (Fig. 4C) and 2 h after (Fig. 4D) the NTG injection. The results showed that the evoked mechanical hypersensitivity on the 1st day was significantly reduced by BHB (Fig. 4D; $F_{\text{time}}(2.581, 25.81) = 6.262$, $P = 0.0035$, $F_{\text{prevent}}(1, 10) = 4.333$, $P = 0.0640$, $F_{\text{prevent}} \times \text{time}(4, 40) = 3.298$, $P = 0.0199$). However, for basal mechanical hypersensitivity, there was no significant difference between the BHB-treated and VEH group (Fig. 4C; $F_{\text{time}}(1.836, 18.36) = 16.44$, $P = 0.0001$, $F_{\text{prevent}}(1, 10) = 2.414$, $P = 0.1513$, $F_{\text{prevent}} \times \text{time}(4, 40) = 1.265$, $P = 0.2997$).

Discussion

BoNT/A therapy prevented, but not reversed, the development of chronic migraine

Two main types of CM animal models are now widely used. One type is based on the repeated stimulation induced neuro-inflammatory reaction of the epidural, such as repeated applications of

inflammatory soup (IS) to the dura mater[14]. The other type is the systemic infusion of vasodilating agents, such as the repeated intraperitoneal administration of NTG[15]. There are also some rare CM models, such as genetically modified chronic migraine model or altering the endogenous pain modulating system.

We adopted the chronic migraine model with repeated NTG injections in mice that was first described by Pradhan et al. Administration of NTG, as a reliable experimental model, demonstrates several behavioral similarities to human migraine[16]. It has been assumed that NTG exerts its pharmacologic effects by generating nitric oxide (NO) and further NO production acts directly on vascular smooth muscle, causing vasodilation[17]. Chronic intermittent administration with NTG not only produced acute mechanical hypersensitivity of the hind paw after each injection but also induced long-lasting basal hyperalgesia. This chronic basal hyperalgesia persisted for several days after the last NTG exposure. In addition to mechanical hyperalgesia, NTG can also induce thermal hypersensitivity of the hind paw. Because of the difficulties in practice and unstable results,in our study, we only measured hind paw mechanical hyperalgesia. In the previous study, prophylactic application of topiramate can reduce acute and chronic hyperalgesia [12]. In this study, we gave a single supraorbital subcutaneous injection of BoNT/A in mice to prevent NTG-induced chronic migraine, BoNT/A can play the same role, and the effect is more stable and lasting.

The roles of CGRP in the therapeutic effects of BoNT/A on chronic migraine

At present, the most classic analgesic mechanism of BoNT/A is to cleave the SNAP25 protein and inhibit the release of pain-related neurotransmitters in vesicles. Multiple studies have shown that CGRP plays a key role in the analgesic mechanism of BoNT/A. CGRP is a 37-amino acid peptide that was discovered in 1982[18].A growing body of evidence indicates that CGRP is a principal mediator of migraine that is widely expressed through both the peripheral and central nervous system (CNS) in the trigeminovascular system. CGRP is released from nerve fibers running along meningeal and cerebral arteries and blood vessels. In the CNS, CGRP-containing neurons can be found in the superficial layers of the spinal trigeminal nucleus[19].

NTG, as a NO donor, injected in mice can excite trigeminal nociceptors and also promote the release of CGRP from trigeminal terminals to promote sensitization of TG neurons. The release of CGRP from the central terminals of TG neurons could repetitively excite second-order neurons in the TNC where the trigeminal ganglion projects to, leading to central sensitization and the manifestation of hyperalgesia and allodynia [20]. CGRP and NO can amplify each other's activity in a reciprocal fashion throughout the trigeminovascular system,thereby maintaining central sensitization and continuous hyperalgesia.

In our study, we have detected the expression of CGRP in the TGs and TNC in mice. We observed that repeated NTG injection elevated CGRP expression in both TGs and TNC, consistent with the immunofluorescence results in the TNC. Pretreatment with BoNT/A reversed NTG induced up-regulation of CGRP in the TGs and TNC. As we usually know, BoNT/A cleaves SNAP25, a part of SNARE complex near the presynaptic membrane, resulting in a blockage of neurotransmitter release, such as CGRP,

substance P, adenosine triphosphate (ATP), glutamate, noradrenaline, serotonin. However, in our research, we assumed that BoNT/A may also reduce the expression of CGRP by regulating the activity of glial cells.

The roles of TRPV1, TRPV4, and TRPA1 in the therapeutic effects of BoNT/A on chronic migraine

Transient receptor potential (TRP) channels are a family of cation channels expressed in cellular membranes, with the exception of the nuclear envelope and mitochondria, of almost every excitable and non-excitable cell type [21, 22]. A TRP channel contribution to migraine are largely due to their expression on meningeal nociceptors and their responsiveness to a variety of endogenous and exogenous stimuli, and activation of TRP channels is well-known to promote the release of CGRP from sensory nerve endings. In addition to inhibiting the release of pain-mediating peptides, BoNT/A may reduce peripheral sensitization by interfering with the integration of relevant sensory receptors and ion channels [e.g. transient receptor potential cation channel vanilloid subfamily member 1 (TRPV1) and transient receptor potential cation channel ankyrin subfamily member 1 (TRPA1)] on nociceptive nerve endings [23]. TRPA1 is expressed in trigeminal neurons and localized on dural afferents, where its activation has been shown to result in headache-like behaviors in mice. Experimental drugs that serve as NO donors, such as nitroglycerine, have been extensively studied for their migraine-inducing properties. Both in humans and animals, NO directly activates TRPA1 via S-nitrosylation, and therefore, could serve as a mechanism for trigeminal excitation and migraine [24]

Expression of TRPV1 and modulation of its function, trafficking and expression by multiple mediators in trigeminal neurons have been proposed to be involved in the development of migraine pathophysiology. TRPV1 activation in neurons leads to release of CGRP, a critical neuropeptide in the development of trigemino-vascular excitation [25]. Experimental studies also showed that NO donors increased TRPV1 expression in an inflammatory pain model [26]. In an ION-CCI-induced TN rat model, changes in TRPV4 in the TNC was investigated. BoNT/A might be transported from the peripheral to the central nervous system by retrograde axonal transport, degrade SNAP-25, block exocytosis, and reduce the protein expression of TRPV4, thus causing the analgesic effect [27]. A research also suggest that facial TRPM8 activation can exert an antimigraine action by inactivating TRPV1 function at the level of TG neurons [28]. Moreover, the activity of IGM-18 on TRPM8 channels can exerte analgesic effect on formalin-induced orofacial pain and chronic constriction injury-induced neuropathic pain, demonstrating the involvement of TRPM8 channels in both acute and chronic pain[29]. In our study, we detected the expression of TRPA1/TRPV1/TRPV4 in the TGs and TNC in the NTG-induced CM mice. However, no significant evidence showed that the expression of these TRP channels are changed during the development of chronic migraine.

The roles of NLRP3 inflammasomes in the therapeutic effects of BoNT/A on chronic migraine

More and more evidence shows that the analgesic effect of BoNT / A is mediated by neurons and glial cells, especially microglia. A study suggests that NLRP3may regulate microglial inflammation in chronic migraine. NLRP3 inflammasome are multi-protein complexes, located in some immune cells such as macrophages and dendritic cells, which are part of the innate immune response against invading

pathogens [30]. NLRP3 activated during cell infection or under stress, regulate the activation of protease caspase-1, and promote the expression, maturation and release of pro-inflammatory cytokines such as IL-1 β and IL-18, triggering a series of inflammatory reactions [31]. A study provides the first evidence showing a novel role of NLRP3-IL-1 β in mediating the transition from acute to chronic neuroinflammation incited by sepsis. Several observations from this study suggest a permissive role for IL-1 β in switching acute self-limiting neuroinflammation to chronic self-propelling neuroinflammation [32]. We assume that brain microglia are the factor for chronic neuroinflammation. Once activated Trigeminal nervous system pathway, microglia release a variety of cytokines, chemokines, and free radicals such as NO and reactive oxygen species (ROS). A research has presented that NLRP3 inflammasome may regulate the inflammatory process of microglia in chronic migraine. The inhibitory action of BoNT/A on synaptic vesicle fusion that blocks the release of miscellaneous pain-related neurotransmitters is known. However, increasing evidence suggests that the analgesic effect of BoNT/A is mediated through neurons and glial cells, especially microglia. In our experiment, pretreatment with BoNT/A in the supraorbital region of CM mice didn't change the expression of SNAP25 in the TNC. BoNT/A may not influence the central nervous system by SNAP25 protein, while microglia may be a promising target. BoNT/A influenced primary microglial cells by inhibiting intracellular signaling pathways, such as p38, ERK1/2, NF- κ B, and the release of pro-inflammatory factors, including IL-1 β , IL-18, IL-6, and NOS2. BoNT/A treatment did not decrease LPS-induced release of pro-inflammatory factors in the astroglia. We detected that in CM mice, BoNT/A inhibited the expression of NLRP3 protein and pro-inflammatory factor IL-1 β caused by NTG in the TNC. Therefore, we speculate that BoNT/A may regulate the activity of microglia in TNC by regulating CGRP. The interaction with microglia is achieved, which plays a role in controlling the release of inflammatory factors. More evidence are required to verify this in vitro experiments.

Conclusions

Clinically, BoNT/A is a relative safe therapy in neurological practice with limited side effects. Thereby, our studies further support that microglia may be a new target for the preventive effect of BoNT/A in chronic migraine.

Abbreviations

BoNT/A: Botulinum neurotoxin A; NTG: nitroglycerin; SNAP25: synaptosomal-associated protein25; CGRP: calcitonin gene-related peptide; TG: trigeminal ganglia; TNC: trigeminal nucleus caudalis; NLRP3: NOD-, LRR- and pyrin domain-containing protein 3; i.p.: intraperitoneal

Declarations

The authors declared no competing interests.

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Figures

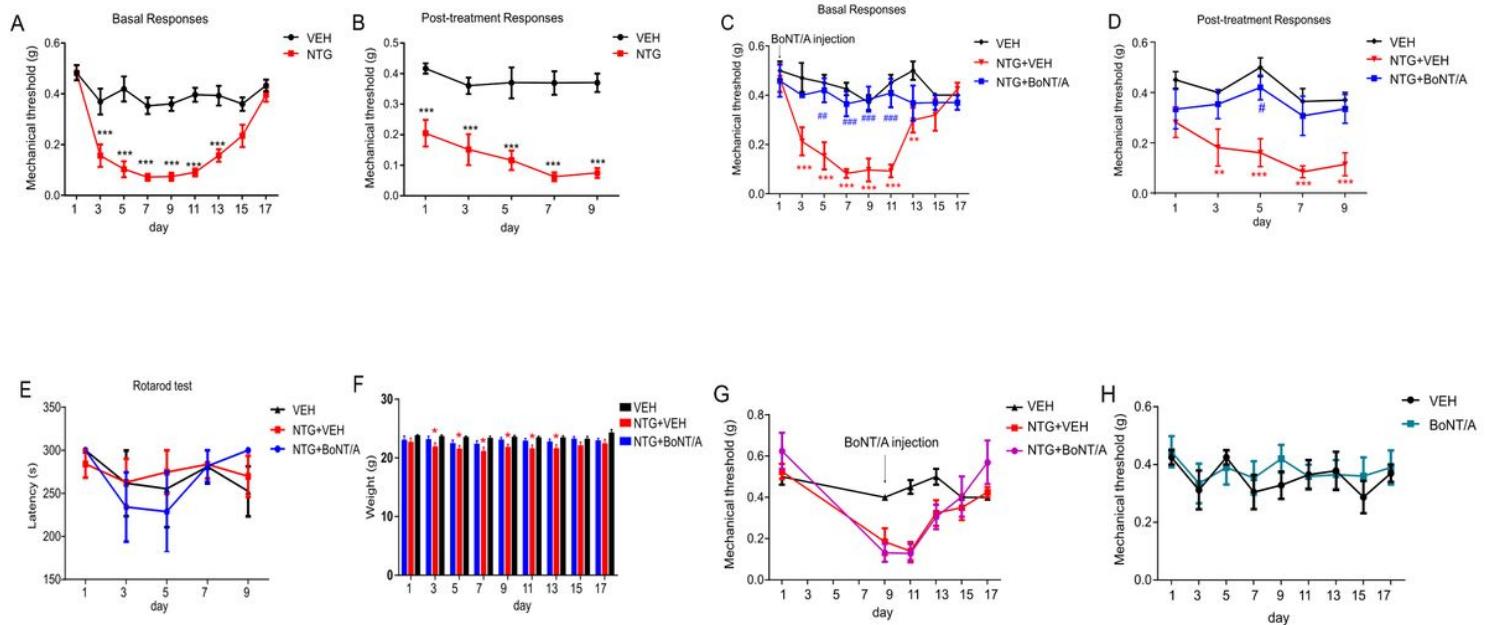


Figure 1

Therapeutic effects of pretreatment of BoNT/A on the development of chronic migraine in mice. (A) The basal mechanical hypersensitivity was detected before the mice received NTG injection on each day. (B) Evoked mechanical hypersensitivity was detected 2 hour after NTG injection in mice ($n = 12$ mice/group). (C-D) Single supraorbital injection of BoNT/A prevented NTG-induced basal (C) and evoked (D) mechanical hypersensitivity ($n = 8$ mice/group). (E) Motor function were assessed by using Rotarod test. (F) Mice weight were measured before drug administration daily. (G) BoNT/A injection on the 9th day didn't reverse NTG-induced basal mechanical hypersensitivity. (H) Facial BoNT/A injection didn't affect mechanical sensitivity in naïve mice compared with VEH group. Data are expressed as the mean \pm SEM,

P < 0.01, *P < 0.001 vs. VEH group. #P < 0.05, ##P < 0.01, ###P < 0.001 vs. NTG +VEH group (two-way ANOVA with post-hoc Bonferroni test).

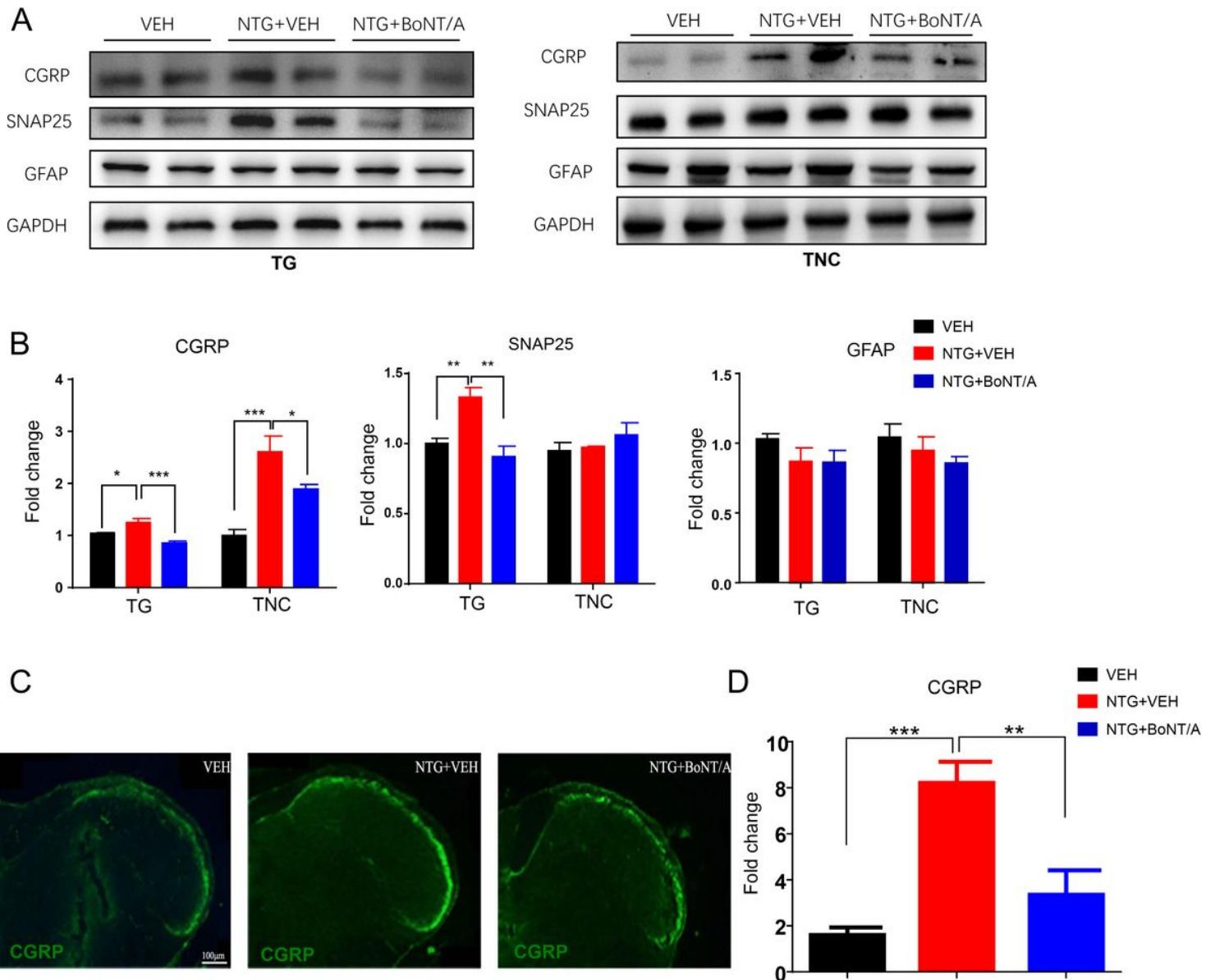


Figure 2

Effects of pretreatment of BoNT/A on the expression of CGRP, SNAP25, and GFAP in the TGs and TNC in mice. (A-B) Representative western blots (A) and semi-quantitative analysis (B) showing the changes of protein expression of CGRP, SNAP25, and GFAP in the TGs and TNC 2 hours after the last NTG injection. (C) Representative immunofluorescence staining for CGRP in the TNC. (D) Repeated NTG administration increased the mean fluorescence intensity of the CGRP-immunoreactive fibres. Scale bar, 100 µm. The data are presented as mean ± SEM, *P<0.05, **P < 0.01, ***P < 0.001 compared with NTG group (one-way ANOVA with post-hoc Bonferroni test). n = 4 mice per group.

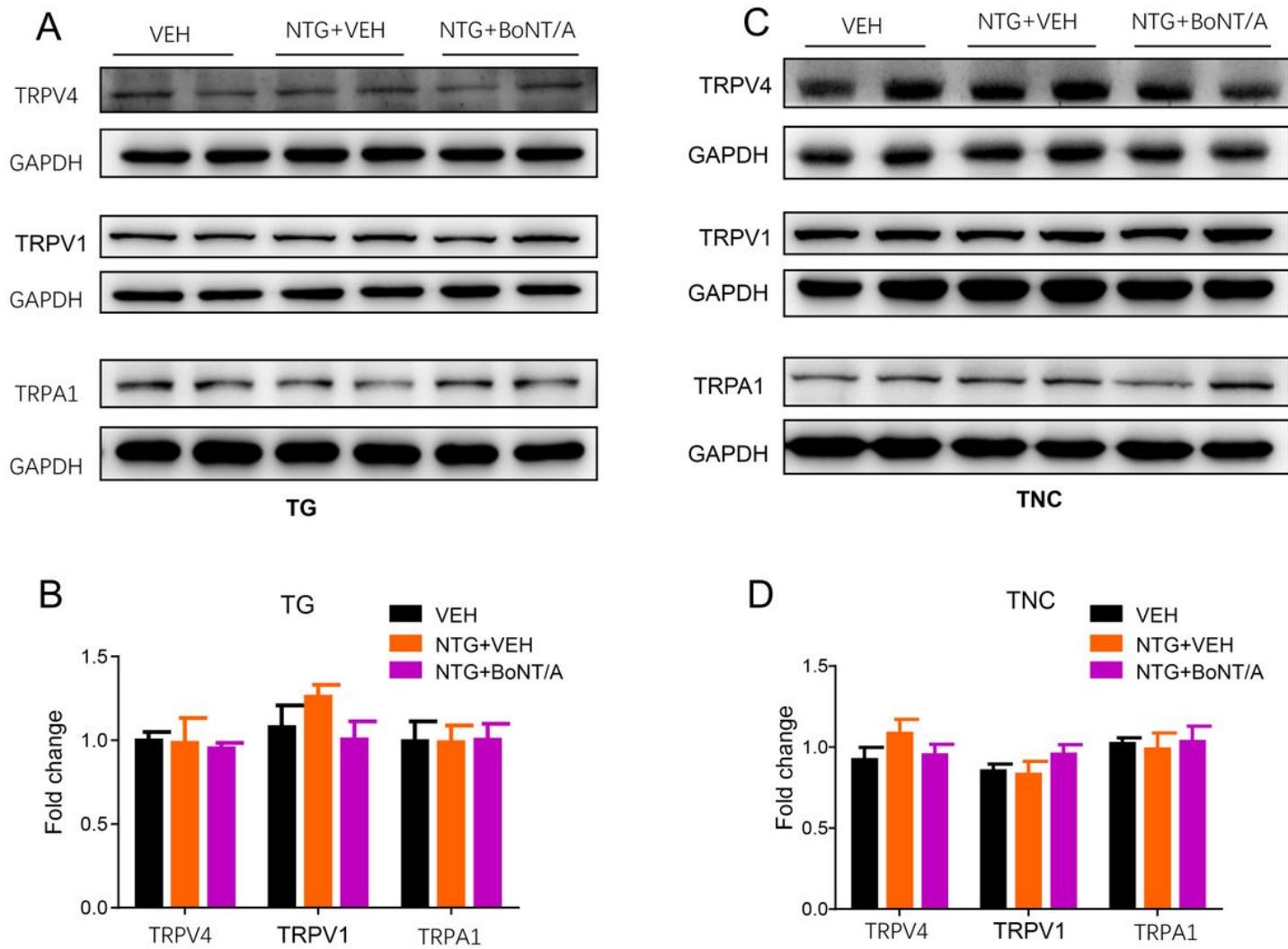


Figure 3

Possible effects of pretreatment of BoNT/A on the expression of TRPV4, TRPV1 and TRPA1 in the TNC in mice. (A-B) Representative western blots (A) and semi-quantitative analysis (B) showing the protein expression of TRPV4, TRPV1 and TRPA1 in the TGs 2 hours after the last NTG injection. (C-D) Representative western blots (C) and semi-quantitative analysis (D) showing the protein expression of TRPV4, TRPV1 and TRPA1 in the TNC 2 hours after the last NTG injection. The data are presented as mean \pm SEM, one-way ANOVA with post-hoc Bonferroni test. n = 4.

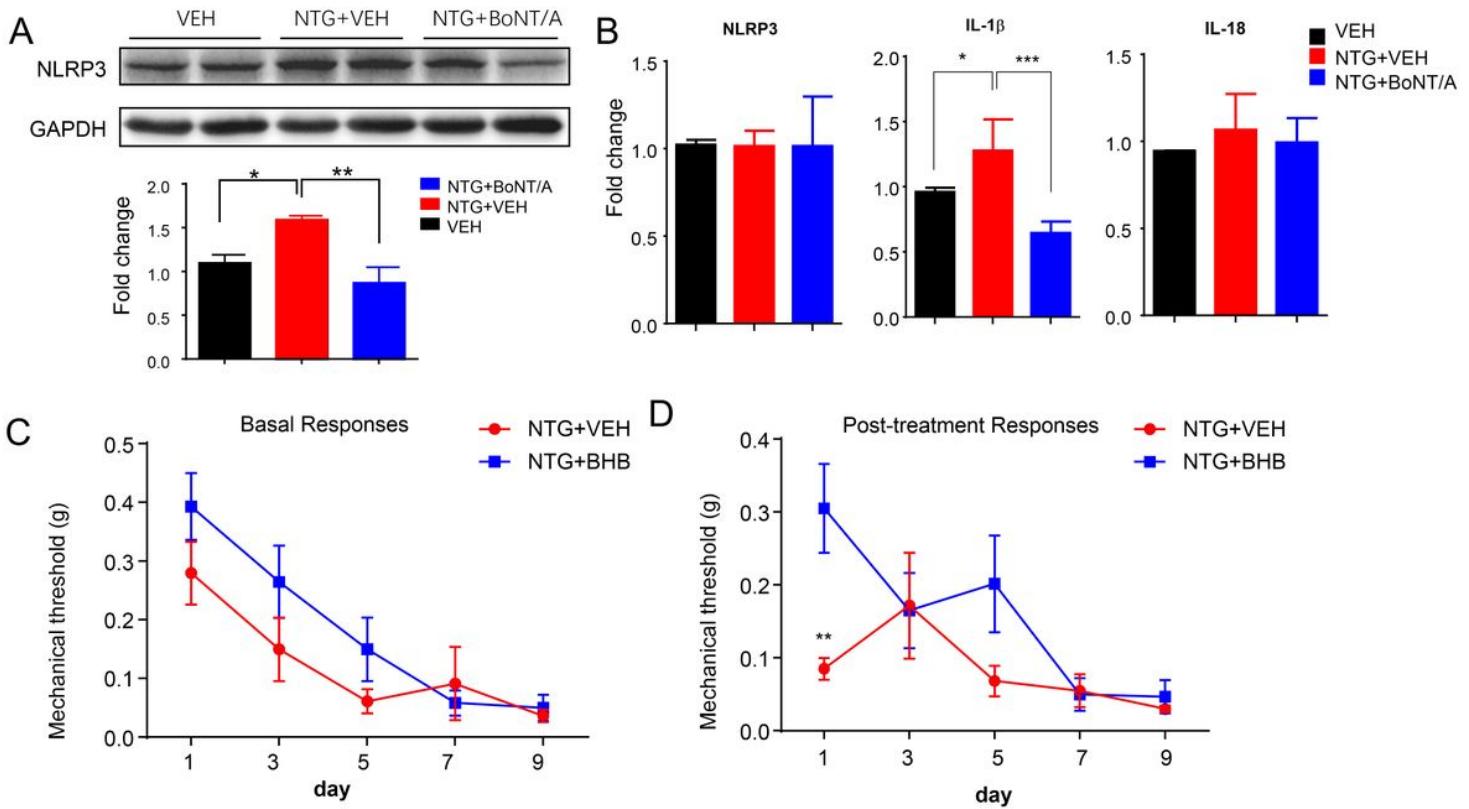


Figure 4

Inhibitory effects of pretreatment of BoNT/A on the expression of NLRP3 in the TNC in mice. (A) Representative western blots showing the protein expression of NLRP3 in the TNC 2 hours after the last NTG injection. Lower panel showing semi-quantitative analysis showing the effects of BoNT/A on the expression of NLRP3. (B) mRNA expression of NLRP3, IL-1 β and IL-18 determined by q-PCR in the TNC. The data are presented as mean \pm SEM, n = 4 per group. (C-D) BHB administration has little effect on NTG-induced basal (C) and evoked (D) mechanical hypersensitivity in mice. *P < 0.05, **P < 0.01, ***P < 0.001 compared with controls (one-way ANOVA with post-hoc Bonferroni test). The data are presented as mean \pm SEM, n = 6 per group.