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Matak, Ivica; Rossetto, Ornella; Lacković, Zdravko

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Botulinum toxin type A selectivity for certain types of pain is associated with capsaicin-sensitive neurons

[1]**Ivica Matak**, MSc; [2]**Ornella Rossetto**, Assist. Prof., [1]**Zdravko Lacković,** Prof.

[1]Laboratory of Molecular Neuropharmacology, Department of Pharmacology and Croatian Brain Research Institute, University of Zagreb School of Medicine, Šalata 11, 10000 Zagreb, Croatia. tel/fax no.: 00385 1 4566843; e-mail (I.M.): ivica.matak@mef.hr; e-mail (Z.L.): lac@mef.hr

[2] Department of Biomedical Sciences, University of Padua, Viale G Colombo 3, 35121 Padua, Italy. Tel. no.: 0039-049-8276077; e-mail (OR): ornella.rossetto@unipd.it

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Corresponding author:

Prof. **Zdravko Lacković**, MD, PhD, Laboratory of Molecular Neuropharmacology, Department of Pharmacology and Croatian Brain Research Institute, University of Zagreb School of Medicine, Šalata 11, 10000 Zagreb, Croatia; tel/fax no.: 00385 1 4566843;

e-mail: lac@mef.hr

ABSTRACT

Unlike most classical analgesics, botulinum toxin type A (BoNT/A) does not alter acute nociceptive thresholds, and shows selectivity primarily for allodynic and hyperalgesic responses in certain pain conditions. We hypothesized that this phenomenon might be explained by characterizing the sensory neurons targeted by BoNT/A in CNS after its axonal transport.

BoNT/A's central antinociceptive activity following its application into the rat whisker pad was examined in trigeminal nucleus caudalis (TNC) and higher level nociceptive brain areas using BoNT/A-cleaved synaptosomal-associated protein 25 (SNAP-25) and c-Fos immunohistochemistry. Occurrence of cleaved SNAP-25 in TNC was examined after non-selective ganglion ablation with formalin or selective denervation of capsaicin-sensitive (vanilloid receptor-1 or TRPV1-expressing) neurons, and in relation to different cellular and neuronal markers. Regional c-Fos activation and effect of TRPV1-expressing afferent denervation on toxin's antinociceptive action were studied in formalin-induced orofacial pain. BoNT/A-cleaved SNAP-25 was observed in TNC, but not in higher level nociceptive nuclei. Cleaved SNAP-25 in TNC disappeared after formalin-induced trigeminal ganglion ablation or capsaicin-induced sensory denervation. Occurrence of cleaved SNAP-25 in TNC and BoNT/A antinociceptive activity in formalin-induced orofacial pain were prevented by denervation with capsaicin. Cleaved SNAP-25 localization demonstrated toxin's presynaptic activity in TRPV1-expressing neurons. BoNT/A reduced the c-Fos activation in TNC, locus coeruleus and periaqueductal gray.

Present experiments suggest that BoNT/A alters the nociceptive transmission at the central synapse of primary afferents. Targeting of TRPV1-expressing neurons might be associated with observed selectivity of BoNT/A action only in certain types of pain.

Key words: botulinum toxin type A; pain; TRPV1-expressing neurons; axonal transport; central afferent terminals; trigeminal nucleus caudalis

1. INTRODUCTION

Botulinum toxin type A (BoNT/A) proteolytically cleaves synaptosomal-associated protein 25 (SNAP-25), part of the soluble N-ethylmaleimide sensitive factor attachment protein receptor (SNARE) complex involved in vesicular neurotransmitter release [13,31]. Subsequent prevention of SNARE-mediated neurotransmitter release mediates BoNT/A's toxicity in botulism and its therapeutic effects associated with hyperactive neuromuscular and autonomic cholinergic synapses. Small amounts of peripherally applied BoNT/A are used for treatment of different painful disorders (review by Jabbari and Machado [30]). In the craniofacial region BoNT/A was approved for chronic migraine treatment [17]. Off-label BoNT/A use may be beneficial in other craniofacial painful disorders, such as temporomandibular joint disorders and trigeminal neuralgia [23,67].

Based on the preclinical model of formalin-induced pain [15], it was suggested that BoNT/A reduces both pain and inflammation by preventing local neurotransmitter release from peripheral sensory nerves [2]. However, further studies questioned the association of BoNT/A antinociceptive activity with its anti-inflammatory-effects. At BoNT/A doses which reduced carrageenan and capsaicin-induced pain, no significant anti-inflammatory effects were observed [5,6,20]. Central antinociceptive activity has been suggested by contralateral BoNT/A effects in experimental bilateral pain after unilateral toxin injection [7,8,20,68,69]. Blockage of axonal transport within sciatic and trigeminal nerve with colchicine prevented the antinociceptive activity of peripherally applied toxin [7,21,39]. BoNT/A-induced SNAP-25 cleavage was demonstrated immunohistochemically in trigeminal nucleus caudalis (TNC) and lumbar dorsal horn [37,39,40]. These observations demonstrated that the BoNT/A's antinociceptive effects are dependent on toxin's axonal transport within sensory neurons directed to central nociceptive regions.

BoNT/A is not active in all forms of pain and does not alter normal acute sensory thresholds [4,5,14,15]. We hypothesized that the antinociceptive effects of BoNT/A might be mediated by capsaicin-sensitive transient receptor potential vanilloid (TRPV1)-expressing neurons,

since this type of neurons does not convey acute responses to innocuous or noxious stimuli [12,44].Therefore, we studied formalin-induced hypersensitivity and the occurrence of cleaved SNAP-25 in TNC after peripheral BoNT/A alone or in combination with capsaicin-induced desensitization. We found that the BoNT/A's antinociceptive action and the occurrence of cleaved SNAP-25 in central nociceptive regions are both associated with capsaicin-sensitive primary afferents, which is consistent with the reduction of hyperalgesia and allodynia by BoNT/A, and the lack of its effects on acute mechanical sensitivity.

2. METHODS

2.1. Animals

Adult male Wistar rats (Department of Pharmacology, University of Zagreb School of Medicine), weighing 300-400 g (12h day/ night cycle, free access to food and water), were used in all experiments. Experiments were performed according to 2010/63/EU Directive on the protection of animals used for scientific purposes and recommendations of International Association for the Study of Pain [71], and approved by the Ethical Committee of University of Zagreb School of Medicine (permit no. 07-76/2005-43).

2. 2. BoNT/A injections

Conscious, restrained animals were injected subcutaneously into the whisker pad with 20 µl of 0.9% saline-diluted BoNT/A (Botox®, Allergan Inc, Irvine, CA, USA), using a 27 1/2 gauge needle. 5 and 15 U/kg doses were chosen based on previous experiments [39,41]. 1 unit (1 U) of BoNT/A preparation contains 48 pg of purified C. botulinum neurotoxin type A complex.

2.3. Intraganglionic denervation of trigeminal nerve with formalin and capsaicin

To study the occurrence of cleaved SNAP-25 in trigeminal central afferent terminals, rats were injected into the whisker pad with 15 U/kg BoNT/A, and formalin was injected intraganglionically (i.g.) 5 days *after* peripheral BoNT/A delivery (sufficient period for cleaved SNAP-25 occurrence in the CNS [39]). Anesthetized animals (chloral hydrate, 300 mg/kg) were administered slowly (~1 µl/min) with 10 µl formalin (37 % aqueous solution of formaldehyde) (Formalin, Kemika, Zagreb, Croatia) into the trigeminal ganglion with a Hamilton syringe, using a percutaeous infraorbital approach [39,44]. Animals were deeply anesthetized and perfused for immunohistochemistry 5 days post formalin-induced denervation (10 days after peripheral BoNT/A).

A procedure similar to the formalin-induced denervation was used to investigate the possible truncated SNAP- 25 occurrence in capsaicin-sensitive central afferent terminals. Anesthetized animals (chloral hydrate, 300 mg/kg) were administered percutaneously into the trigeminal ganglion (~1 µl/min) with two injections of 10 µl 2% capsaicin (Sigma, St. Louis, MO, USA) or vehicle (0.9% saline + 10% ethanol + 10% Tween-80), separated 48 h. First injection of capsaicin was administered i.g. 5 days after BoNT/A (15 U/kg) peripheral treatment. In comparison to 0.5% and 1% doses of capsaicin which evoked gradual recovery of eye-wipe response within one week, 2% capsaicin was chosen for further experiments due to the long-term loss of response (monitored up to 12 days after denervation). Animals were sacrificed 10 days post peripheral BoNT/A (3 days post second capsaicin i.g. injection).

We examined if the occurrence of BoNT/A enzymatic activity in TNC is dependent on capsaicin-sensitive neurons. In a separate experiment, the denervation of TRPV1-expressing primary sensory neurons was performed *before* the peripheral BoNT/A injection. Animals were subjected to chemical denervation with 2% capsaicin 5 and 3 days prior to BoNT/A (15 U/kg) treatment, and sacrificed by perfusion 5 days post peripheral BoNT/A.

2.4 Behavioral assessment of the effects of trigeminal primary afferent denervation

We assessed the effects of trigeminal denervation procedures on the animal response to mechanical innocuous and noxious stimuli, as well as TRPV1-sensitive sensory function. Measurements were performed 3-4 days following the trigeminal ganglion ablation with formalin or desensitization of TRPV1-expressing neurons with capsaicin. Prior to behavioral measurements, rats were allowed to accommodate to testing cage environment until normal sniffing/no locomotion posture was assumed. The observer was blinded to the animal treatment.

Whisker pad mechanical or nociceptive sensitivity was first monitored with Von Frey filaments (2 and 8 g bending forces), and then followed by pin-prick test (5-10 min. interval between each stimulus). Von Frey filament bending forces (2 and 8 g) were chosen based on the preliminary experiment with a series of Von Frey filaments (1-15 g) in intact animals. Within the 2 to 8 g range, the filaments elicited a non-painful response in all control animals (non aversive behavior, few animals reacted by slow head withdrawal). Von Frey filaments with bending forces higher than 8 g (10 and 15 g) elicited head deflection (filament bending force was stronger than the rat neck muscles). Pin prick test was employed by using a sterile 27 1/2 gauge needle pressed gently against the whisker pad without penetrating the dermis. Response to innocuous and nociceptive mechanical stimuli in the facial area was quantified by using a semi-quantitative behavioral scoring paradigm, originally devised and described in details by Vos et al. [65]. Aversive behavior was quantified by the following descriptive categories: a) no response, b) non-aversive response, c) mild-aversive response, d.) strong aversive response, e) prolonged aversive behavior, which consist of a sum of following response elements: i) detection (exploratory / sniffing behavior directed to stimulating object), ii) withdrawal (animal slowly the moves head away from stimulating object), iii) escape/attack (avoids further contact / biting and grabbing movement towards stimulation object), iv) facial grooming (three or more asymmetric grooming movements).

Each descriptive category, based on sum of present response elements, was assigned with a score [65]:

0 = no response (no detection);

1 = non-aversive response (detection);

2 = mild-aversive response (detection + withdrawal)

3 = strong aversive response (detection + withdrawal + escape/attack);

4 = prolonged aversive behavior (detection + withdrawal + escape/attack + facial grooming);

Corneal reflex was employed to check for the normal sensitivity of corneal surface to tactile stimuli prior to capsaicin eye-wipe test. Corneal reflex was examined bilaterally by briefly applying a tipped sterile cotton wisp to the cornea, which elicited a blinking response. To prevent the visual contact-evoked reaction, the rat's head was approached by the experimenter's hand from posterolateral side, and the cotton tip was gently applied to the cornea across the lateral eye corner. Cotton tip was applied 5 times (>30 second interval between consecutive applications), and the percentage of elicited blinking responses was used as a measure of behavioral response.

Capsaicin eye-wipe test was used to examine the sensory function of TRPV1-expressing trigeminal neurons. Small drop (~10 µl) of saline-diluted 0.01% capsaicin was released on the corneal surface, and the number of ipsilateral eye-wipes was counted [16,44]. TRPV1-expressing neurons are considered to be desensitized if the wiping response is greatly reduced or prevented [16,44].

To study the possible role of TRPV1-expressing sensory neurons in BoNT/A antinociceptive activity, the effect of BoNT/A on orofacial formalin test was examined in animals desensitized with i.g. capsaicin. Four days after the completion of capsaicin i.g.-induced desensitization (2 injections within 24 h), animals were injected into the whisker pad with saline/5 U/kg BoNT/A. Orofacial formalin test was performed 5-6 days after peripheral saline/BoNT/A treatment.

Formalin test was employed as described previously [39,41,51]. Animals were injected into the whisker pad with 50 µl of saline-diluted 2.5% formalin and observed for 45 min in a transparent cage. Total duration of ipsilateral facial rubbing and grooming evoked by facial formalin was assessed during 3 min periods divided in phase I (0-12 min) and phase II (12-45 min). Observer was blinded to the animal treatment.

2.5. Immunohistochemistry of cleaved SNAP-25 in the brain

For the assessment of cleaved SNAP-25 localization, animals were injected with BoNT/A subcutaneously into the whisker pad, and sacrificed after 5-6 days. Apart from TNC, possible occurrence of cleaved SNAP-25 was studied in thalamus, hypothalamus, sensory cortex, locus coeruleus and periaqueductal gray. Since Marinelli et al. [37] reported the occurrence of cleaved SNAP-25 in lumbar spinal astrocytes of neuropathic mice, we examined the colocalization of cleaved SNAP-25 with marker of astrocytes in animals with trigeminal neuropathy induced by infraorbital nerve constriction (IoNC), as previously described [21].

Anesthetized animals (chloral hydrate 300 mg/kg i.p.) were perfused transcardially with saline, followed by 4% paraformaldehyde in phosphate-buffered saline (PBS). Brain tissue was excised, cryoprotected with sucrose, and kept on -80 ºC as previously described [39,40] Cryostat-cut 40 µm coronal sections of brainstem and diencephalon were collected for free floating in PBS with 0.25% Triton X-100 (PBST), washed and blocked with 10 % normal goat serum (NGS) in PBST. Sections were incubated overnight at room temperature in 1% NGS with 1: 1500 rabbit polyclonal antibody to cleaved SNAP-25 (produced by O.R.), which was previously well characterized and recognizes specifically the BoNT/A-truncated SNAP-25 [39]. Following day the sections were incubated with fluorescent secondary antibody (Goat anti-rabbit Alexa Fluor 555, Molecular Probes, Invitrogen, Carlsbad, CA, USA). Tissue was then incubated overnight at 4ºC with mouse monoclonal antibodies to synaptophysin (1:500, Sigma, St Louis, MO, USA) microtubule-associated protein 2 (MAP-2) (1:1000, Sigma) glial

fibrillary acidic protein (GFAP) (1:1000, Sigma), and NeuN (1:500, Millipore, Temecula, CA, USA). Next day the sections were incubated with goat anti-mouse Alexa fluor 488. Co-staining of cleaved SNAP-25 and TRPV1 was performed with goat anti-vanilloid receptor 1 (TRPV1) polyclonal antibody (1:400, Santa Cruz, Dallas, TX, USA), and donkey anti-rabbit Alexa 488/ donkey anti goat Alexa 555 secondary antibodies.

Co-staining of cleaved SNAP-25 with CGRP was performed with rabbit polyclonal anti-CGRP (Sigma). To prevent the cross-reactivity of primary antibodies raised in rabbit, a modified antibody elution procedure was used [48]. In brief, after incubation with antibodies to cleaved SNAP-25 and secondary goat anti rabbit Alexa 555, sections were washed, transferred to Superfrost Plus glass slides and allowed to adhere and dry. Cleaved SNAP-25 immunoreactivity was photographed in glycerol-coverslipped slides for later comparison. Coverslips were then removed. Slides were washed in PBS, and incubated in dark in pre-heated acidic elution buffer (50°C, pH=2) containing 1% SDS and 25 mM glycine for 30 min without shaking. After elution, sections were blocked again and incubated overnight at 4ºC with CGRP antibody (1:5000). Cross reactivity controls were incubated with 1% NGS. Next day the sections were incubated with goat anti-rabbit Alexa Fluor 488. In cross-reactivity controls, no binding of Alexa fluor 488-labeled secondary antibody was observed. Morphology of cleaved SNAP-25 fibers before elution and after completed immunostaining remained the same.

In studies involving cleaved SNAP-25 immunostaining, sections from 3-4 animals per group (15-25 sections/animal) were examined. Immunostained sections were visualized with Olympus BX-51 epifluorescent microscope coupled to DP-70 digital camera (Olympus, Tokyo, Japan) or TCS SP2 AOBS confocal microscope (Leica, Wetzlar, Germany). Double label images were composed using cellSens Dimension software. Images were processed for brightness and contrast with Adobe Photoshop.

2.6 C-Fos immunohistochemistry after orofacial formalin test

BoNT/A effects on neural activation evoked by orofacial formalin were assessed by quantifying the c-Fos expression in different brain regions of animals injected with 5U/kg BoNT/A or saline. Immunohistochemical staining for c-Fos was performed on coronal sections from caudal medulla, pons, mesencephalon and diencephalon, using rabbit anti c-Fos primary antibody (Santa Cruz, Dallas, TX, USA, dilution 1:500, incubation overnight at room temperature) and goat anti-rabbit Alexa Fluor 488 fluorescent secondary antibody. Immunostained sections were visualized with Olympus BX-51 fluorescent microscope coupled to DP-70 digital camera (Olympus, Tokyo, Japan). C-Fos-positive neuronal fluorescent profiles were automatically counted using cellSens Dimension software (Olympus, Tokyo, Japan). In each region, c-Fos-positive profiles were counted from 4 randomly selected sections per animal. Brain regions were indentified in coronal sections using the rat stereotaxic atlas [47] and appropriate landmarks for each region (central canal, obex, aqueduct, ventricles, etc.).

2.7. Immunohistochemistry of CGRP-expressing central afferent terminals and brainstem neurons after trigeminal ganglion denervation

Denervation of primary afferents in the TNC after formalin-induced ablation of trigeminal ganglion was verified using the immunohistochemistry of calcitonin gene-related peptide (CGRP), which is present in central afferent terminals [25]. Since approximately 70% of the CGRP-expressing trigeminal sensory neurons are TRPV1-positive [50], we checked for the reduced CGRP expression after desensitization of capsaicin-sensitive primary afferents. Ipsilateral and contralateral TNC of each coronal section were visualized with epifluorescent microscope by employing the low-magnification objective (4x) to obtain microphotographs containing the entire TNC region. Images were processed using cellSens Dimension software. Surface area of TNC containing green CGRP immunoreactivity was quantified by

using green channel pixel thresholding. To quantify the extent of degeneration, surface area of ipsilateral, denervated side was divided by surface area of contralateral side which served as a control.

To assess the possible postsynaptic degeneration of central neurons in theTNC region after i.g. treatment with formalin or capsaicin, neuronal nuclear (NeuN) and dendritic (MAP-2) staining was performed.

2.8 Statistical analysis

Parametric data were represented as mean ± standard error mean (SEM), and analyzed by unpaired t-test (for comparison between two groups) or one-way ANOVA followed by Newman-Keuls post hoc test (multiple group comparisons). Non-parametric data (response scores of aversive behavior to mechanical stimuli) were represented by scatter plot and median, and analyzed by Kruskall-Wallis test, followed by Dunn's post hoc. $p<0.05$ was considered significant.

3. RESULTS

3.1 Intraganglionic denervation of trigeminal nerve with formalin and capsaicin

3.1.1 Behavioral effects of trigeminal primary afferent denervation

The animals injected with i.g. formalin showed no response to the ipsilateral whisker pad stimulation with Von Frey filaments, independently of the filament bending force (2 or 8 g) (Fig. 1A , Fig.1B). In addition, formalin i.g. treated-animals did not respond to the pin prick test ipsilaterally to formalin-induced ablation (Fig. 1C). Ipsilateral response to capsaicin eye-wipe test in i.g.formalin-treated animals was abolished (Figure 2A). Corneal reflex (blinking response to cotton whip stimulation of cornea) was almost completely prevented (not shown). Contralaterally, the animals responded to whisker pad and corneal mechanical

stimulation similarly to control animals (not shown). In addition, capsaicin-evoked eye-wipe response was preserved on the non-denervated side (not shown). Formalin is a chemical fixative which immediately kills the living cells by cross-linking of biological molecules and protein precipitation [57]. In line with that, insensitivity to mechanical and capsaicin-induced stimulation after i.g. formalin suggested a non-selective denervation of trigeminal primary afferents.

Acute mechanical sensitivity was unaltered after i.g. capsaicin-evoked desensitization. Capsaicin i.g.-treated animals showed non-aversive response to whisker pad mechanical stimulation with Von Frey filaments (Fig. 1A, Fig. 1B), responded to noxious pin prick stimulus with strong aversive behavior, (Fig. 1C), and exhibited 100% preserved corneal reflex response, similarly to vehicle-treated animals (not shown). Facial BoNT/A pretreatment did not significantly alter the mechanical responses in either vehicle i.g. or capsaicin i.g. –treated animals.

Animals desensitized with 2% capsaicin had a largely reduced response to capsaicin eye wipe test on the ipsilateral side (Figure 2), in line with the effects of capsaicin-induced desensitization of TRPV1-expressing neurons [16,44,57]. On the contralateral side, animals responded similarly to vehicle-treated controls (not shown). Present data indicated that the unilateral i.g. capsaicin selectively desensitized TRPV1-expressing neurons only, without altering primary afferents which mediate the acute mechanical sensitivity.

Figure 1

Figure 2

In animals injected i.g. with vehicle, BoNT/A reduced phase II of formalin-induced orofacial pain, whereas phase I pain was not affected, as previously described [15,39,41]. Capsaicin i.g.–induced denervation prevented the antinociceptive activity of BoNT/A in orofacial formalin-induced pain, while the denervation itself did not influence the duration of

nocifensive behavior in formalin test (Fig. 3). These data suggest that the BoNT/A antinociceptive efficacy is dependent on TRPV1-expressing sensory neurons.

Figure 3

3.1.2 Effects of trigeminal ganglion denervation on CGRP-expressing central afferent terminals and brainstem neurons

In line with the abolished unilateral sensory response, trigeminal ganglion ablation with formalin resulted in almost complete unilateral disappearance of CGRP immunoreactivity in the TNC, which is expressed in a subpopulation of central afferent terminals (Fig. 4A). Capsaicin-evoked denervation induced a large, but in contrast to formalin-induced denervation, incomplete reduction of CGRP immunoreactivity (Figure 5B, Fig. 5C). Decrease of CGRP immunostaining of the ipsilateral TNC in response to i.g. capsaicin is in line with previous studies which reported reduced neuropeptide content in the dorsal horn after desensitization of capsaicin-sensitive central afferent terminals with high dose TRPV1 agonists [22,32]. Remaining CGRP staining possibly corresponded to the peptidergic afferent population not expressing TRPV1 [50].

Quantification of CGRP immunoreactivity supports the loss of CGRP in i.g. formalin-treated animals (Fig. S1A), and CGRP reduction in capsaicin i.g.-treated animals (Fig S1B). Immunostaining of dendrites (MAP-2) and cell bodies (NeuN) of brainstem neurons in the TNC was unaltered by i.g. formalin (Fig. S2A, Fig. S2B). Dendritic and somatic staining of central neurons in the TNC was unaffected by i.g. capsaicin (not shown), similarly to i.g. formalin.

3.1.3 Occurrence of BoNT/A enzymatic activity in the TNC after denervation of trigeminal nerve with formalin and capsaicin

Previously, we found the occurrence of BoNT/A-cleaved SNAP-25 in the TNC after toxin injection into the whisker pad [39]. By employing the trigeminal nerve ablation we examined if

the BoNT/A's enzymatic activity in TNC was located within primary afferent terminals. Formalin-induced ganglion ablation performed 5 days following BoNT/A peripheral injection induced complete disappearance of cleaved SNAP-25 staining in the TNC (Fig. 4B), indicating that the BoNT/A-cleaved SNAP-25 was located in central afferent terminals. Double labeling of cleaved SNAP-25 and TRPV1 in TNC demonstrated the occurrence of products of BoNT/A enzymatic activity in TRPV1-expressing neurons (Fig. 5A). Animals subjected to chemical denervation with capsaicin 5 days following peripheral BoNT/A lacked the immunoreactivity for cleaved SNAP-25 in TNC (Fig. 5B), which suggests that BoNT/A enzymatic activity occurs in capsaicin-sensitive central afferent terminals. Hypothetically, some other types of afferents, which are capsaicin insensitive, might mediate the occurrence of cleaved SNAP-25 in the TNC when the capsaicin-sensitive afferents are desensitized with capsaicin before injection of BoNT/A. However, animals subjected to i.g. capsaicin-induced denervation prior to BoNT/A injection lacked the BoNT/A-cleaved SNAP-25 in TNC (Figure 5C), suggesting that the occurrence BoNT/A enzymatic activity in the TNC is dependent solely on capsaicin-sensitive neurons.

Figure 4

Figure 5

3.2 Immunohistochemical localization of cleaved SNAP-25 in the brain

Cleaved SNAP-25 immunoreactivity appeared either as punctate immunoreactivity or fiber-like profiles. Punctate immunoreactivity colocalized with synaptophysin, a presynaptic marker. On the other hand, fiber-like profiles showed no colocalization with synaptophysin (Fig. 6A). Cleaved SNAP-25 was absent from MAP-2-stained dendrites of TNC neurons (Fig. 6B). In BoNT/A-injected naïve (Fig. 6C) and infraorbital nerve constriction-induced neuropathic animals (not shown), cleaved SNAP-25 was detected outside of GFAP-immunoreactive astrocytes.

Cleaved SNAP-25 mainly did not colocalize with neuropeptide CGRP, except in few neuronal terminals (Fig. S3). After 5 U/kg peripheral BoNT/A injection, cleaved SNAP-25 was detected in TNC only, but not in other sensory regions (not shown).

Figure 6

3.3. BoNT/A effects on regional c-Fos expression in the orofacial formalin test

In present study we have examined the effect of BoNT/A on c-Fos expression in the TNC and upstream sensory regions after formalin injection into the orofacial area (Fig. 7, Table 1). Formalin-evoked c-Fos expression in TNC, locus coeruleus, periaqueductal gray, medial thalamus (paraventricular nucleus), amygdala and hypothalamus was increased 3-9 times compared to saline controls (Table 1, middle column). Increased c-Fos expression in examined regions is in agreement with previous studies involving peripheral formalin test [11].

Similarly to previous findings in spinal cord dorsal horn [2,18,64], in present experiment BoNT/A lowered the pain-evoked neural activation (measured by c-Fos expression) in the TNC. Additionally, BoNT/A reduced the formalin-evoked neural activation in locus coeruleus and periaqueductal gray. BoNT/A did not affect the expression of c-Fos in paraventricular nucleus of thalamus, ipsilateral and contralateral hypothalamus and contralateral central amygdala (Fig. 7, Table 1).

Figure 7

Table 1

4. DISCUSSION

In contrast to classical analgesics such as opioids, BoNT/A does not alter the acute nociceptive thresholds, but it selectively reduces the allodynic and hyperalgesic responses in

certain pain conditions [4,5,14,15]. We previously discovered that the antinociceptive activity of BoNT/A is mediated by its axonal transport to central sensory nociceptive nuclei [7,21,39]. In present study we investigated the possibility that the selectivity of BoNT/A antinociceptive action is mediated by specific subtypes of sensory neurons targeted by BoNT/A.

Enzymatic activity of BoNT/A in TNC occurs in central afferent terminals. Occurrence of cleaved SNAP-25 in TNC and lumbar dorsal horn, the regions which receive afferent nociceptive input, suggests that BoNT/A alters central nociceptive transmission [39]. However, the localization of this action in TNC was, up to now, unknown. In present study we examined whether BoNT/A's enzymatic activity in the TNC is located in primary sensory neurons. Loss of cleaved SNAP-25 in the TNC after formalin-induced ablation of primary afferents demonstrated that the BoNT/A enzymatic activity occurs in central primary afferent terminals.

In present experiments we did not observe any truncated SNAP-25 remaining after ganglionic denervation, thus, our results do not support possible transcytosis to second order synapses in the TNC. However, transcytosis of BoNT/A in rats was demonstrated after both anterograde and retrograde axonal transport in the optic system [52,53]. Recently, a decrease of spontaneous and evoked inhibitory glycinergic potentials in isolated rat lumbar substantia gelatinosa neurons following peripheral BoNT/A injection was reported [1]. The authors suggested toxin's transcytosis to glycinergic interneurons.

BoNT/A's antinociceptive activity is associated with capsaicin-sensitive neurons. After demonstrating that BoNT/A's proteolytic activity in TNC was located within central afferent terminals of trigeminal neurons, we found that the terminals involved are sensitive to capsaicin and express TRPV1 (Fig. 4A, Fig. 4B). Moreover, chemical denervation with i.g. capsaicin prevented the occurrence of cleaved SNAP-25 in the TNC, as well as the antinociceptive activity of BoNT/A in formalin-induced orofacial pain (Fig. 4C, Fig. 5). Mentioned experiments demonstrate that the BoNT/A's antinociceptive activity, mediated by

toxin's axonal transport to CNS [7,21,39], involves capsaicin sensitive (TRPV1-expressing) central afferent terminals.

Enzymatic activity of BoNT/A in capsaicin-sensitive neurons supports the reduction of capsaicin-evoked pain [5,24,55]. It was reported that BoNT/A reduces TRPV1 expression in peripheral sensory neurons, possibly by preventing SNARE-mediated receptor translocation to the cell membrane [3,56,69,70]. Similar effect may occur in central afferent terminals, where BoNT/A might regulate the TRPV1 receptor-mediated central nociceptive transmission.

TRPV1-expressing neurons are primarily glutamatergic [27] but might contain other transmitters such as Substance P, CGRP etc. [9,25,32,50]. Thus, BoNT/A might prevent glutamate as well as other co-transmitters' release from a distinct set of nerve endings [19,21]. Recently, it was proposed that BoNT serotype B reduces spinal substance P release from TRPV1-expressing neurons in mice [38].

BoNT/A selectivity for hyperalgesia and allodynia is associated with capsaicin-sensitive neurons. Since only 16- 20% of trigeminal neurons express TRPV1 [9,26,28,50], our observations might suggest a preferential targeting of BoNT/A to TRPV1-expressing central terminals in the TNC. Selective targeting of TRPV1-expressing nerve endings might explain the activity of BoNT/A only in certain types of pain. Comparison between the antinociceptive effects of BoNT/A and suppressed function of TRPV1-expressing neurons in different types of experimental acute nociceptive, inflammatory and neuropathic pain indicates a considerable agreement of the effects of BoNT/A and TRPV1-mediated antinociceptive effects:

-BoNT/A and suppression of TRPV1-expressing neurons (evoked by denervation of TRPV1-expressing neurons, or TRPV1 antagonists) do not affect acute mechanical thresholds [4,5,14,15,32,43,44,59]. In present study we observed preserved acute mechanical sensitivity upon either BoNT/A treatment or denervation of capsaicin-sensitive primary afferents (Fig. 1). Transmission of acute mechanical stimuli by neurons which are not

capsaicin-sensitive might explain the lack of effect of BoNT/A on acute innocuous or nociceptive mechanical thresholds.

- BoNT/A, denervation of TRPV1-expressing neurons, and TRPV1 agonists, reduce the nocifensive behavior and mechanical hyperalgesia evoked by capsaicin [5,6,24,32,49,55,59], and thermal hyperalgesia evoked by inflammatory or neuropathic pain [4,5,6,16,33,36,49,56,59,60,66].
- BoNT/A and TRPV1 antagonists reduce the inflammatory and neuropathic mechanical allodynia and hyperalgesia [4,18,21,33,46,49,66]. The results are ambiguous after denervation with high dose TRPV1 agonists: some studies report the reduction of mechanical allodynia [36,60], while others do not [35].
- BoNT/A and TRPV1 receptor antagonists reduce formalin-induced pain [15,18,34,39,41,59,63]. However, in present experiments, 2.5% formalin-induced nocifensive response was unaltered by i.g. capsaicin (Fig. 3). This is in accordance with a recent similar study employing i.g. resiniferatoxin (a more potent capsaicin analog) and 2.5% orofacial formalin [16]. Effect of desensitization of TRPV1-expressing neurons on the duration of formalin-evoked nociceptive behavior in mice was shown to be dependent on formalin concentration [54]. While intrathecal capsaicin reduced the 0.5% formalin-evoked behavior, it did not reduce the behavior evoked by higher formalin dose (2%) [54]. Unaltered response to formalin test might be associated with central plastic changes occurring after denervation of TRPV1-expressing afferents, such as the abnormally increased receptive fields of dorsal horn neurons [42]. Another theoretical possibility is that the denervation of TRPV1-expressing neurons might result in compensatory nociceptive activation of other primary afferent types in the formalin test.

Cleaved SNAP-25 cellular and regional localization. Herein we examined the localization of truncated SNAP-25 in relation to cellular markers in the TNC. Cleaved SNAP-25 punctate immunoreactivity colocalized with presynaptic terminals immunolabeled with synaptophysin, consistent with well known BoNT/A activity in synapses [10]. Cleaved SNAP-25 fiber-like

profiles, most likely corresponding to axons, were not immunoreactive to synaptophysin (Fig 6A). This is in line with extrasynaptic occurrence of SNAP-25 along the axons [61]. Cleaved SNAP-25 did not colocalize either with MAP-2-positive dendrites of secondary neurons, or with GFAP, marker of astrocytes (Fig. 6B, Fig. 6C). Recent study of Marinelli et al. [37] reported BoNT/A-truncated SNAP-25 occurrence in spinal astrocytes of neuropathic mice. Differences between the studies might be associated with experimental setup, animal species (mice vs. rats) and sensory region examined (lumbar spinal dorsal horn vs. TNC). Following BoNT/A subcutaneous injection into the whisker pad area, we did not observe convincing cleaved SNAP-25 colocalization with CGRP-containing peptidergic afferents (Fig. 4, Supplement Fig. S3). In rats, significant portion of TRPV1-expressing trigeminal neurons (~30-56%) does not express CGRP [9,50]. Lack of colocalization could be associated with the site of toxin administration, since TRPV1-expressing afferents which innervate cutaneous structures are primarily non-peptidergic [9,27,62]. Our results suggest that BoNT/A's antinociceptive action, at least in present experimental setup, is not mediated primarily by direct prevention of central CGRP release.

Cleaved SNAP-25 in sensory regions examined above the level of TNC (locus coeruleus, periaqueductal gray, thalamus, hypothalamus, sensory cortex) was not observed. However, pain-evoked neural activity (assessed with c-Fos expression) was decreased by BoNT/A in locus coeruleus and periaqueductal gray (but not in thalamus, hypothalamus and amygdala) (Fig. 7; Table 1). Reduction of pain-evoked neural activity in regions where BoNT/A enzymatic activity was not observed suggests that the toxin's indirect effects in CNS may be more widespread compared to its direct effects mediated by central SNAP-25 cleavage.

5. CONCLUSION

Present results suggest the association of BoNT/A's antinociceptive activity with capsaicin-sensitive central afferent terminals. This could explain selective action of BoNT/A on some forms of pain, only.

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FIGURE CAPTIONS

Fig.1 Mechanical sensitivity of facial area in rats is unaltered by capsaicin-induced desensitization of TRPV1-expressing neurons, and it is abolished after non-selective ablation of trigeminal primary afferents.

5 days following the peripheral BoNT/A (15 U/kg) or saline injection into the whisker pad, rats were injected intraganglionically (i.g.) with either vehicle, 2% capsaicin (double vehicle or capsaicin treatment separated 24-48 h), or formalin (single i.g. treatment). Mechanical sensitivity of the facial area was examined 3-4 days after ganglion treatments.

A. response to ipsilateral whisker pad stimulation with 2 g filament; B. response to ipsilateral whisker pad stimulation with 8 g filament; C. response to ipsilateral whisker pad pin-prick stimulation

N(animals per group)=5-6. Behavioral scores are represented as median (horizontal line) and individual values were represented by scatter plot (dots). * - $p<0.05$ in comparison to vehicle i.g.; + - $p<0.05$ in comparison to capsaicin i.g.; ++ - $p<0.01$ in comparison to capsaicin i.g. (Kruskal-Wallis test followed by Dunn's post hoc, $p<0.05$).

Fig. 2 Capsaicin-induced eye-wipe response after capsaicin or formalin-induced denervation of trigeminal nerve.

5 days following peripheral BoNT/A (15 U/kg) or saline injection into the whisker pad, rats were injected intraganglionically (i.g.) with vehicle, 2% capsaicin (double vehicle or capsaicin treatment), or formalin (single i.g. treatment). Capsaicin-evoked sensitivity of the eye corneal surface was examined 3-4 days after ganglion treatment.

Eye-wipe response (number of eye wipes) was measured after ipsilateral capsaicin application to corneal surface (0,01%, 10 µl).

N(animals per group)=5-6. Results are represented as mean ±SEM. *** - $p<0.001$ in comparison to vehicle i.g.; (one way ANOVA followed by Newman-Keuls post hoc, $p<0.05$).

Fig. 3. Antinociceptive activity of BoNT/A in orofacial formalin-induced pain is mediated by capsaicin-sensitive sensory neurons.

Chemical denervation with 2% i.g. capsaicin prevents BoNT/A's antinociceptive activity in phase II of orofacial formalin-induced pain. Capsaicin/vehicle pretreatment was completed 4 days prior to peripheral saline or BoNT/A (5 U/kg) injection, and formalin test was performed 5-6 days after saline/BoNT/A injection. Number of animals per group = 4-6. Results are represented as mean ±SEM. ** - $p<0.01$ in comparison to vehicle control; + - $p<0.05$ in comparison to capsaicin i.g. + BoNT/A; # - $p<0.05$ in comparison to capsaicin i.g.+ vehicle (one way ANOVA followed by Newman-Keuls post hoc, $p<0.05$).

Fig. 4 Proteolytic activity of BoNT/A in TNC is located in central afferent terminals of primary sensory neurons.

A.) Immunoreactivity for CGRP (green), marker of peptidergic primary afferents, is almost completely eliminated from TNC ipsilaterally to formalin intraganglionic (i.g.) treatment, in comparison to i.g. saline treatment (right sides of coronal sections). Scale bar=200 μm

B.) Formalin i.g. abolishes cleaved SNAP-25 in medullary dorsal horn (red immunofluorescent staining, arrows). Saline or formalin (10 μl) was administered into the trigeminal ganglion 5 d following peripheral BoNT/A injection into the whisker pad (15 U/kg). N(animals per group)=4 (15-25 sections were examined per each animal). Scale bar=50 μm

Fig. 5 BoNT/A's proteolytic activity in TNC is associated with TRPV1-expressing (capsaicin-sensitive) primary afferents.

A.) Fluorescent images of cleaved SNAP-25 (SNAP-25(c)) and TRPV1-double labeling in ipsilateral TNC 5 days after peripheral injection of BoNT/A (15 U/kg). Cleaved SNAP-25 immunoreactivity (green) in the dorsal horn is localized within TRPV1-expressing neurons (red). Scale bar= 20 μm.

B.) Capsaicin 2% i.g. treatments performed 5 and 7 days after administration of peripheral BoNT/A (15 U/kg) eliminates cleaved SNAP-25 (red immunostaining, arrows) in the TNC and reduces CGRP immunostaining (green). Animals were sacrificed 10 d post BoNT/A. Scale bar= 50 μm

C.) Chemical denervation with 2% i.g. capsaicin prior to BoNT/A treatment prevents the occurrence of cleaved SNAP-25 in the TNC. Capsaicin 2%/vehicle double pretreatment was completed 3 days before BoNT/A injection (15 U/kg) into the whisker pad, and animals were sacrificed 5 days post peripheral BoNT/A. Red immunostaining represents cleaved SNAP-25 (arrows). CGRP staining (green) was lower in capsaicin i. g. pretreated animals, in comparison to vehicle control. N(animals per group)=3-4 (15-25 sections were examined per each animal). Scale bar= 50 μm

Fig. 6 Cleaved SNAP-25 localization in relation to presynaptic terminals, dendrites and astrocytes.

Confocal images of ipsilateral TNC 5 days after BoNT/A (15 U/kg) injection into the rat whisker pad. Cleaved SNAP-25 (SNAP-25(c)- red immunofluorescence) partially colocalizes with synaptophysin (arrows), a presynaptic marker (A.). Cleaved SNAP-25 did not colocalize with MAP-2, marker of dendrites (B.), and GFAP, marker of astrocytes (C.). Images are representative of confocal microphotographs obtained from 4 animals. Scale bars=20 μm.

Fig. 7 BoNT/A reduces pain-evoked neural activity in trigeminal nucleus caudalis and locus coeruleus, but not in thalamus. Fluorescent images of orofacial formalin-induced neural activity (assessed with c-Fos expression (green)) in A.) ipsilateral trigeminal nucleus caudalis; B) ipsilateral locus coeruleus and C.) paraventricular thalamic nucleus. 5 U/kg BoNT/A or saline was applied into the whisker pad 5-6 days prior to formalin injection into the whisker pad. N(animals per group)=3-4. Scale bar = 200 μm.

Fig. S1 Quantification of reduction of CGRP immunoreactivity after unilateral trigeminal ganglion ablation with formalin (A.) or desensitization with capsaicin B.). Surface areas of ipsilateral and contralateral trigeminal nucleus caudalis covered by CGRP were calculated using pixel thresholding. Surface area of ipsilateral CGRP immunoreactivity was divided by the surface area of CGRP immunoreactivity from contralateral side of the same coronal section. CGRP immunoreactivity was almost completely eliminated by formalin .g. treatment, and largely reduced by i.g. capsaicin.

N(animals per group)=3-4, 4-6 coronal sections per animal were analyzed.

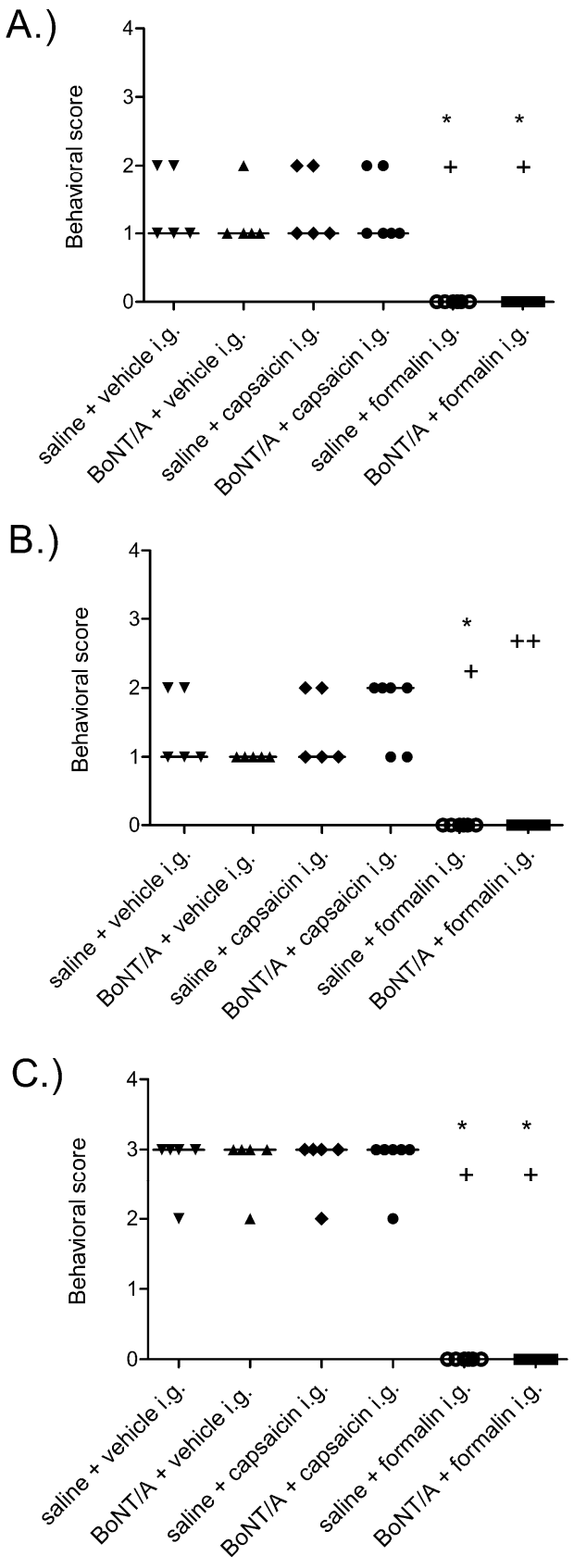
Data are represented as mean ± SEM; ***- $p<0.001$ in comparison to saline or vehicle i.g. treatment (A. t-test or B. one-way ANOVA followed by Newman-Keuls post hoc, $p<0.05$).

Fig. S2 Ablation of primary afferents does not alter secondary brainstem neurons.

A.) and B.) Formalin i.g. does not alter the immunoreactivities of dendrites (MAP-2) or neuronal bodies (NeuN) of secondary neurons in the TNC (green). C. Immunoreactivity for CGRP (red) is almost completely eliminated from TNC ipsilaterally to formalin i.g. treatment (right). N(animals)=3, 10-15 coronal sections per animal were examined. Scale bar=200 µm.

Fig. S3 SNAP-25 cleavage occurs outside of CGRP-expressing peptidergic terminals after BoNT/A injection into the whisker pad. Fluorescent microphotographs of ipsilateral TNC 5 days after BoNT/A (15 U/kg) injection into the rat whisker pad. Cleaved SNAP-25 localization (red) was studied in relation to CGRP (green), marker of peptidergic primary afferents. Although the majority of BoNT/A-cleaved SNAP-25 did not colocalize with CGRP (upper panel), occasionally, cleaved SNAP-25 profiles appeared to colocalize with bright fluorescent CGRP fibers (lower panel, arrow). Images are representative of microphotographs obtained from 4 animals (10-15 sections per animals were examined). Scale bar (upper panel = 50 μm, lower panel =25 μm

Fig. 1


A.)
Behavioral score
0
1
2
3
4
*
+
*
+
saline + vehicle i.g.
BoNT/A + vehicle i.g.
saline + capsaicin i.g.
BoNT/A + capsaicin i.g.
saline + formalin i.g.
BoNT/A + formalin i.g.
B.)
Behavioral score
0
1
2
3
4
*
+
++
saline + vehicle i.g.
BoNT/A + vehicle i.g.
saline + capsaicin i.g.
BoNT/A + capsaicin i.g.
saline + formalin i.g.
BoNT/A + formalin i.g.
C.)
Behavioral score
0
1
2
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+
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+
saline + vehicle i.g.
BoNT/A + vehicle i.g.
saline + capsaicin i.g.
BoNT/A + capsaicin i.g.
saline + formalin i.g.
BoNT/A + formalin i.g.

Fig. 2

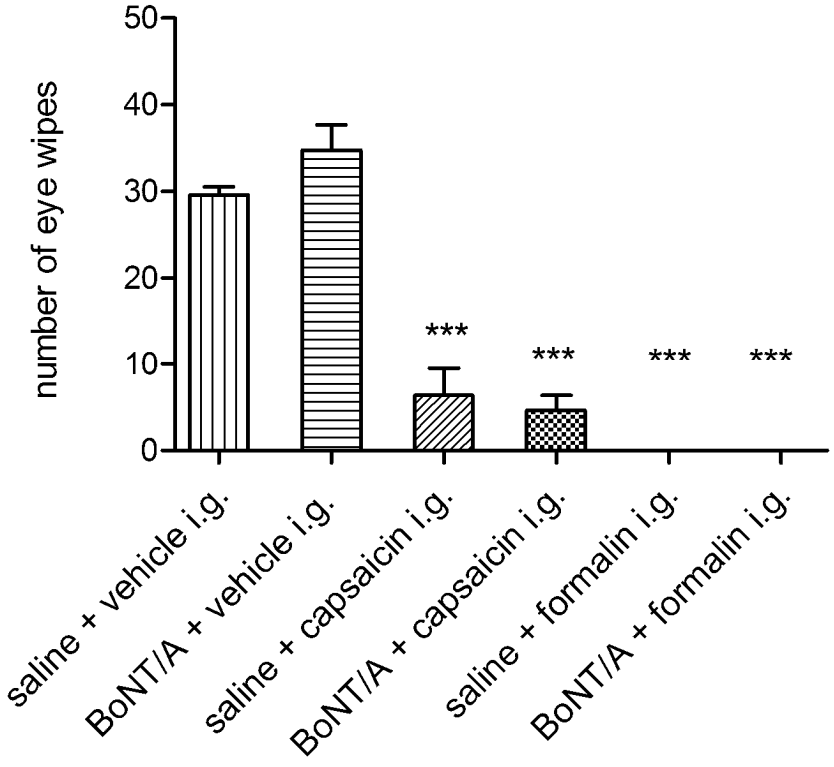

number of eye wipes
50
40
30
20
10
0
***
***
***
***
saline + vehicle i.g.
BoNT/A + vehicle i.g.
saline + capsaicin i.g.
BoNT/A + capsaicin i.g.
saline + formalin i.g.
BoNT/A + formalin i.g.

Fig. 3

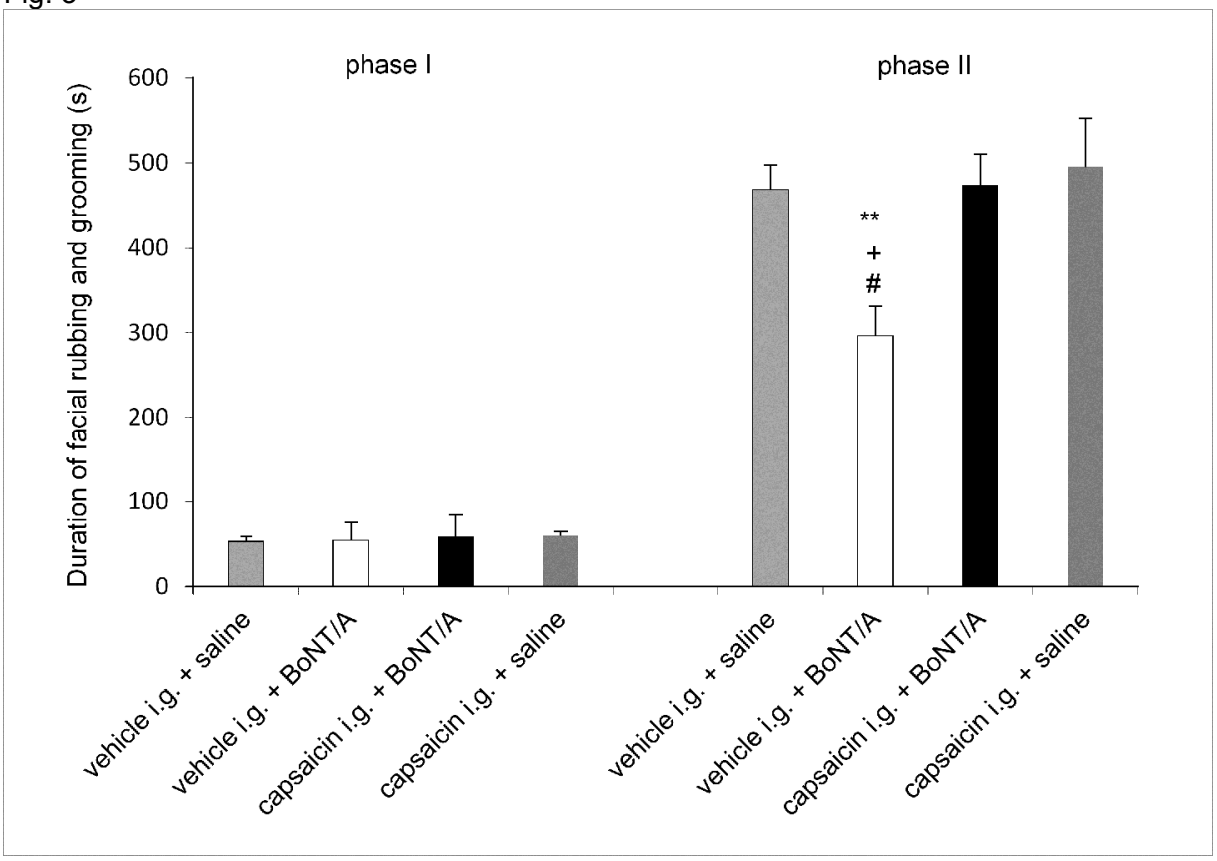

phase I
phase II
Duration of facial rubbing and grooming (s)
600
500
400
300
200
100
0
**
+
#
vehicle i.g. + saline
vehicle i.g. + BoNT/A
capsaicin i.g. + BoNT/A
capsaicin i.g. + saline
vehicle i.g. + saline
vehicle i.g. + BoNT/A
capsaicin i.g. + BoNT/A
capsaicin i.g. + saline

Fig. 4

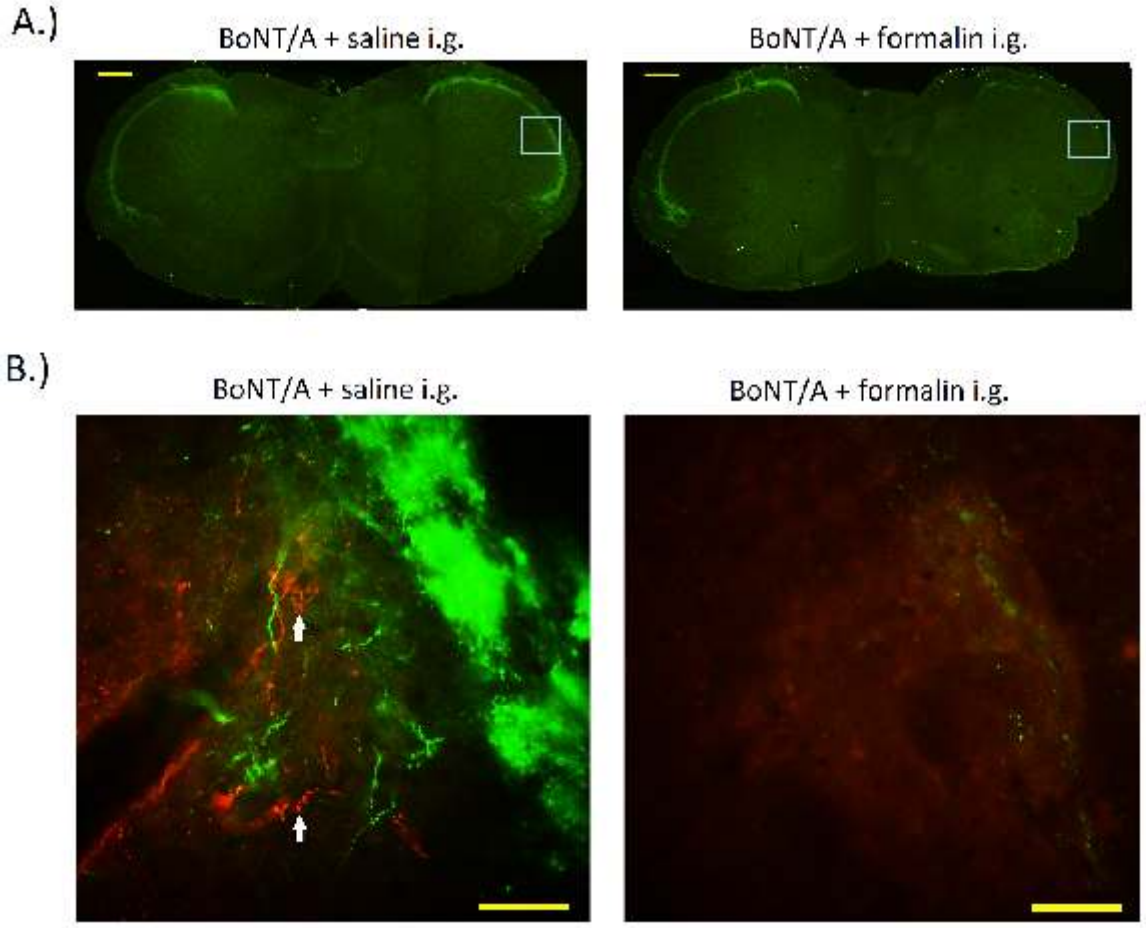

A.)
BoNT/A + saline i.g.
BoNT/A + formalin i.g.
B.)
BoNT/A + saline i.g.
BoNT/A + formalin i.g.

Fig. 5

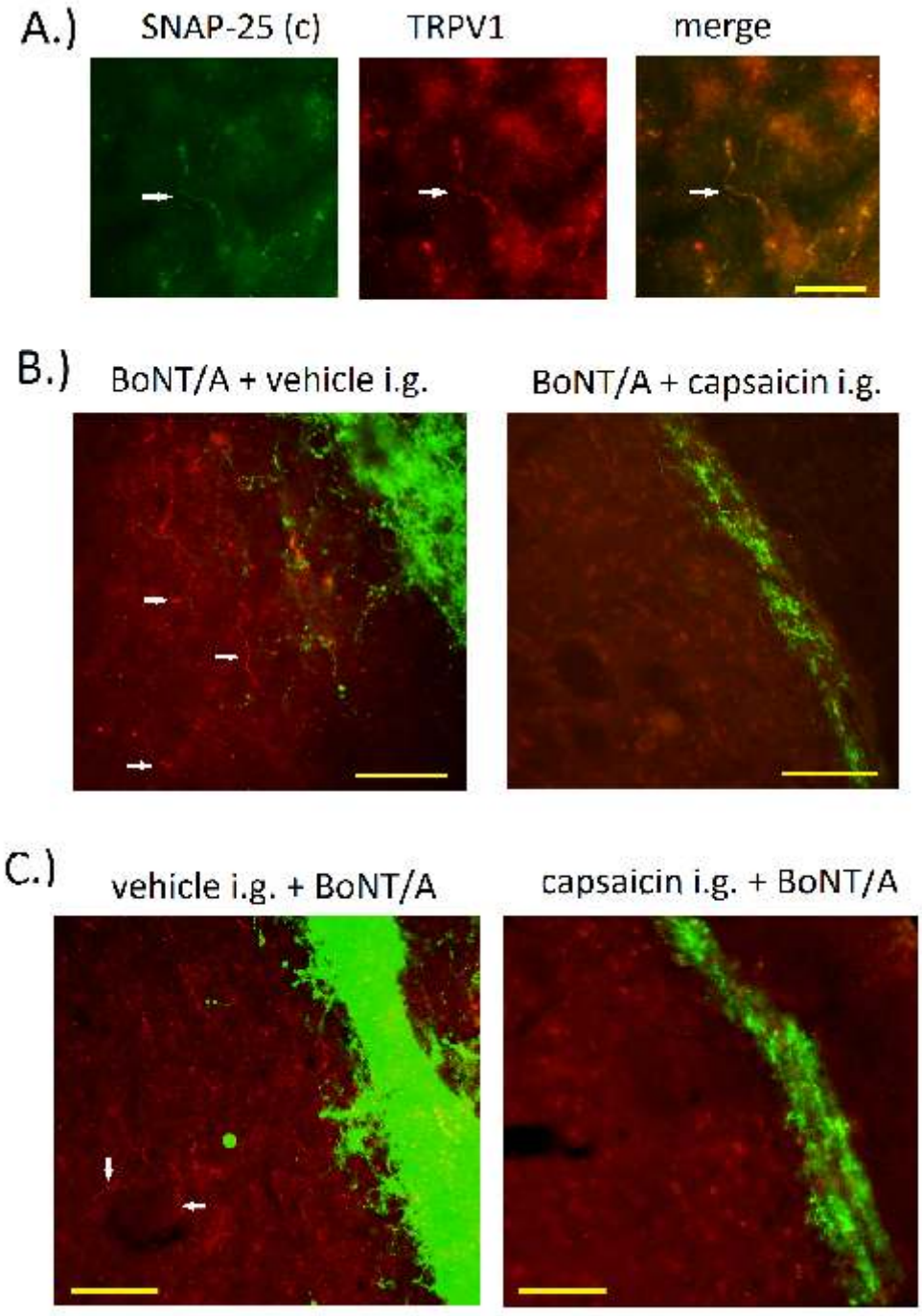

A.)
SNAP-25 (c)
TRPV1
merge
B.)
BoNT/A + vehicle i.g.
BoNT/A + capsaicin i.g.
C.)
vehicle i.g. + BoNT/A
capsaicin i.g. + BoNT/A

Fig. 6

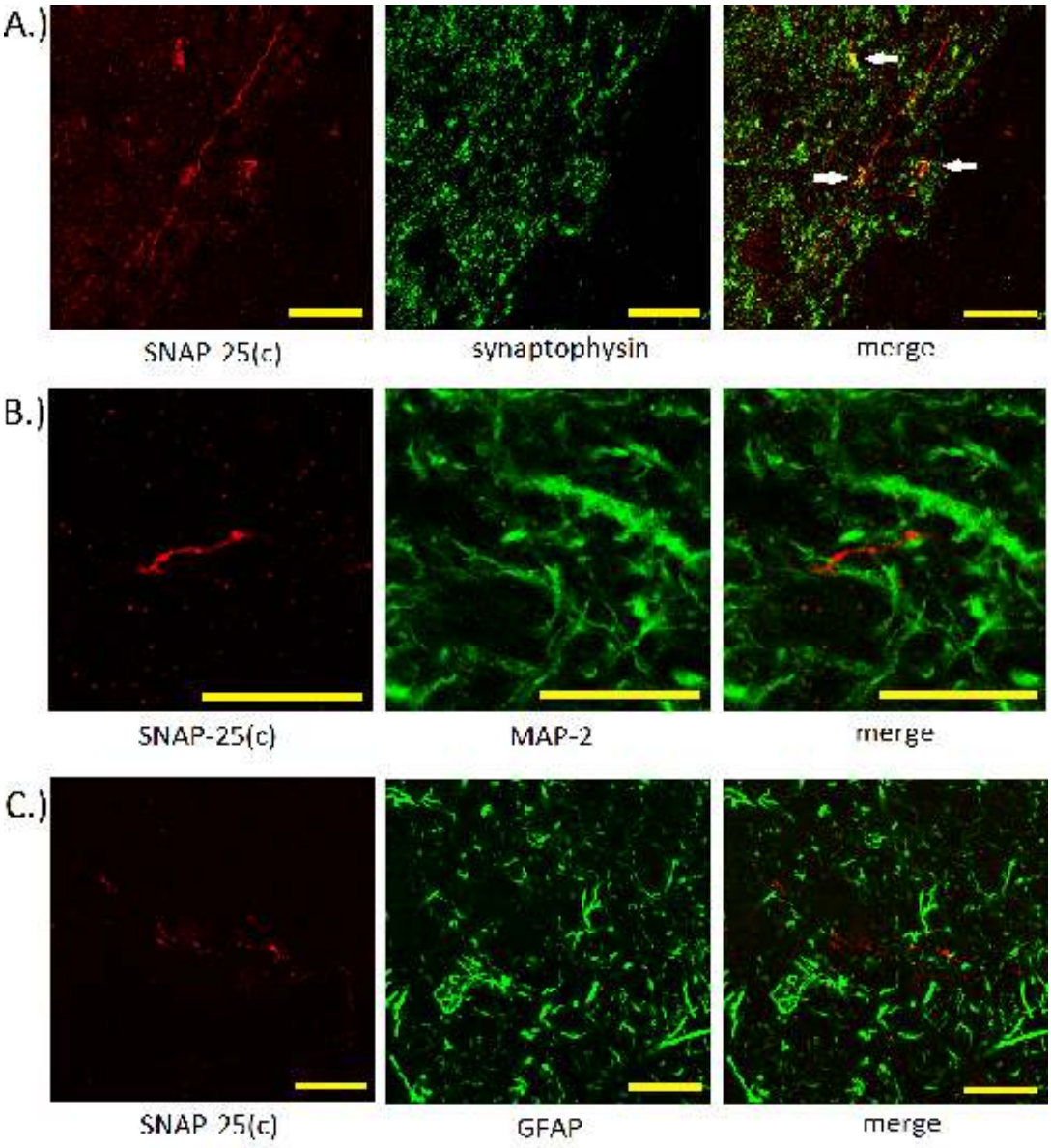
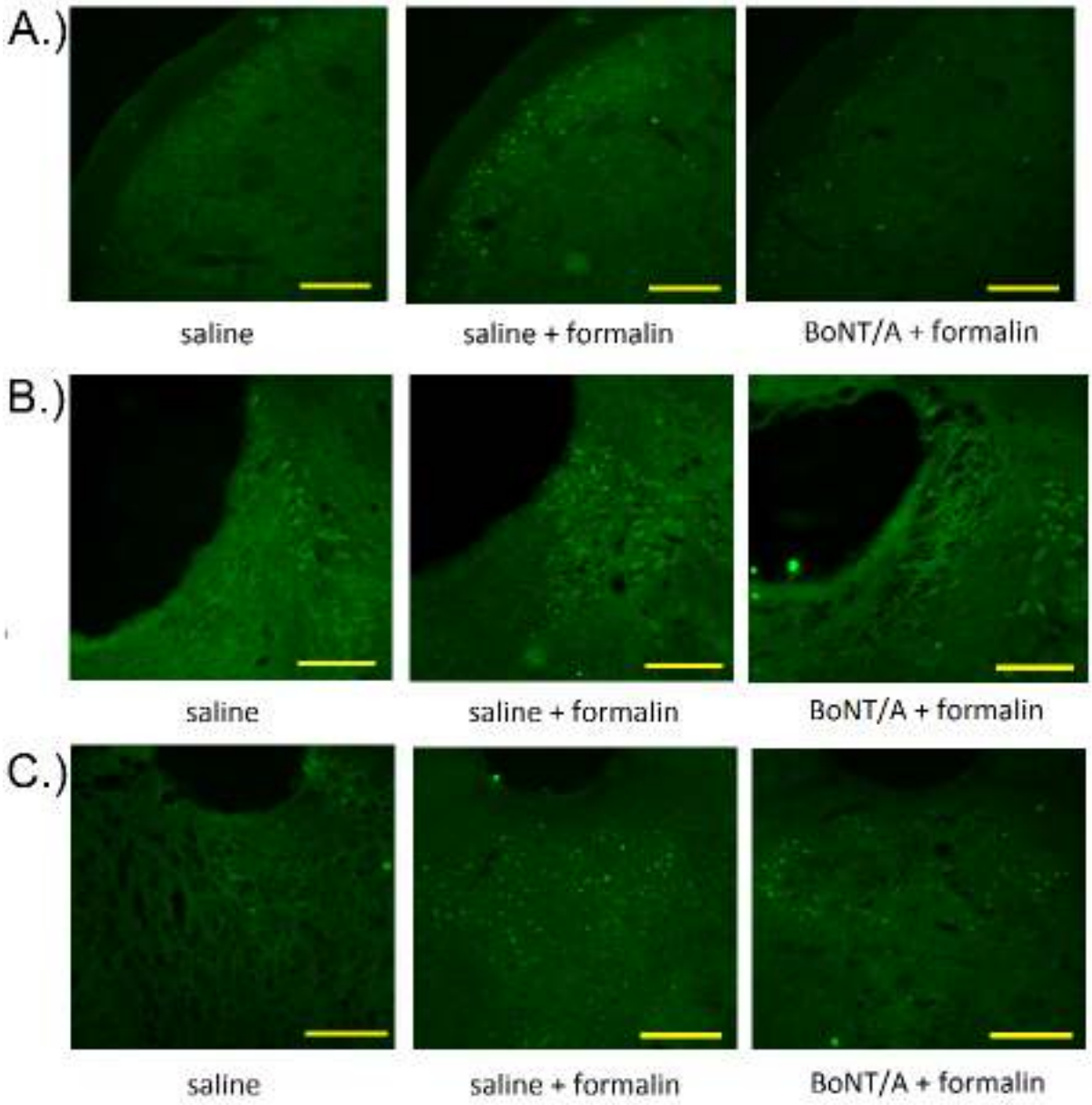
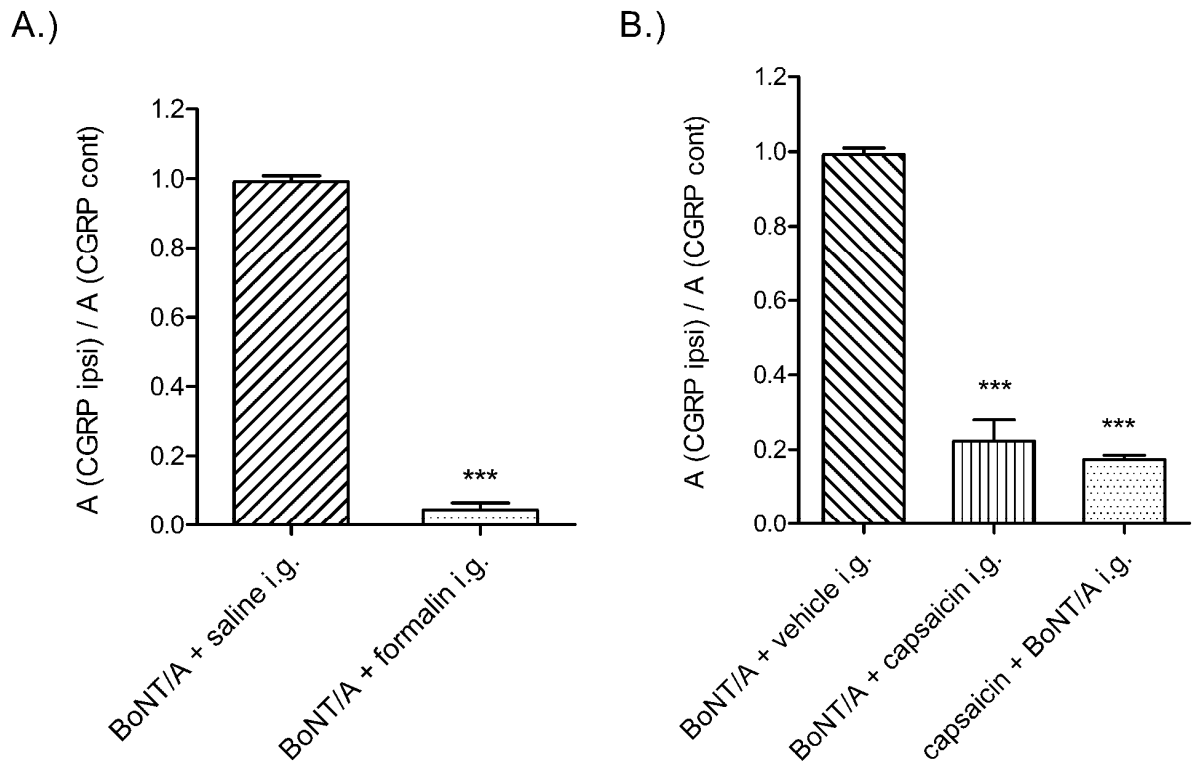

A.)
SNAP 25(c)
synaptophysin
merge
B.)
SNAP-25(c)
MAP-2
merge
C.)
SNAP 25(c)
GFAP
merge

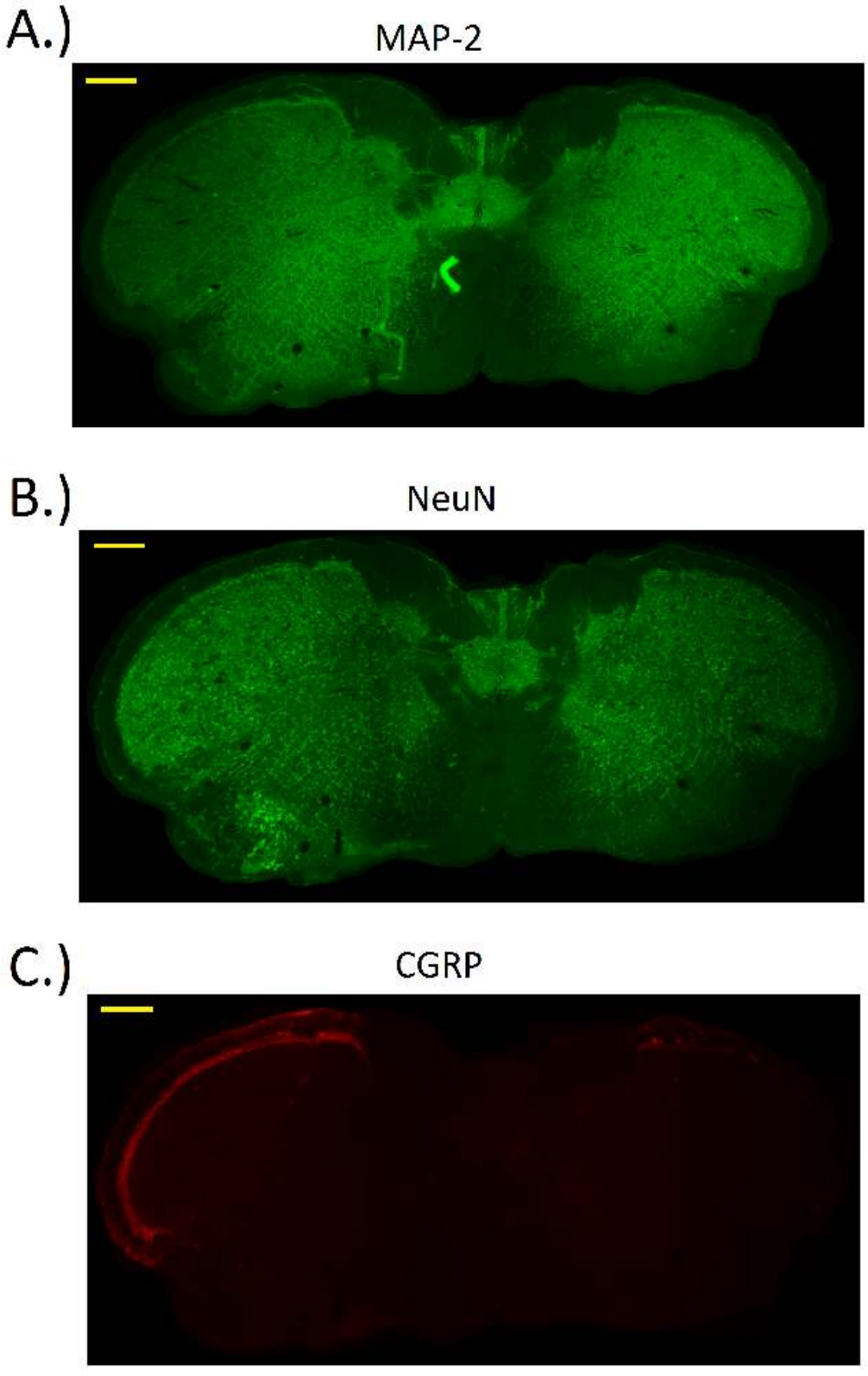
Fig. 7


A.)
saline
saline + formalin
BoNT/A + formalin
B.)
saline
saline + formalin
BoNT/A + formalin
C.)
saline
saline + formalin
BoNT/A + formalin

Supplement. Fig.S1



Supplement. Fig. S2



Supplement. Fig. S3

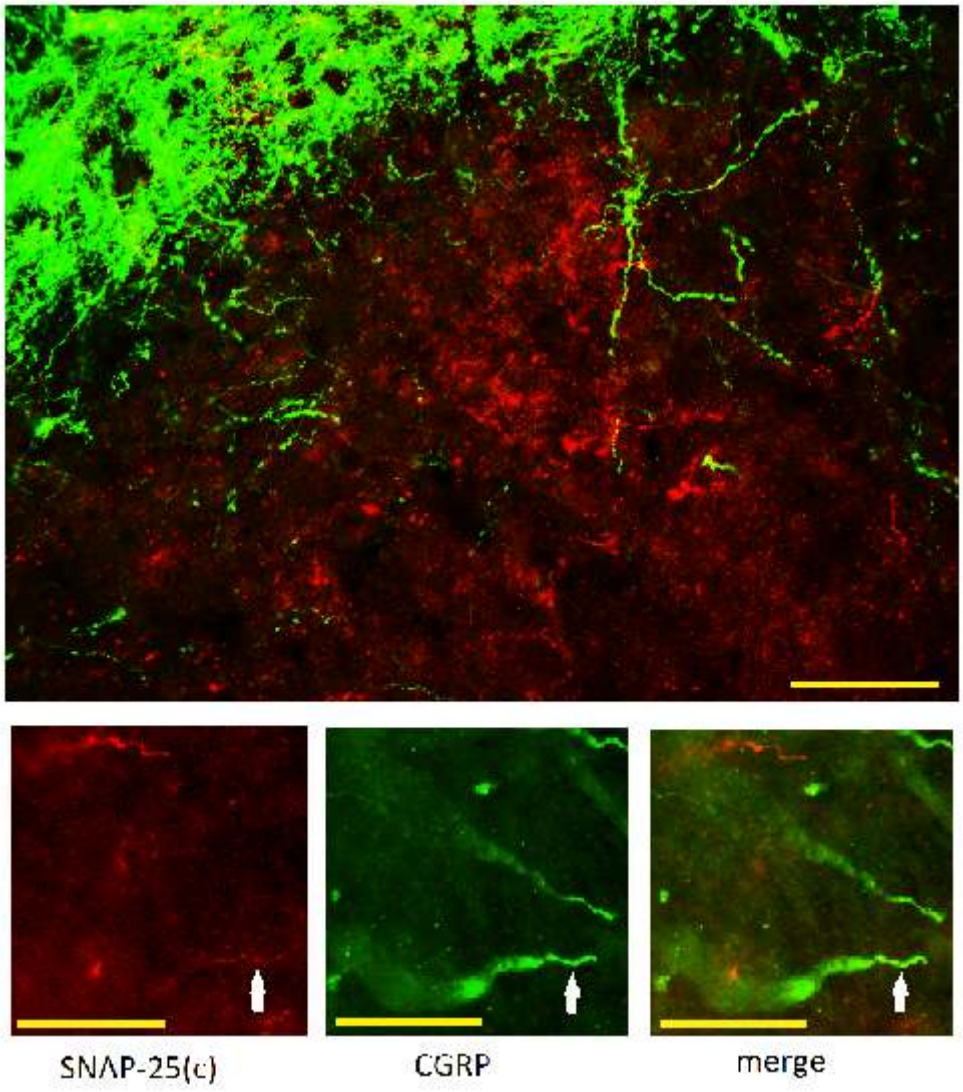


Table 1. BoNT/A differentially alters regional c-Fos activation in orofacial formalin test. Orofacial formalin test was performed 5 days following the saline or 5 U/kg BoNT/A injection into the whisker pad, and animals were perfused 2 h after formalin injection. Number of immunofluorescently stained c-Fos-positive neuronal profiles in examined regions was automatically quantified in 4 randomly selected sections per animal.

	saline (N=3)	saline + formalin (N=4)	BoNT/A + formalin (N=4)
trigeminal nucleus caudalis (ipsilateral)	14.7±0.7	138.5±14.0	75.7±9.3 (p=0.003)
locus coeruleus (ipsilateral)	4.7±2.8	21.2 ±2.4	13.7±1.7 (p=0.045)
locus coeruleus (contralateral)	3.0±1.5	24.6±3.3	15.3±1.5 (p=0.023)
periaqueductal gray	90.7±26.4	290.9±20.4	149.7±8.9 (p=0.001)
hypothalamus (ipsilateral)	40.7±5.4	342±15.6	338.2±24.3 (n.s.)
hypothalamus (contralateral)	44.7 ±16.1	341.8±27.3	294.9 ±20.7 (n.s.)
paraventricular thalamic nucleus	19.2±2.5	132.5±17.7	110.1±11.8 (n.s.)
central amygdaloid nucleus (contralateral)	7.4±2.3	36.0±6.3	45.9±3.9 (n.s.)

Data are represented as mean ± SEM. N=number of animals per group. For BoNT/A + formalin group p values are shown in comparison to saline + formalin group (one-way

ANOVA followed by Newman-Keuls post-hoc, $p<0.05$ was considered significant); n.s.= non significant.