Long-term anti-itch effect of botulinum neurotoxin A is associated with downregulation of TRPV1 and TRPA1 in the dorsal root ganglia in mice

Lei-Fang Cao[a,*], Meng Si[a,*], Ya Huang[b], Li-Hua Chen[c], Xiao-Yan Peng[d], Ya-Qin Qin[a], Teng-Teng Liu[b], Yan Zhou[b], Tong Liu[b] and Wei-Feng Luo[a]

Itch is a common symptom in patients with skin and systemic diseases, but the effective treatment is limited. Here, we evaluated the anti-itch effects of the botulinum toxin type A (BoNT/A) using acute and chronic dry skin itch mouse models, which were induced by compound 48/80, chloroquine, and a mixture of acetone–diethylether–water treatment, respectively. Pretreatment of intradermal BoNT/A exerted long-term inhibitory effects on compound 48/80-induced and chloroquine-induced acute itch on days 1, 3, 7, and 14, but not on day 21, in mice. Furthermore, a single injection of BoNT/A reduced the expression of the transient receptor potential cation channel, subfamily V, member 1 (TRPV1), and the transient receptor potential cation channel, subfamily A, member 1 (TRPA1) at both transcriptional and translational levels in the dorsal root ganglia (DRG) in mice. Pretreatment of BoNT/A also attenuated chronic itch induced by acetone–diethylether–water treatment and abolished the upregulation of TRPA1 in the DRG. Thus, it was suggested that downregulation of the expression of TRPA1 and TRPV1 in the DRG may contribute toward the long-term anti-itch effects of a single injection of BoNT/A in mice and BoNT/A treatment may serve as an alternative strategy for anti-itch therapy. *NeuroReport* **00:000–000**

NeuroReport 2017, **00**:000–000

Keywords: botulinum neurotoxin A, itch, TRPA1, TRPV1

[a]Department of Neurology, the Second Affiliated Hospital of Soochow University, [b]Institute of Neuroscience, [c]Jiangsu Key Laboratory of Preventive and Translational Medicine for Geriatric Diseases, Department of Nutrition and Food Hygiene, School of Public Health, Soochow University, Suzhou and [d]Suqian First Hospital, Suqian, China

Correspondence to Wei-Feng Luo, Department of Neurology, the Second Affiliated Hospital of Soochow University, Suzhou, Jiangsu Province 215004, China
Tel: +86 512 677 84177; fax: +86 512 658 80187; e-mail: lwfwxx@126.com

*Lei-Fang Cao and Meng Si contributed equally to the writing of this article.

Received 16 January 2017 **accepted** 19 January 2017

Introduction

Itch (pruritus) is a common unpleasant sensation that elicits the reflex to scratch [1]. Acute itch serves as a warning and self-protective mechanism to prevent potentially harmful irritations. However, chronic itch is a challenging and significant clinical problem, which is often associated with skin diseases, systemic diseases, and metabolism disorders [2,3]. Although scratching transiently relieves acute itch, persistent itch-scratch cycles often exacerbates skin problems, disrupts sleep, and markedly reduces the quality of life in chronic itch patients [2]. Primary sensory neurons in dorsal root ganglia (DRG) are responsible for detecting pruritogenic stimuli through their peripheral terminals in the skin and transducing itch signals to the spinal cord through their central terminals [1]. Histamine, a well-known itch mediator, is mainly released from mast cells and activates sensory nerve fibers by binding on histamine H1/H4 receptors [1]. Although antihistamines are often prescribed for treating allergic itch, most types of chronic itch are resistant to antihistamines [1,2]. In addition, steroids and immunosuppressants, which are also used for chronic itch treatment, have several side effects, such as severe nephrotoxicity and neurotoxicity [2]. The incomplete understanding of the molecular mechanisms underlying itch, especially histamine-independent itch, is a major hurdle in the development of novel anti-itch therapy.

Botulinum toxin type A (BoNT/A) is a poisonous biological substance derived from *Clostridium botulinum* and a member of the clostridial neurotoxin family, including BoNT/A-G and tetanus neurotoxin [4]. BoNT/A is composed of the heavy chain with the receptor-binding site, the translocation domain, and the light chain with endopeptidase activity, which cleaves synaptosomal-associated protein 25 (SNAP-25), an essential molecule for membrane fusion [4]. Intriguingly, it was found that catalytically active BoNT/A is retrogradely transported in neurons and was transcytosed to afferent synapses, in which it cleaves SNAP-25 [5]. This novel pathway for BoNT/A trafficking in neurons may have important implications for the activity of BoNT/A. However, it was also observed that the SNAP-25-cleavage activity may not necessarily be involved in BoNT/A's several actions, including neuroexocytosis, cell cycle and apoptosis, neuritogenesis, and gene expression [6]. Although the well-known activity of BoNT/A was to block acetylcholine release at the neuromuscular junction, recent studies also showed that BoNT/A can inhibit the release of other

 DOI: 10.1097/WNR.0000000000000779

neuromodulators/transmitters, such as glutamate, substance P, serotonin, noradrenaline, dopamine, enkephalin, glycine, γ-aminobutyric acid, and calcitonin gene-related peptide [4]. In clinic, BoNT/A was first used and approved over two decade ago for the treatment of strabismus (or misaligned eyes) [7]. So far, botulinum toxin has shown efficacy in many disorders characterized by abnormal muscle contraction, such as hemifacial spasm, blepharospasm, and spastic lower eyelid entropion [7]. In addition, BoNT/A has also been used widely as a powerful treatment for chronic pain, such as trigeminal neuralgia, low-back pain, urologic pain, neuropathic pain, musculoskeletal pain, diabetic neuropathy, and migraine [4]. Interestingly, clinical evidence has provided some clues for the anti-itch effects of BoNT/A, possibly through inhibition of the release of acetylcholine as well as other mediators involved in itch [8,9]. However, the precise mechanisms underlying the anti-itch effect of BoNT/A remain unclear.

Transient receptor potential (TRP) channels play pivotal roles in multiple somatosensations, such as thermal and mechanical sensation, pain, and itch [10,11]. The transient receptor potential cation channel, subfamily V, member 1 (TRPV1) and the transient receptor potential cation channel, subfamily A, member 1 (TRPA1) belonging to the TRP channels superfamily are the non-selective ligand-gated cation channels [12]. It is generally considered that TRPV1 is required for histamine-dependent itch [13], whereas TRPA1 is required for histamine-independent itch, such as chloroquine (CQ)-induced itch [14], bile acids-induced cholestatic itch [15], and oxidative stress-induced itch [16,17]. The aim of the present study was to investigate the effects of BoNT/A on acute and chronic itch in the mice, and we further examined the possible association of the regulation of TRP channels and the anti-itch effects of BoNT/A.

Methods

Animals

All male CD1 (ICR) mice (6–8 weeks old, total 525 mice) were purchased from the Shanghai SLAC Laboratory Animal Company (Shanghai, China) and maintained on a 12 h light/dark cycle, with enough food and water available *ad libitum*, and kept at standard room temperature (22 ± 2° C) and humidity (60–80%). All animal experiments and procedures were according to the guidelines of the International Association for the Study of Pain. The animal procedures used in this work were evaluated and approved by the Animal Use and Ethic Committee of the Soochow University. Animal experiments were conducted in a sound-attenuated cabin between 9:00 a.m. and 16:00 p.m. and were conducted in a blinded manner with respect to the drug treatment.

Drugs and administration

BoNT/A (GMP nos S10970037) was obtained 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 10 mmol/l Na Hepes and 150 mmol/l NaCl (pH = 7.2). To obtain respective doses, BoNT/A was reconstituted in an adequate volume of 0.9% NaCl. Compound 48/80, CQ, and formaldehyde solution were purchased from Sigma-Aldrich (St Louis, Missouri, USA). The reagents were dissolved in sterile saline if not specialized.

BoNT/A was injected in three different ways: (a) for the itch model, BoNT/A (0.03, 0.1, 0.3, and 1.0 U) was single intradermally (i.d.) injected at three sites at the nape of the neck in a total volume of 100 µl under a brief anesthesia with isoflurane; (b) for the formalin-induced pain model, BoNT/A (0.1 U/mice) was subcutaneously injected into the plantar surface of the hindpaw in a volume of 20 µl; and (c) for the compound 48/80-induced itch model, BoNT/A (0.01, 0.03, 0.1 U) were also repeated i.d. injected at the nape of the neck in a total volume of 100 µl three times every 3 days under a brief anesthesia with isoflurane.

Mouse model of acute itch

The nape of the neck of mice was shaved 2 day before the experiments. On the day of testing, mice were individually placed in small plastic chambers (10 × 10 × 12.5 cm) on an elevated metal mesh floor and allowed at least 30 min for habituation. Under brief anesthesia of isoflurane, compound 48/80 (100 µg) and CQ (200 µg) in a volume of 50 µl mice were i.d. injected into the nape of the neck through a 26 G needle. After the injection, mice were immediately returned to their chambers and videorecorded for 30 min (Sony HDR-CX610; Shanghai, China). The video was played back offline and the scratching behavior was quantified in a blinded manner. A scratch was counted when a mouse lifted its hindpaw to scratch the injected skin and returned the paw to the floor or the mouth.

Dry skin-induced mouse model of chronic itch

As described previously [17], the hair of the nape of the neck of mice was shaved with electric clippers and depilatory paste 3 day before the treatments. The dry skin was induced by the treatment with a 1 : 1 mixture of acetone and diethylether for 15 s, followed by clean water for 10 s [acetone–diethylether–water (AEW)] twice a day (10:00 and 17:00) under HisofluraneH anesthesia for 7 days. Control animals were treated with water only. The drug treatment group received BoNT/A (i.d., 0.03 U/site, total three sites) at 1 day before AEW treatment. The spontaneous scratching behavior was videorecorded for 1 h before the next treatment. Bouts of

scratching were quantified for 1 h by experimenters in a blinded manner.

Mouse model of formalin-induced pain

Formalin-induced pain was tested after intraplantar injections of BoNT/A (0.1 U) at 30 min, 1, 3, 7, 14, and 21 days in mice. Mice were habituated for 3 days (1 h/day) in the plastic cage ($10 \times 10 \times 12.5$ cm). After pretreatment with BoNT/A, the mice were subcutaneously injected with 20 μl of 0.5% formalin into the same paw using a 26 G needle. Following the injection, animals were videotaped immediately for 60 min in the plastic enclosure. The cumulative amount of time that the animal spent licking or flinching the injected hindpaw was calculated in blocks of consecutive 5 min periods as described previously. Behavioral studies were carried out by an experimenter who was not aware of the treatments.

Rota-rod test

To assess the effect of BoNT/A on the motor function in mice, we used DXP-2 Rota-Rod equipment (Institute of Materia Medica, Chinese Academy of Medical Sciences). Each mouse was pretrained for 3–5 consecutive days with the rod rotating at an accelerating speed from 4 to 25 rpm until they maintained a stable baseline performance. One day after the last training session, mice were tested at the speed of the rotor (25 rpm) three times and the average duration of running time was recorded.

RNA isolation and quantitative real-time polymerase chain reaction

We collected cervical DRGs following an i.d. injection of BoNT/A (0.1 U) at different time points. Total RNA was extracted by homogenizing tissues using Trizol Reagent (Invitrogen, Carlsbad, California, USA) according to the protocol supplied by the manufacturer. Chloroform (Sigma-Aldrich) was added after homogenization and the tubes were vortexed, followed by incubation at room temperature for 5 min and centrifugation at 14 000 rpm for 20 min at 4°C. The supernatant was transferred to a new tube and isopropanol was added. The aqueous phase was centrifuged at 14 000 rpm for 20 min at 4°C. Pellets were washed using 70% ethanol and resuspended in diethylpyrocarbonate treated water. The purity and concentration of RNA were determined using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, Delaware, USA) with absorbance at 260 and 280 nm. One microgram of total RNA was reverse transcribed to synthesize cDNA using the RevertAid First Strand cDNA Synthesis Kit according to the protocol supplied by the manufacturer (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The Q-PCR experiment was conducted by SYBR Green PCR Master Mix (Roche, Basle, Switzerland) using the Opticon real-time PCR Detection System (ABI 7500; Life Technologies, Carlsbad, California, USA). Data were normalized to the housekeeping gene β-actin. The $2^{-\Delta\Delta C_t}$ method was used to analyze the relative level of gene expression. The specific fragment was amplified with the following primers: TRPV1: forward: ACCACGGCTGCTTACTATCG, reverse: GCTGGAATCCTCGGGTGTAG; TRPA1: forward: GATGTCCACGTGTGCCAAAG, reverse: ACCTGAAAATGCTGCCTGGT.

Western blotting

After an i.d. injection of BoNT/A (0.1 U) into the nape of the neck at 30 min, 1, 3, 7, 14, and 21 days in mice or after the behavior test of the AEW model, mice were terminally anesthetized with 4% chloral hydrate (intraperitoneally 10 ml/kg) and transcardially perfused with saline; the cervical DRGs were rapidly removed and homogenized in a lysis buffer containing phosphatase inhibitors and a cocktail of protease inhibitors for total protein extraction and assay according to our previous report. The protein concentrations were determined using the BCA Protein Assay (Pierce, Rockford, Illinois, USA). SDS-PAGE was performed on 10% polyacrylamide gels at 90 V for 30 min and at 110 V for 2 h. After transfer onto a PVDF membrane, the blots were blocked with 5% nonfat milk in TBS and incubated overnight at 4°C with anti-TRPV1 (rabbit, 1 : 1000; Alomone Labs, Jerusalem, Israel), anti-TRPA1 (rabbit, 1 : 1000; Alomone Labs), and anti-α-tubulin (mouse, 1 : 1000; Vazyme, Suzhou City, China), followed by the secondary antibody conjugated to horseradish peroxidase (1 : 2000; Santa Cruz Biotechnology, Santa Cruz, California, USA). Protein bands were visualized using an enhanced chemiluminescence detection kit (Pierce) and the band densities were detected and analyzed using the Molecular Imager ChemiDoc XRS + System (Bio-Rad, Hercules, California, USA).

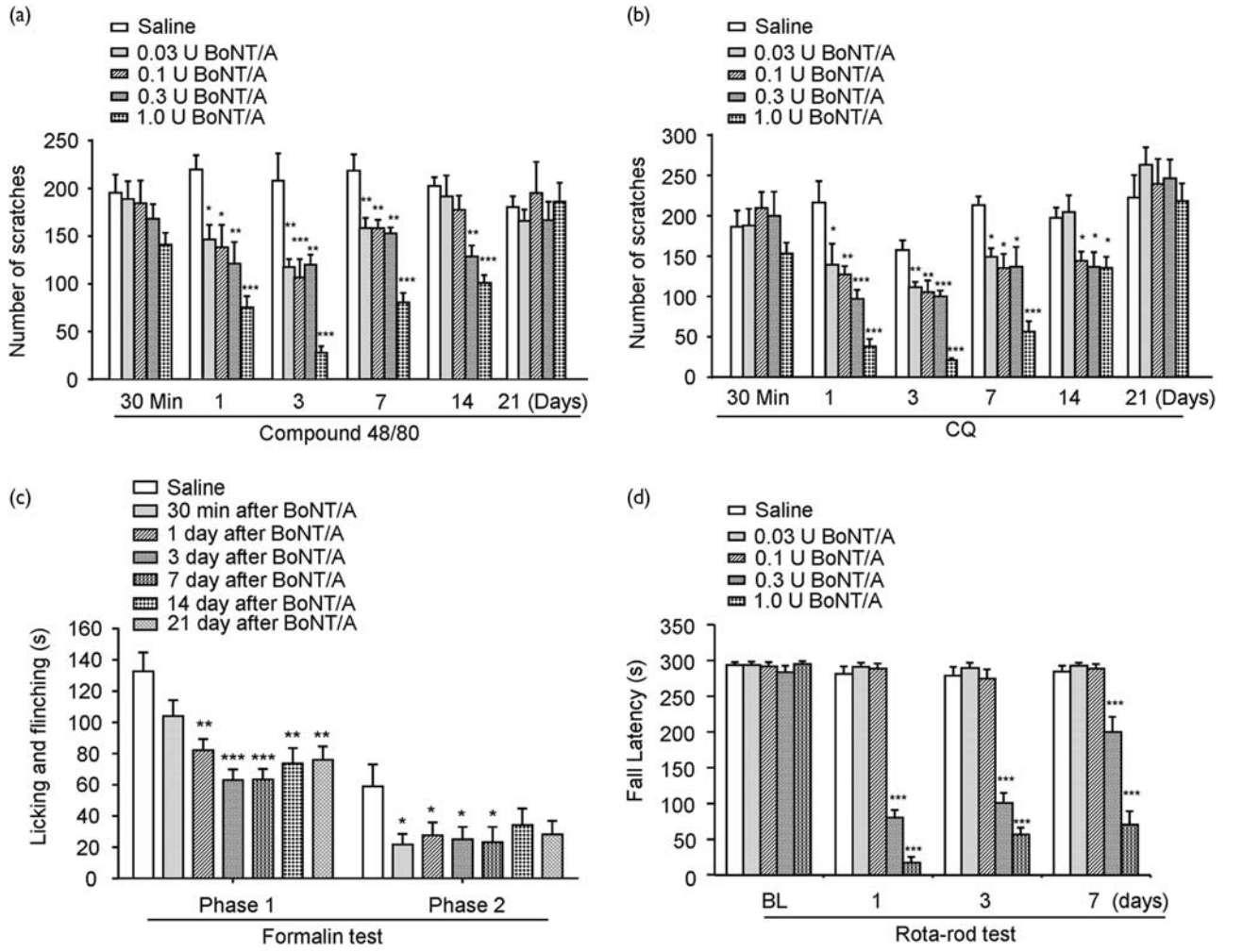
Statistical analysis

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 ± SEM. An unpaired Student's *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* value of less than 0.05 was considered statistically significant.

Results

To explore the potential effects of BoNT/A on acute itch, we first used a mouse model of acute histamine-dependent itch by an i.d. injection of compound 48/80, which induced itch through mast cell degranulation and histamine release [18]. Consistent with previous studies, scratching behavior was induced by an i.d. injection of compound 48/80 into the nape of the neck in mice

Fig. 1



Long-term inhibitory effects of a single injection of botulinum toxin type A (BoNT/A) on acute itch in mice. (a) Effects of a single intradermal (i.d.) injection of BoNT/A on compound 48/80-induced histaminergic itch in mice. (b) Effects of a single i.d. injection of BoNT/A on chloroquine (CQ)-induced nonhistaminergic itch in mice. BoNT/A (0.03, 0.1, 0.3, and 1.0 U) was i.d. injected 30 min, 1, 3, 7, 14, and 21 days before compound 48/80 (100 μg) and CQ (200 μg) injections. Control mice were treated with the same volume of saline ($n = 5$–6 mice/group). (c) Total time of licking and flinching responses during phase 1 (0–15 min) and phase 2 (15–60 min) after formalin injection. Intraplantar injection of BoNT/A (0.1 U/paw) or saline injection was administered 30 min and 1, 3, 7, 14, and 21 days before the injection of 20 μl formalin (0.5%) ($n = 7$–10 mice/group). (d) Effects of a single injection of BoNT/A on motor function evaluated by the rota-rod test in mice. The rota-rod test was performed after BoNT/A (0.03, 0.1, 0.3, and 1.0 U) injection at 30 min, 1, 3, and 7 days into the nape of the neck of mice ($n = 6$–8 mice/group). BL: base line. Data are presented as mean ± SEM; $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus the control group (one-way analysis of variance, followed by the Bonferroni post-hoc test).

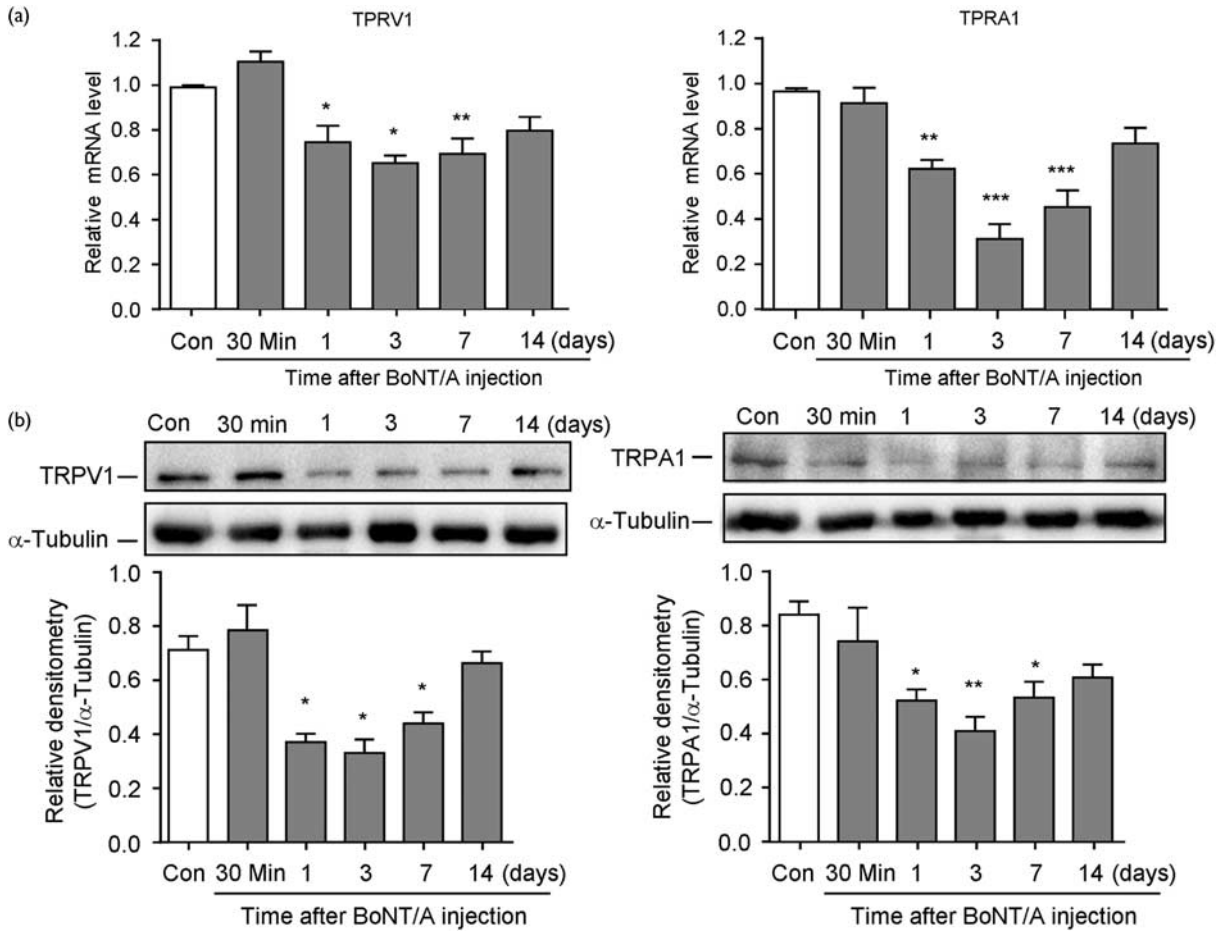
(Fig. 1a). A single i.d. administration of different doses of BoNT/A (0.03, 0.1, 0.3, and 1.0 U) 30 min before the i.d. injection of compound 48/80 had no significant effects on compound 48/80-induced acute itch in mice (Fig. 1a). However, pretreatment with BoNT/A (0.03, 0.1, 0.3, and 1.0 U) for 1, 3, 7, and 14 days before injection of compound 48/80 significantly attenuated compound 48/80-induced acute itch [Fig. 1a; 1 day: $F_{(4, 25)} = 8.255$, $P = 0.0002$; 3 days: $F_{(4, 25)} = 14.24$, $P < 0.0001$; 7 days: $F_{(4, 25)} = 19.61$, $P < 0.0001$; 14 days: $F_{(4, 25)} = 11.45$, $P < 0.0001$]. After BoNT/A injection at 3 days, the anti-itch effect seemed to be the most obvious. After BoNT/A injection at 21 days, there were no significant differences between pretreatment with the saline and all doses of BoNT/A (Fig. 1a). These results suggested that BoNT/A could inhibit compound 48/80-induced acute histaminergic itch for a long period.

CQ (an antimalaria drug) could induce histamine-independent itch by activation of Mas-related G protein-coupled receptor A3 and TRPA1 in primary sensory neurons in mice [14]. We subsequently investigated whether BoNT/A treatment could reduce CQ-induced acute histamine-independent itch in mice. 30 min before the i.d. injection of CQ, a single i.d. injection of BoNT/A (0.03, 0.1, 0.3, and 1.0 U) could not

suppress CQ-induced acute itch in mice (Fig. 1b). However, 1–14 days before the injection of compound 48/80, pretreatment with BoNT/A significantly attenuated CQ-induced acute itch [Fig. 1b; 1 day: $F_{(4, 24)} = 12.57$, $P < 0.0001$; 3 days: $F_{(4, 25)} = 16.81$, $P < 0.0001$; 7 days: $F_{(4, 24)} = 10.83$, $P < 0.0001$; 14 days: $F_{(4, 25)} = 4.676$, $P = 0.0059$]. After BoNT/A injection at 3 days, the anti-itch effect of BoNT/A seemed to be the most robust (Fig. 1b). After BoNT/A injection at 21 days, all doses of BoNT/A had no significant effects on CQ-induced itch in mice (Fig. 1b). These results suggested that BoNT/A could also inhibit CQ-induced acute non-histaminergic itch for a long period, similar to that of compound 48/80-induced itch.

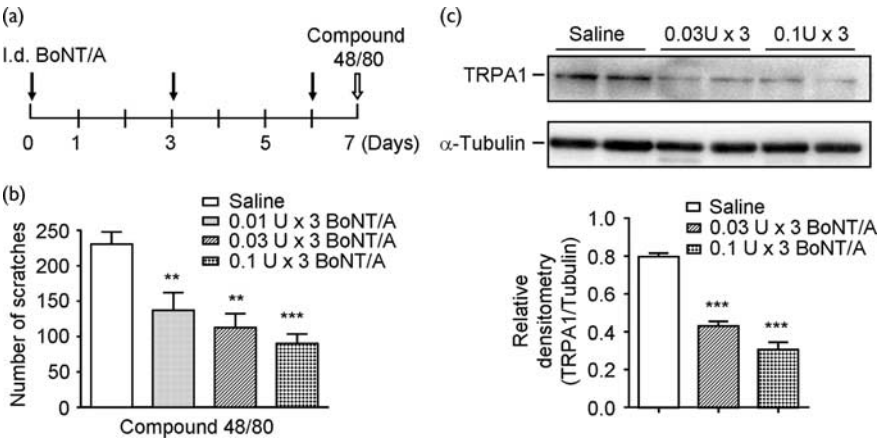
Next, we investigated whether pretreatment of BoNT/A inhibited formalin-induced pain in mice. At 30 min and 1, 3, 7, 14, and 21 days after an intraplantar injection of BoNT/A (0.1 U), mice received a 0.5% formalin injection into the same hindpaw [19]. Formalin-evoked nocifensive behaviors consisted of phase 1 (0–10 min) and phase 2 (10–60 min) (Fig. 1c). For phase 1, pretreatment with the BoNT/A group significantly decreased the time of licking and flinching, with maximal effect after 3 days and lasted at least 21 days [Fig. 1c; $F_{(6, 47)} = 8.796$, $P < 0.0001$]. For phase 2, pretreatment of BoNT/A significantly decreased the time of licking and flinching from 30 min to 7 day. However, after BoNT/A treatment for 21 days, the inhibitory effects did not reach statistical significance [Fig. 1c;

Fig. 2



Long-term inhibitory effects of a single injection of botulinum toxin type A (BoNT/A) on the expression of transient receptor potential cation channel, subfamily V, member 1 (TRPV1) and transient receptor potential cation channel, subfamily A, member 1 (TRPA1) in cervical dorsal root ganglia (DRG) of mice. (a) Quantitative RT-PCR analysis showed that the mRNA expression of TRPV1 and TRPA1 in DRG was downregulated by pretreatment of BoNT/A. The results of TRPA1 and TRPV1 were normalized to β-actin gene expression level. (b) Western blotting data showed that the protein expression level of TRPV1 and TRPA1 in DRG was downregulated by pretreatment of BoNT/A. The representative western blotting pictures are shown in upper layers. The quantitative data on the expression of TRPV1 and TRPA1 are shown in the lower layers. Results are representative of three independent experiments. Cervical DRGs were collected after a BoNT/A (0.1 U) intradermal injection into the nape of the neck at 30 min, 1, 3, 7, and 14 days. Con: control treatment. Data are expressed as the mean ± SEM; $n = 3$–4 mice/group. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus the control group (one-way analysis of variance, followed by the Bonferroni post-hoc test).

Fig. 3



Inhibitory effect of repeated injections of botulinum toxin type A (BoNT/A) on the compound 48/80-induced acute itch model. (a) BoNT/A (0.01, 0.03, and 0.1 U for three times) was administered intradermally (i.d.) before compound 48/80 (100 μg) injection. (b) Compound 48/80-induced itch was dose dependently reduced by repeated injections of BoNT/A. Mice in the control group were treated with the same volume of saline. Data are presented as mean ± SEM; $n = 5$–6 mice. $^{**}P < 0.01$, $^{***}P < 0.001$ versus the control group (one-way analysis of variance, followed by the Bonferroni post-hoc test). TRPV1, transient receptor potential cation channel, subfamily V, member 1.

$F_{(6, 46)} = 2.498$, $P = 0.0355$]. Thus, the results suggested that BoNT/A attenuated inflammatory pain for a much longer duration compared with its anti-itch activity. In addition, we used the rota-rod test to explore the potential effects of BoNT/A on motor function. The results showed that an i.d. injection of BoNT/A into the nape of the neck at higher doses (0.3 and 1.0 U) significantly decreased the fall latency of mice in the rota-rod test [Fig. 1d; $F_{\text{treatment}(4, 28)} = 135.9$, $P < 0.0001$; $F_{\text{time}(3, 84)} = 118.7$, $P < 0.0001$; $F_{\text{treatment} \times \text{time}(12, 84)} = 41.02$, $P < 0.0001$], suggesting that higher doses of BoNT/A impaired motor function in mice. Thus, we used lower dose (0.1 U) of BoNT/A in the following experiments.

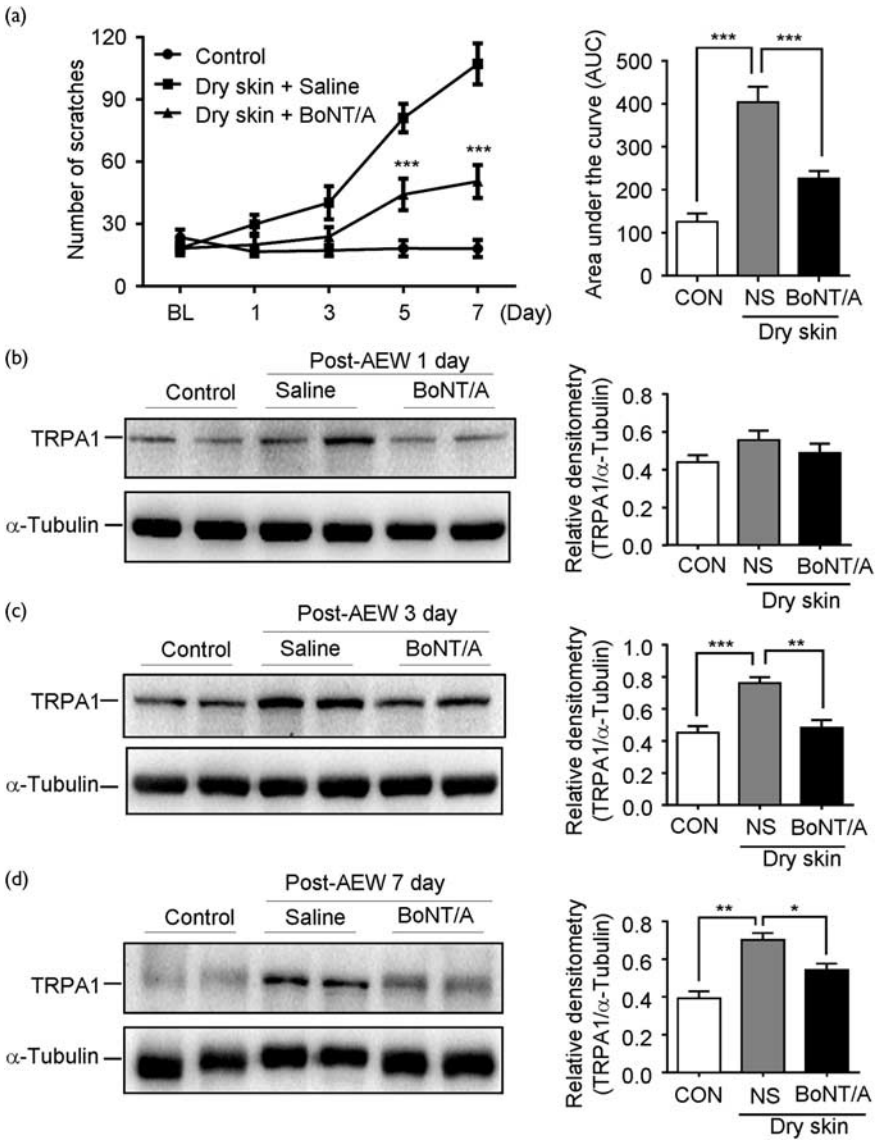
Increasing evidence supported the concept that TRPV1 mediates histaminergic itch and TRPA1 mediates non-histaminergic itch, respectively [20]. We subsequently asked whether administration of BoNT/A could regulate the expression of TRPA1 and TRPV1 on the mRNA and protein levels in the DRG level. After an i.d. single injection of BoNT/A (0.1 U) into the nape of the neck at 30 min and 1, 3, 7, and 14 days, cervical DRGs were harvested for analysis. The results showed that the mRNA expression of TRPV1 and TRPA1 was notably decreased by a single injection of BoNT/A, and this effect lasted for at least 7 days [Fig. 2a; For TRPV1: $F_{(5, 17)} = 11.95$, $P < 0.0001$; For TRPA1: $F_{(5, 17)} = 20.29$, $P < 0.0001$]. To determine whether pretreatment of BoNT/A affects the protein expression of TRPA1 and TRPV1 in cervical DRGs, a western blotting assay was performed in the cervical DRGs at 30 min and 1, 3, 7, and 14 days after BoNT/A treatment. BoNT/A significantly decreased TRPA1 and TRPV1 protein expression, which lasted for at least 7 days compared with that of control mice [Fig. 2b; for TRPV1: $F_{(5, 15)} = 9.437$, $P = 0.0003$; for TRPA1: $F_{(5, 13)} = 5.104$, $P = 0.0083$]. Thus, pretreatment of BoNT/A could decrease the expression of TRPV1 and TRPA1 at both the mRNA and protein levels, which may be associated with the anti-itch effects of BoNT/A.

We further tested whether repeated injections of BoNT/A could induce more dramatic effects on acute itch in mice. Pretreatment of BoNT/A repeated three times significantly decreased compound 48/80-induced scratching compared with the saline group [Fig. 3a and b; $F_{(3, 19)} = 10.34$, $P = 0.0003$]. After repeated injections of BoNT/A, the protein expression of TRPA1 in cervical DRGs was also significantly decreased [Fig. 3c; $F_{(2, 6)} = 78.40$, $P < 0.0001$]. The results indicated that repeated injections of BoNT/A also exerted similar anti-itch effects in mice.

To further investigate the effects of BoNT/A on dry skin-induced chronic itch, which was produced by AEW treatment twice daily for 7 days in mice, AEW treatment caused intense scratching in mice [Fig. 4a; $F_{\text{time}(4, 56)} = 37.05$, $P < 0.0001$; $F_{\text{group}(1, 14)} = 44.73$, $P < 0.0001$; $F_{\text{time} \times \text{group}(4, 56)} = 41.06$, $P < 0.0001$]. Treatment of BoNT/A (i.d., 0.1 U) significantly suppressed the development of chronic itch on days 5 and 7 [Fig. 4a; $F_{\text{time}(4, 56)} = 40.4$, $P < 0.0001$; $F_{\text{group}(1, 14)} = 21.32$, $P = 0.0004$; $F_{\text{time} \times \text{group}(4, 56)} = 7.622$, $P < 0.0001$]. Thus, we concluded that BoNT/A also produced antipruritic activity under a chronic itch condition.

Previous work showed that upregulation of TRPA1 expression in DRG contributed toward the development of chronic itch related to dry skin. We further evaluated the protein levels of TRPA1 expression by western blotting. The results showed that protein expression of TRPA1 was significantly increased after AEW treatment

Fig. 4



Long-term inhibitory effect of a single injection of botulinum toxin type A (BoNT/A) on dry skin-induced chronic itch and the upregulation of transient receptor potential cation channel, subfamily A, member 1 (TRPA1) expression in mice. (a) BoNT/A significantly inhibited dry skin-induced scratching behavior in mice. (b–d) Western blotting data showed that chronic itch-induced upregulated expression of TRPA1 in dorsal root ganglia (DRG) was suppressed by pretreatment of BoNT/A. The cervical DRGs were collected after acetone–diethylether–water (AEW) treatment 1 day (b), 3 day (c), and 7 days (d). The representative western blotting pictures are shown in the left layers. The quantitative data on the expression of TRPA1 are shown in the right layers. Results are representative of three independent experiments. botulinum toxin type A (BoNT/A) (0.1 U) was administered intradermally 1 day before AEW treatment. Control mice were treated with the same volume of saline instead of BoNT/A. Data are presented as mean ± SEM; $n = 6$–7 mice. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus the corresponding groups (one-way analysis of variance, followed by the Bonferroni post-hoc test).

for 3 days [$t_{(10)} = 5.414$, $P = 0.0003$] and 7 days [$t_{(6)} = 5.860$, $P = 0.0011$] compared with the sham group (Fig. 4b–d). After an injection of BoNT/A, the protein expression of TRPA1 was significantly decreased after AEW treatment for 3 days [$t_{(8)} = 4.571$, $P = 0.0018$] and 7 days [$t_{(7)} = 3.169$, $P = 0.0157$] compared with the saline group (Fig. 4b–d). Thus, the results indicated that pretreatment of BoNT/A abolished the upregulation of TRPA1 expression induced by dry skin.

Discussion

In the present study, we found that a single pretreatment with BoNT/A induced long-term inhibitory effects on acute and chronic itch in mice. Pretreatment with BoNT/A also resulted in the downregulated expression of TPRV1 and TPRA1 at transcriptional and translational levels in the cervical DRGs of mice. Thus, on the basis of the critical roles of TRPV1 and TRPA1 in acute and chronic itch, we postulated that the anti-itch

effects of BoNT/A might be associated with the downregulated expression of TPRV1 and TPRA1 in the DRGs of mice.

To understand the anti-itch activity of BoNT/A, we evaluated the effects of BoNT/A on compound 48/80 and CQ-induced acute itch behavior and dry skin-induced chronic itch in mice. Compound 48/80 is known to induce itch behavior in mice, mainly through the release of histamine from mast cells. Histamine binds to the H1R or the H4R receptor, which couples to the TRPV1 channel, to induce acute histaminergic itch [13,21]. CQ, an antimalaria drug, induced nonhistaminergic itch through action on the Mas-related G protein-coupled receptor A3 receptor on sensory nerves and then induced the opening of TRPA1 [14]. Thus, it is suggested that TRPV1 mediates histamine-dependent itch and TRPA1 mediates histamine-independent itch, respectively. In addition, we used the AEW model to induced chronic itch in mice, which mimicked the dry skin condition [22]. In our study, both compound 48/80-induced and CQ-induced acute scratching behavior and dry skin-induced chronic scratching behavior were suppressed by pretreatment with BoNT/A. These results indicated that a single injection of BoNT/A exerts long-term anti-itch effects in mouse itch models.

In the present study, we found that pretreatment with BoNT/A induced the downregulation of the TRPV1 and TRPA1 expression at transcriptional and translational levels in the DRGs of mice. In the chronic itch model, we also found that pretreatment of BoNT/A inhibited the upregulation of TRPA1 expression induced by AEW treatment. In contrast, the expression level of TRPV1 in the DRGs was not altered by AEW treatment (data not shown). Previous studies had shown that TRPA1 is required for both transduction of chronic itch signals to the central nerve system and for the considerable skin changes triggered by dry-skin-evoked itch [23]. On the basis of the key roles of TRPV1 and TRPA1 in acute and chronic itch, the anti-itch effects of BoNT/A may be attributed to the downregulation of TRPV1 and TRPA1 in the DRGs in mice. Previous reports also showed that BoNT/A reduced the expression of TRPV1 in DRG and produced analgesic effects in a rat neuropathic pain model [24]. Recently, it was shown that the upregulated membrane surface-expressed TRPV1 and TRPA1 channels by tumor necrosis factor-α were abolished by BoNT/A treatment [25]. However, the mechanisms underlying the regulation of the mRNA expression of TRPV1 and TRPA1 by BoNT/A remain unclear.

In summary, our study suggests that BoNT/A exerts long-term inhibitory effects in histamine-dependent and histamine-independent acute itch and dry skin-induced chronic itch in mice. The anti-itch effects of BoNT/A may be because of the downregulation of TRPV1 and TRPA1 expression at transcriptional and translational levels in the DRGs. Thus, BoNT/A may be a novel, natural, and effective therapeutic agent for the treatment of acute and chronic itch clinically.

Acknowledgements

This work was supported by grants from the NSFC (National Natural Science Foundation of China) to Tong Liu (31371179, 81300968) and by a grant from the Nature Science Foundation of Jiangsu province to Li-Hua Chen (BK20140372). Tong Liu was also supported by funding from Jiangsu Province (2015-JY-029). This project is also supported by the Second Affiliated Hospital of Soochow University Preponderant Clinic Discipline Group Project Funding (XKQ2015002). This project is also supported by A Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions.

Author contributions: Wei-Feng Luo and Tong Liu designed and supervised this study. Lei-Fang Cao, Meng Si, Ya Huang, Li-Hua Chen, Xiao-Yan Peng, Ya-Qin Qin, Teng-Teng Liu, and Yan Zhou conducted the experiments, and collected and analyzed the data. Lei-Fang Cao, Wei-Feng Luo, and Tong Liu prepared the manuscript.

Conflicts of interest

There are no conflicts of interest.

References

1 Han L, Dong X. Itch mechanisms and circuits. *Annu Rev Biophys* 2014; **43**:331–355.
2 Yosipovitch G, Bernhard JD. Clinical practice. Chronic pruritus. *N Engl J Med* 2013; **368**:1625–1634.
3 Liu T, Gao YJ, Ji RR. Emerging role of toll-like receptors in the control of pain and itch. *Neurosci Bull* 2012; **28**:131–144.
4 Rossetto O, Pirazzini M, Montecucco C. Botulinum neurotoxins: genetic, structural and mechanistic insights. *Nat Rev Microbiol* 2014; **12**:535–549.
5 Binz T, Rummel A. Cell entry strategy of clostridial neurotoxins. *J Neurochem* 2009; **109**:1584–1595.
6 Matak I, Lackovic Z. Botulinum neurotoxin type a: actions beyond snap-25? *Toxicology* 2015; **335**:79–84.
7 Dressler D. Clinical applications of botulinum toxin. *Curr Opin Microbiol* 2012; **15**:325–336.
8 Wallengren J, Bartosik J. Botulinum toxin type a for neuropathic itch. *Br J Dermatol* 2010; **163**:424–426.
9 Akhtar N, Brooks P. The use of botulinum toxin in the management of burns itching: preliminary results. *Burns* 2012; **38**:1119–1123.
10 Sun S, Dong X. TRP channels and itch. *Semin Immunopathol* 2016; **38**:293–307.
11 Wang H, Woolf CJ. Pain TRPs. *Neuron* 2005; **46**:9–12.
12 Chung MK, Jung SJ, Oh SB. Role of TRP channels in pain sensation. *Adv Exp Med Biol* 2011; **704**:615–636.
13 Shim WS, Tak MH, Lee MH, Kim M, Kim M, Koo JY, *et al.* TRPV1 mediates histamine-induced itching via the activation of phospholipase a2 and 12-lipoxygenase. *J Neurosci* 2007; **27**:2331–2337.
14 Wilson SR, Gerhold KA, Bifolck-Fisher A, Liu Q, Patel KN, Dong X, Bautista DM. TRPA1 is required for histamine-independent, mas-related g protein-coupled receptor-mediated itch. *Nat Neurosci* 2011; **14**:595–602.
15 Lieu T, Jayaweera G, Zhao P, Poole DP, Jensen D, Grace M, *et al.* The bile acid receptor TGR5 activates the TRPA1 channel to induce itch in mice. *Gastroenterology* 2014; **147**:1417–1428.
16 Liu T, Ji RR. Oxidative stress induces itch via activation of transient receptor potential subtype ankyrin 1 in mice. *Neurosci Bull* 2012; **28**:145–154.
17 Zhou FM, Cheng RX, Wang S, Huang Y, Gao YJ, Zhou Y, *et al.* Antioxidants attenuate acute and chronic itch: peripheral and central mechanisms of oxidative stress in pruritus. *Neurosci Bull* 2016. [Epub ahead of print].

18 Schlosburg JE, Boger DL, Cravatt BF, Lichtman AH. Endocannabinoid modulation of scratching response in an acute allergenic model: a new prospective neural therapeutic target for pruritus. *J Pharmacol Exp Ther* 2009; **329**:314–323.
19 McNamara CR, Mandel-Brehm J, Bautista DM, Siemens J, Deranian KL, Zhao M, *et al*. TRPA1 mediates formalin-induced pain. *Proc Natl Acad Sci USA* 2007; **104**:13525–13530.
20 McNeil B, Dong X. Peripheral mechanisms of itch. *Neurosci Bull* 2012; **28**:100–110.
21 Jian T, Yang N, Yang Y, Zhu C, Yuan X, Yu G, *et al*. TRPV1 and PLC participate in histamine h4 receptor-induced itch. *Neural Plast* 2016; **2016**:1682972.
22 Miyamoto T, Nojima H, Shinkado T, Nakahashi T, Kuraishi Y. Itch-associated response induced by experimental dry skin in mice. *Jpn J Pharmacol* 2002; **88**:285–292.
23 Wilson SR, Nelson AM, Batia L, Morita T, Estandian D, Owens DM, *et al*. The ion channel TRPA1 is required for chronic itch. *J Neurosci* 2013; **33**:9283–9294.
24 Shimizu T, Shibata M, Toriumi H, Iwashita T, Funakubo M, Sato H, *et al*. Reduction of TRPV1 expression in the trigeminal system by botulinum neurotoxin type-a. *Neurobiol Dis* 2012; **48**:367–378.
25 Meng J, Wang J, Steinhoff M, Dolly JO. TNFα induces co-trafficking of TRPV1/TRPA1 in VAMP1-containing vesicles to the plasmalemma via MUNC18-1/syntaxin1/SNAP-25 mediated fusion. *Sci Rep* 2016; **6**:21226.