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INVOLVEMENT OF SUBSTANCE P IN THE ANTINOCICEPTIVE EFFECT OF BOTULINUM TOXIN TYPE A: EVIDENCE FROM KNOCKOUT MICE

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Abstract—The antinociceptive action of botulinum toxin type A (BoNT/A) has been demonstrated in behavioral animal studies and clinical settings. It was shown that this effect is associated with toxin activity in CNS, however, the mechanism is not fully understood. Substance P (SP) is one of the dominant neurotransmitters in primary afferent neurons transmitting pain and itch. Thus, here we examined association of SP-mediated transmission and BoNT/A antinociceptive action by employing gene knockouts. Antinociceptive activity of intraplantarly (i.pl.) injected BoNT/A was examined in mice lacking the gene encoding for SP/neurokinin A (*tac1*^{-/-}) or SP-preferred receptor neurokinin 1 (*tac1r*^{-/-}), compared to control C57Bl/6 J wild type animals. BoNT/A action was assessed in inflammatory pain induced by formalin and CFA, and neuropathic pain induced by partial sciatic nerve ligation. BoNT/A activity in CNS was examined by c-Fos and BoNT/A-cleaved SNAP-25 immunohistochemistry. In wild type mice, acute (formalin-evoked) and chronic pain (neuropathic and inflammatory) was reduced by peripherally injected BoNT/A. In *tac1*^{-/-} and *tac1r*^{-/-} knockout mice, BoNT/A exerted no analgesic effect. In control animals BoNT/A reduced the formalin-evoked c-Fos expression in lumbar dorsal horn, while in knockout mice the c-Fos expression was not reduced. After peripheral toxin injection, cleaved SNAP-25 occurred in lumbar dorsal horn in all animal genotypes. BoNT/A antinociceptive activity is absent in animals lacking the SP and neurokinin 1 receptor encoding genes, in spite of presence of toxin's enzymatic activity in central sensory regions. Thus, we conclude that the integrity of SP-ergic system is necessary for the antinociceptive activity of BoNT/A. © 2017 The Authors. Published by Elsevier Ltd on behalf of IBRO. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

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Keywords: botulinum toxin type A; antinociceptive action; substance P; neurokinin 1 receptor; synaptosomal-associated protein 25.

INTRODUCTION

Botulinum neurotoxin type A (BoNT/A), derived from *Clostridium botulinum*, selectively enters neurons and inhibits synaptic neurotransmitter exocytosis by proteolytic cleavage of synaptosomal-associated protein 25 (SNAP-25) (Schiavo et al., 1993). Local paralysis with low peripheral BoNT/A doses is the basis for treatment of neuromuscular and autonomic disorders like spasticity, dystonia, axillary hyperhidrosis, neurogenic bladder, etc. (Jabbari, 2016). In addition, BoNT/A has been used in different chronic pain conditions: diabetic neuropathy, trigeminal neuralgia, arthritis, low back pain, and chronic migraine (review by Matak and Lacković, 2014). It was suggested that BoNT/A reduces pain by inhibiting the release of pro-nociceptive neurotransmitters like glutamate, calcitonin gene-related peptide, and substance P (SP), from primary sensory neurons (Göbel et al., 2001; Freund and Schwartz, 2003). Initial animal data led to hypothesis that BoNT/A exerts its antinociceptive activity by preventing the sensory transmitter release in periphery (Aoki, 2005). However, more recent data demonstrated the necessity of axonal transport for BoNT/A antinociceptive action (Bach-Rojecky and Lacković, 2009). Evidence of toxin enzymatic activity in brainstem and spinal cord sensory regions suggest that BoNT/A inhibits central pain transmission (Matak et al., 2011).

SP is an 11-aminoacid neuropeptide present in peripheral and central terminals of non-myelinated primary sensory neurons. SP is encoded by tachykinin 1 (*tac1*) gene, which also encodes neurokinin A (NKA) by alternate splicing (Steinhoff et al., 2014). Actions of SP are mainly mediated by neurokinin 1 receptor (NK1R), encoded by tachykinin1 receptor (*tac1r*) gene. NK1R is a G protein-coupled receptor which mediates stimulatory effects of SP in epithelial and mast cells in peripheral tissue, as well as neurons and glia in CNS (Pintér et al., 2014; Todd et al., 2002). In the brain, SP has been involved in stress, anxiety, depression, emesis etc. In the periphery, SP-ergic system is implicated in autonomic functions such as cardiovascular responses, intestinal motility, bladder functions, inflammation and transmission of pain and itch (Mistrova et al., 2016). SP activates mast cell degranulation and increases permeability of blood

vessel epithelium leading to neurogenic inflammation in the peripheral tissue. In the spinal cord, SP is involved in transmission of hyperalgesia and allodynia via NK1R located at second order-sensory neurons (Pintér et al., 2014; Todd et al., 2002).

BoNT/A may prevent the release of SP both in vitro and in vivo (Welch et al., 2000; Ishikawa et al., 2000; Lucioni et al., 2008; Carmichael et al., 2010; Filipović et al., 2012). It prevents neurogenic plasma protein extravasation in the rat hind-limb skin evoked by sciatic nerve stimulation (Carmichael et al., 2010), and dural plasma protein extravasation evoked by different types of trigeminal pain (Filipović et al., 2012; Lacković et al., 2016). It was suggested that peripherally injected botulinum toxin serotype B (BoNT/B) prevents central SP release (Marino et al., 2014; Ramachandran et al., 2015). Although BoNT/A may prevent the SP transmission, the causal role of this effect in the mechanism of its antinociceptive action has not been assessed so far. Thus, in the present study we examined the effect of BoNT/A on acute and chronic experimental pain in *tac1* and *tac1r* homozygous mouse knockouts lacking SP and NK1R.

EXPERIMENTAL PROCEDURES

Animals & ethics statement

Experiments were performed on adult male mice lacking the SP and NKA (*tac1*^{-/-}), and the tachykinin receptor NK1R (*tac1r*^{-/-}) backcrossed for 8–10 generations to the C57BL/6 J line. The *tac1*^{-/-} and *tacr1*^{-/-} mice were generated as previously described (De Felipe et al., 2000; Laird et al., 2000). Original breeding pairs of *tac*^{-/-} and *tacr1*^{-/-} were provided by Prof. John P. Quinn (University of Liverpool, UK). C57Bl/6J mice were used as wild type controls and the original breeding pairs were purchased from Innovo Ltd. (Hungary). The animal weights ranged from 25 to 28 g. The mice were bred at the Department of Pharmacology and Pharmacotherapy (University of Pécs, Hungary), provided with unlimited access to standard mouse chow and drinking water, and maintained under a 12-h light–dark cycle at 24–25 °C room temperature. Animal procedures were performed according to European Communities Council Directive (86/609/EEC) and recommendations of International Association for the Study of Pain (Zimmerman, 1983), and approved by the Ethics Committee on Animal Research of University of Pécs. All efforts were made to reduce the number of animals used.

BoNT/A treatment

BoNT/A (Botox®, Allergan Inc, Irvine CA, USA) was injected intraplantarly (i.pl.) into right hind paw pad of conscious, restrained animals (20 µl of 0.9% saline-diluted, using a 30-gauge needle). 1 unit (1 U) of BoNT/A preparation contains 48 pg of purified botulinum neurotoxin type A complex. The applied doses (0.2 and 0.4 U) were equivalent to previously employed rat doses (7 and 15 U/kg) which did not induce any measurable paralytic effects (Cui et al., 2004; Bach-Rojecky and

Lacković, 2005). BoNT/A injected i.pl. did not impair the hind limb toe spreading reflex. Normal 0/5 digit abduction score upon tail suspension (Brown et al., 2013) was observed in all animals.

Neuropathic hyperalgesia model

Traumatic mononeuropathy was induced by partial sciatic nerve ligation model introduced by Seltzer et al. (1990). Animals were deeply anesthetized by i.p. ketamine-xylazine (100 mg/kg ketamine and 5 mg/kg xylazine). The right sciatic nerve was exposed at the mid-thigh level. Under magnifying binoculars, approximately 1/3 to 1/2 of the nerve diameter was tightly ligated with 9–0 non-absorbable atraumatic suture. Seven days following the nerve injury, BoNT/A (0.2 U) or saline was injected i.pl. into the operated leg. Mechanical pain threshold was behaviorally assessed by dynamic plantar esthesiometry (Ugo Basile, Varese, Italy), as previously described (Borbély et al., 2013; Botz et al., 2013). In brief, mice were allowed to accommodate in individual testing cubes with wire mesh floor for 10 min. Thin metal probe with gradually increasing pressure (0–10 g) was applied to the plantar surface of mouse hind-paw, until a lifting response was elicited. The mechanonociceptive threshold was noted as the pressure value in grams at which the animal withdrew its paw. The cut-off value was set to 10 g. Hyperalgesia was determined as compared to the pre-treatment self-control values. The contralateral, non-operated side served as a control value at different time points (7, 10 and 14 days after nerve injury).

Adjuvant-induced chronic inflammatory hyperalgesia model

Three days after BoNT/A (0.2U) administration, 20 µl of complete Freund's adjuvant (CFA, killed mycobacteria suspended in paraffin oil, 1 mg/ml; Sigma, St. Louis, MO, USA) was injected i.pl. into the ipsilateral hind paw. Paw volume was measured by the displacement of liquid induced by the hind paw immersion into the chamber of plethysmometer (Ugo Basile, Varese, Italy). The mechanonociceptive threshold was measured by dynamic plantar esthesiometry. The mechanonociceptive threshold and paw volume parameters obtained on the contralateral, non-inflamed side served as control values at different time points (0, 1, 3 and 5 days after CFA treatment).

Formalin-evoked acute inflammatory nocifensive behaviors, neuronal c-Fos activation and cleaved SNAP-25 immunohistochemistry

In this experiment mice were treated with saline or 0.4 U BoNT/A. This dose was chosen based on previously employed BoNT/A dose (15 U/kg) which suppressed formalin-evoked wide dynamic range neuronal excitation and c-Fos expression in rats (Aoki, 2005). To determine the acute inflammatory nocifensive behavior, formalin test was used 7 days after the BoNT/A or saline treatment. Conscious, restrained mice were injected i.pl. with 20 µl of 0.9% saline-diluted 5% formalin (ipsilateral to BoNT/A

or saline) by 30-gauge needle. Nocifensive behavior was quantitatively evaluated by the duration of paw liftings and lickings in each 5-min examination period during 1 h (Bölcseki et al., 2005). The observation period was divided into two phases: phase I (0–15 min) and phase II (15–60 min). Phase I behavior represents a quick onset response evoked by direct chemical stimulation of nerve endings by formalin, while phase II represents delayed inflammatory hyperalgesic behavior associated with peripheral and central sensitization (Tjølsen et al., 1992). Two hours after formalin injections, the animals were deeply anesthetized with Euthasol® (Virbac AH, Fortworth, TX, USA) and perfused for immunohistochemistry with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde fixative. Following the perfusion, the lumbar spinal cord was excised and kept for 24 h in 15% sucrose + fixative, followed by 30% sucrose in PBS for 24 h. The samples were then removed and kept on -80 °C until further use.

The lumbar spinal cord L4 segment was cut with a cryostat to 35- μ m coronal slices, which were immunostained for c-Fos by immunohistochemistry, as previously described (Matak et al., 2014). In brief, free floating sections were washed with PBS and blocked with 10% normal goat serum for 1 h. Sections were then incubated with 1:500 rabbit anti-c-Fos primary antibody (sc-52, Santa Cruz, Dallas, TX, USA) overnight at room temperature. The following day, sections were washed and incubated with the secondary fluorescent antibody goat-anti rabbit Alexa Fluor 488 (Invitrogen, Carlsbad, CA, USA), transferred onto the glass slides and coverslipped with anti-fading agent. Tissue sections were then photographed with a fluorescent microscope equipped with a digital camera (Olympus, Tokyo, Japan). C-Fos-positive neurons in ipsilateral and contralateral dorsal horns were automatically counted in 5 randomly selected sections per single animal using cellSens Dimension software (Olympus, Tokyo, Japan), as previously described (Matak et al., 2014). Cleaved SNAP-25 immunohistochemistry was performed similarly as previously described in rats (Matak et al., 2014). In brief, free floating sections were washed with PBS and incubated 1 h in 10% normal donkey serum, followed by polyclonal rabbit antibody to

cleaved SNAP-25 (provided by Ornella Rossetto, University of Padua, Italy), 1:2000 dilution in 1% normal donkey serum overnight at room temperature. The next day the sections were washed and incubated in donkey anti rabbit Alexa Fluor 555. Neuronal counterstain was similarly performed with mouse anti-NeuN antibody (Millipore, Temecula, CA, USA, 1:500 dilution overnight at 4 °C) and donkey anti-mouse Alexa Fluor 488 secondary antibody.

Statistical analysis

The data are represented as mean \pm SEM and analyzed by one-way ANOVA or two-way ANOVA for repeated measurements followed by Bonferroni's post hoc test. $P < 0.05$ was considered significant.

RESULTS

Deletion of *tac1* and *tac1r* genes prevented the reduction of neuropathic mechanical hyperalgesia by BoNT/A

Seven days after sciatic nerve partial ligation injury, animals developed ipsilateral mechanical hyperalgesia, evident as approximately 35–40% reduction of the mechanonociceptive thresholds compared to the contralateral side ($p < 0.001$). Deletion of SP/NKA or the NK1R-encoding genes did not affect the development of neuropathic mechanical hyperalgesia, which was similar to WT mice. BoNT/A significantly reduced the hyperalgesia in wild type animals 7 days after its injection. In contrast, BoNT/A did not counteract the nerve ligation-evoked mechanical hyperalgesia in the gene-deleted groups at any investigated time-points (Fig. 1).

Deletion of *tac1* and *tac1r* genes prevented the reduction of CFA-induced inflammatory mechanical hyperalgesia by BoNT/A

In saline-treated animals, CFA-induced inflammation decreased the mechanonociceptive thresholds in all groups ($p < 0.001$ in comparison to contralateral non-inflamed paws) (Fig. 1). BoNT/A pretreatment 3 days

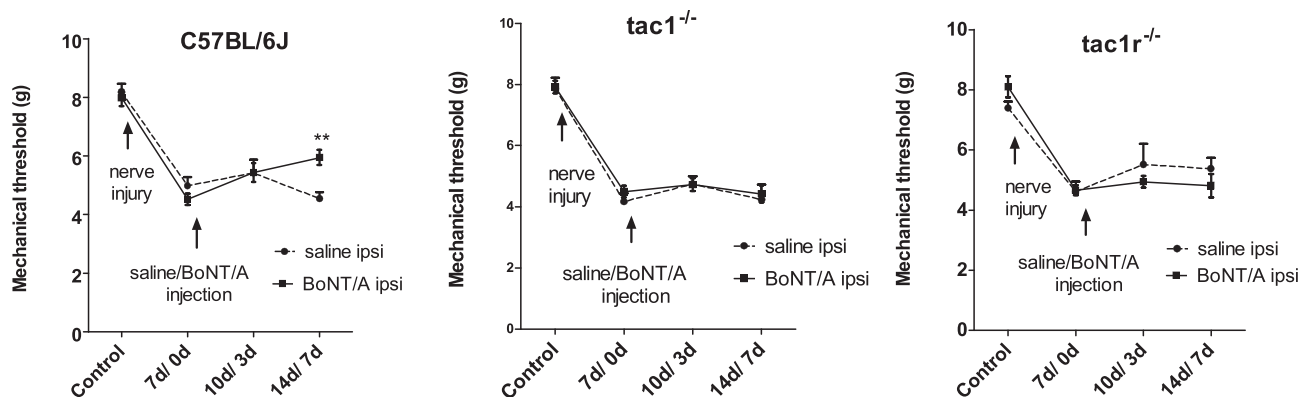


Fig. 1. Time course of development of ipsilateral mechanical hyperalgesia following nerve injury induced by partial sciatic nerve ligation 7 days prior to BoNT/A treatment in wild type (C57BL/6 J), *tac1*^{-/-}, and *tac1r*^{-/-} knockout animals (time points indicate days after nerve injury operation / days after BoNT/A; ** $p < 0.01$ in comparison to saline ipsi; n (mice/group) = 6–8; mean \pm SEM, two-way ANOVA for repeated measurements followed by Bonferroni's post hoc test).

prior to CFA significantly reduced the inflammatory mechanical hyperalgesia in wild type mice at days 1 and 3 post CFA ($p < 0.001$). In wild type animals, at day 6 the ipsilateral mechanical hyperalgesia started to diminish, and the statistical difference between saline and BoNT/A treatment was no longer observed. The gene deletion of *tac1* and *tac1r* did not alter the development of mechanosensitivity, however, it prevented the antinociceptive action of BoNT/A (Fig. 2).

Ipsilateral hind paw swelling resulting in an approximately 2-fold increase of the hind paw volume was observed 1, 3 and 6 days after CFA injection in all genotypes (Fig. 3). BoNT/A treatment slightly attenuated the development of edema formation in wild type and *tac1r*-deleted mice on day 1 post CFA, but the effect was not significant at later time points (Fig. 3).

Tac1 and tac1r gene deletion prevents the BoNT/A antinociceptive effect on formalin-evoked nocifensive behavior and neuronal activation

In wild type and knockout mice, formalin injection into the hind paw induced biphasic nocifensive behavior and

increased number of c-Fos-labeled neurons in the ipsilateral spinal dorsal horn. BoNT/A treatment significantly reduced the phase II nocifensive behavior and spinal c-Fos activation related to acute neurogenic inflammatory mechanisms only in wild type animals (Figs. 4–6). In *tac1* and *tac1r* knockouts, BoNT/A pretreatment affected neither the phase II nocifensive behavior, nor the activation of c-Fos (Figs. 5 and 6). Cleaved SNAP-25-immunoreactive fibers were observed in ipsilateral lumbar spinal cord dorsal horn in all animal genotypes (Fig. 7), as well as in spinal cord ventral horn (not shown).

DISCUSSION

In the present study we examined the influence of SP and NK1R knockouts on the antinociceptive activity of BoNT/A. We found that the deletion of genes encoding SP/NKA and NK1R in mice prevented the BoNT/A antinociceptive activity in acute and chronic inflammatory pain, as well as neuropathic pain (Fig. 1, Fig. 2, Fig. 4). These observations suggest the

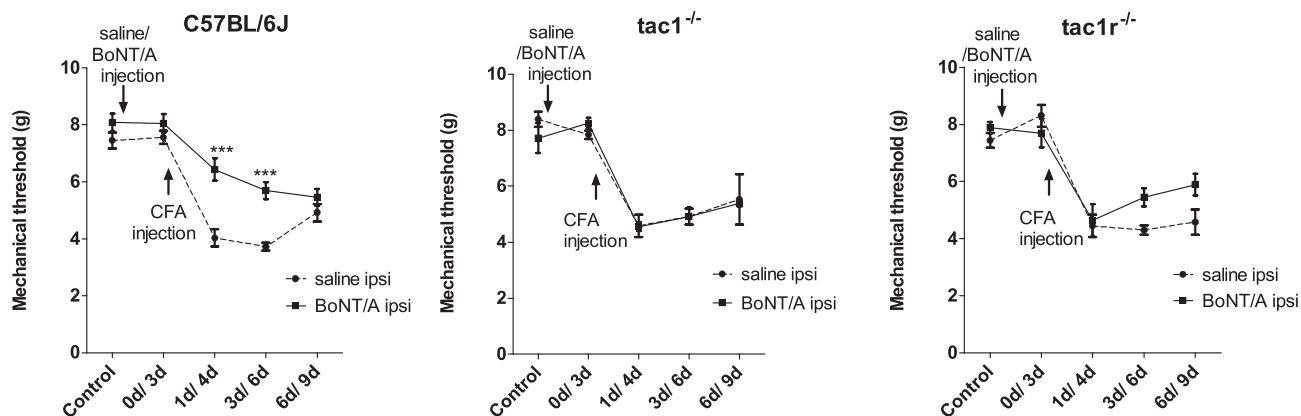


Fig. 2. Time course of development of ipsilateral mechanical hyperalgesia following complete Freund's adjuvant (CFA) subcutaneous injection into the hind paw at day 3 post BoNT/A pretreatment in wild type (C57BL/6 J), *tac1*^{-/-}, and *tac1r*^{-/-} mice (time points indicate days after CFA / days after BoNT/A; *** $p < 0.001$ in comparison to saline ipsi; n (mice/group) = 5–8, mean \pm SEM, two-way ANOVA for repeated measurements followed by Bonferroni's post hoc test).

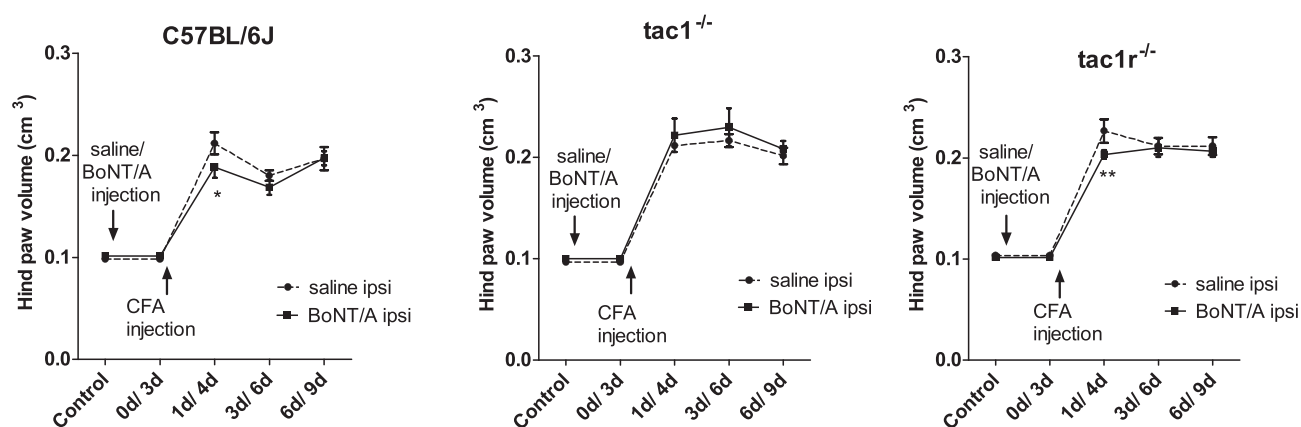


Fig. 3. Time course of development of ipsilateral hindpaw edema following complete Freund's adjuvant (CFA) subcutaneous injection into the hind paw at day 3 post BoNT/A pretreatment in wild type (C57BL/6 J), *tac1*^{-/-}, and *tac1r*^{-/-} mice (time points indicate days after CFA/days after BoNT/A; * $p < 0.05$ in comparison to saline ipsi; ** $p < 0.01$ in comparison to saline ipsi; n (mice/group) = 5–8, mean \pm SEM, two-way ANOVA for repeated measurements followed by Bonferroni's post hoc test).

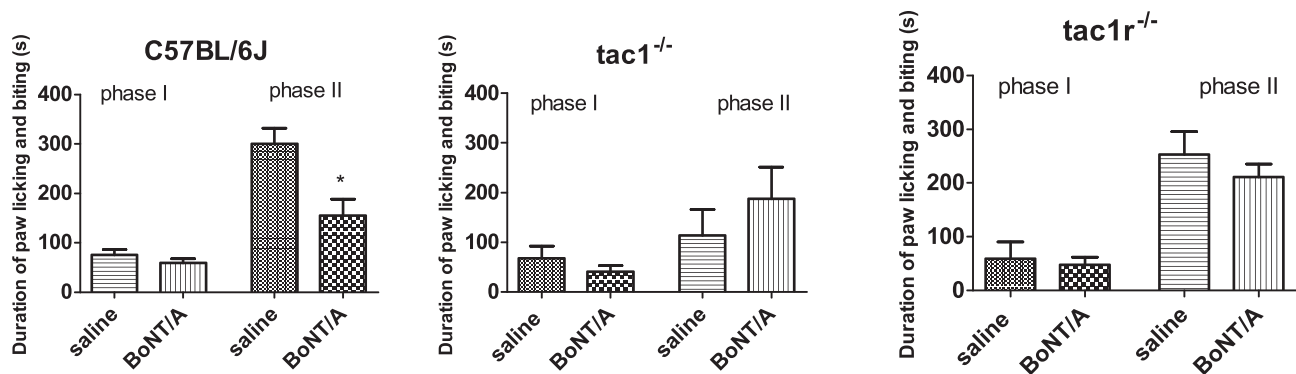


Fig. 4. Lack of the effect of BoNT/A on 5% formalin-evoked nociceptive behavior in $tac1^{-/-}$ and $tac1r^{-/-}$ mice. Phase I behavior represents the total duration of nociceptive behavior during first 15 min following formalin injection, while phase II represents the duration of nociceptive behavior from 15 to 60 min following formalin challenge. (mean \pm SEM. ** $p < 0.01$ in comparison to saline treatment; n (mice/group) = 4–5; mean \pm SEM, one-way ANOVA followed by Bonferroni's post hoc test).

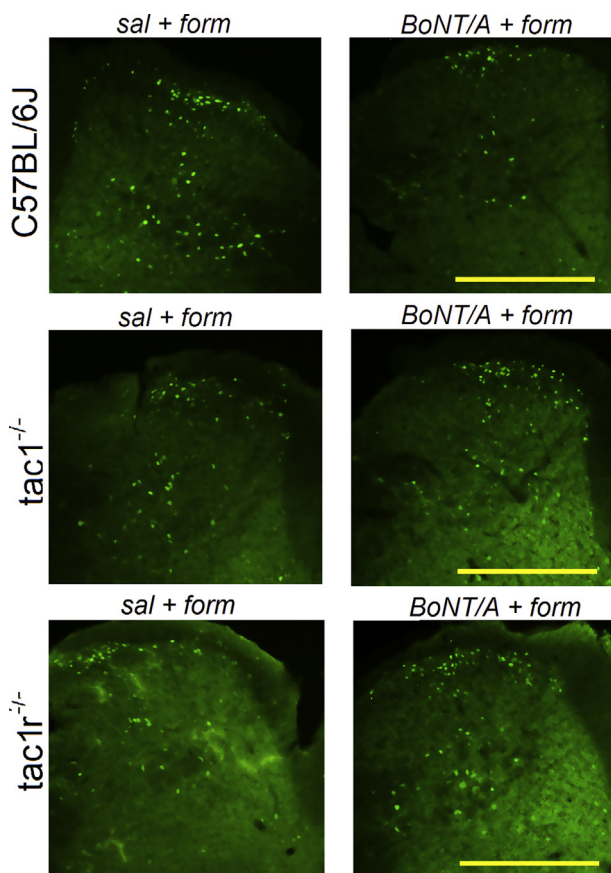


Fig. 5. Lack of effect of BoNT/A on c-Fos expression in $tac1^{-/-}$ and $tac1r^{-/-}$ mice. Neuronal activation was evoked by unilateral 5% formalin injection into the hind paw, and assessed by c-Fos immunohistochemistry. The image is representative of 4–5 animals per group. Green punctate immunofluorescent signal represents c-Fos-expressing neuronal profiles. Scale bar = 250 μ m.

involvement of SP-NK1R signaling in the antinociceptive action of BoNT/A.

As the underlying mechanism of its analgesic activity it was suggested that BoNT/A prevents peripheral neurotransmitter and pro-inflammatory mediator release at peripheral sensory nerve endings (Aoki, 2005). More

recent observations support central site of BoNT/A antinociceptive action: 1. bilateral effect after unilateral BoNT/A in mirror and polyneuropathic pain, 2. prevention of BoNT/A antinociceptive effect by disruption of axonal transport within peripheral neurons, and 3. occurrence of BoNT/A proteolytic products in central sensory regions (Bach-Rojecky and Lacković, 2009; Favre-Guilmond et al., 2009; Bach-Rojecky et al., 2010; Matak et al., 2011; Matak et al., 2012; Favre-Guilmond et al., 2017). Enzymatic activity of BoNT/A at central afferent terminals most likely involves prevention of sensory neurotransmitter release (Matak and Lacković, 2014). Present data suggest that BoNT/A analgesic effect in mice is dependent on the integrity of SP/NK1R-mediated nociceptive transmission. Along with reduction of pain behavior, BoNT/A antinociceptive action is accompanied by prevention of neuronal activation and c-Fos expression in the dorsal horn (Aoki, 2005; Drinovac et al., 2013). Thus, to support the behavioral findings we assessed the c-Fos expression evoked by formalin-induced pain. In accordance with behavioral data, BoNT/A did not reduce the dorsal horn c-Fos activation in $tac1$ and $tac1r$ knockout animals (Figs. 5 and 6). Since c-Fos activation is a reliable marker of increased nociceptive transmission from primary afferents to second order sensory neurons, these results are in line with the possible BoNT/A interaction with SP/NK1R transmission at the spinal level. Peripherally applied BoNT/B reduced the internalization of NK1R in the dorsal horn induced by formalin test or TRPV1 agonist capsaicin in mice, indicative of inhibition of central SP release (Marino et al., 2014; Ramachandran et al., 2015). The lack of antinociceptive action of BoNT/A in knockout mice might result from altered susceptibility of sensory neurons to BoNT/A action, and possible lack of BoNT/A enzymatic activity in CNS. Thus, we examined the occurrence of BoNT/A-mediated SNAP-25 cleavage in the spinal dorsal horn, where the sensory neurons which innervate the site of BoNT/A injection terminate centrally. Individual fibers expressing cleaved SNAP-25 occurred in both wild type and knockout animals pretreated with BoNT/A i.pl. (Fig. 7). This observation confirms that peripherally applied BoNT/A is enzymatically active in

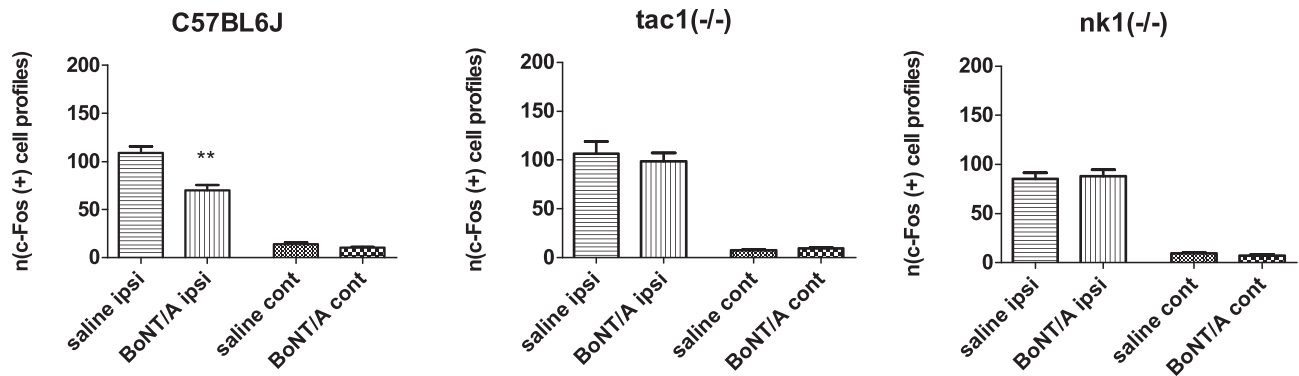


Fig. 6. Lack of effect of BoNT/A on c-Fos expression in $tac1^{-/-}$ and $tac1r^{-/-}$ mice. Neuronal activation was evoked by unilateral 5% formalin injection into the hind paw and assessed by c-Fos immunohistochemistry. Number of c-Fos-expressing neurons was automatically counted in ipsilateral (ipsi) and contralateral (cont) lumbar dorsal horn. (mean \pm SEM. ** $p < 0.01$ in comparison to saline treatment; n (mice/group) = 4–5; mean \pm SEM, one-way ANOVA followed by Bonferroni's post hoc test).

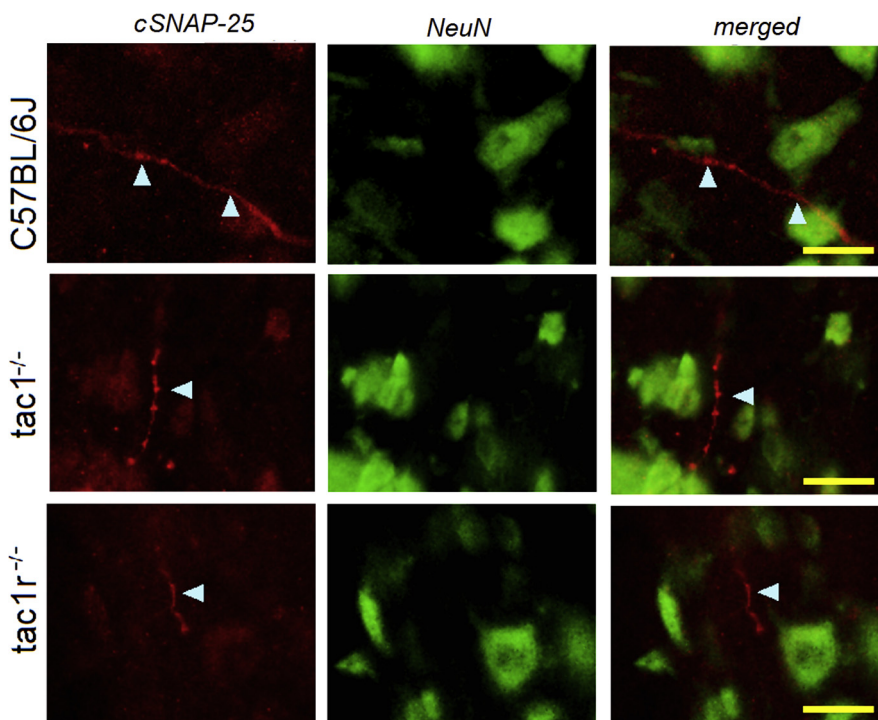


Fig. 7. Occurrence of individual BoNT/A-cleaved SNAP-25 (cSNAP-25) immunofluorescent fibers in dorsal horn of i.pl. BoNT/A-injected wild type and $tac1^{-/-}$ and $tac1r^{-/-}$ mice. Red fluorescent fibers (indicated by arrow heads) represent cleaved SNAP-25, while green staining represents neurons (NeuN marker). Scale bar = 20 μ m. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

sensory regions, although it had no antinociceptive effect in SP/NKA and NK1R knockouts.

In the CFA-induced hind paw inflammation, BoNT/A attenuated the edema formation in wild type and NK1R knockout mice at day 1 post CFA treatment, while at later time points the effect was not significant (Fig. 3). Since the BoNT/A anti-inflammatory action does not parallel the time-course of its antinociceptive action (Figs. 2 and 3), these data do not support causal association of peripheral anti-inflammatory actions of BoNT/A in its antinociceptive effect. In line with present

observation carrageenan- or capsaicin-induced hind paw inflammatory hyperalgesia, the BoNT/A antinociceptive effect was not associated with detectable anti-inflammatory effect (Bach-Rojecky and Lacković, 2005; Bach-Rojecky et al., 2008; Favre-Guilmarde et al., 2009). Human studies involving experimental inflammatory pain reported variable results: BoNT/A reduced the pain and vasomotor responses evoked by capsaicin and glutamate in human trigeminal area (Gazerani et al., 2006, 2009; da Silva et al., 2014), while in studies involving capsaicin application to the forearm skin, the results were divergent (Tugnoli et al., 2007; Schulte-Mattler et al., 2007; Voller et al., 2003). In a recent large multicentric study it was shown that BoNT/A beneficial action in peripheral neuropathic pain was not associated with reduction of CGRP and SP peptide concentration in patient skin biopsy samples (Attal et al., 2016).

In present experiments, the antinociceptive effect of BoNT/A in neuropathic pain (Fig. 1) seems modest compared to the effect in CFA or formalin-induced inflammatory pain (Fig. 2, Fig. 4). We observed low reduction of pain threshold induced by neuropathy (only 35–40%), and inability of BoNT/A to fully restore the initial mechanical pain threshold (Fig. 1). This result is in line with other studies which report modest effect of BoNT/A in chronic constriction injury (CCI)-induced neuropathic pain in mice (Marinelli et al., 2010; Mika et al., 2011; Vacca et al. 2013). We did not assess the effect of BoNT/A beyond day 7 after peripheral toxin treatment, however, the effect of BoNT/A in previous mice studies was prolonged, and did not increase significantly with time (Marinelli et al., 2010;

Mika et al., 2011; Vacca et al., 2013). Smaller effect of BoNT/A on nerve injury-induced mechanical hyperalgesia, in comparison to carrageenan or diabetic neuropathy-evoked mechanical hyperalgesia, was observed in rats (Bach-Rojecky et al., 2010; Drinovac et al., 2013; Favre-Guilmond et al., 2017). It was speculated that this might be associated with different neurochemical mediators involved in pain of different origins (Favre-Guilmond et al., 2017). In the CFA-evoked inflammatory pain model BoNT/A effect was slightly more pronounced compared to neuropathic pain (Fig. 2). On day 6 following the CFA treatment, the mechanical pain thresholds in control animals started to recover, and the antinociceptive effect of BoNT/A was no longer statistically significant. Lack of significant action of BoNT/A on day 6 post CFA was probably due to recovery of pain threshold in control animals, rather than seemingly short duration of BoNT/A action.

In summary, 1.) gene knockout of SP and NK1R has no major effect on pain sensitivity in different models (Figs. 1, 2, 4), 2.) we found the evidence of BoNT/A enzymatic activity in CNS (Fig. 7), however 3.) BoNTA had no antinociceptive effect in these gene knockouts (Fig. 1, 2, 4, and 6). It is widely accepted that SP and NK1R are one of the key elements in transmission of pain. In support of this, NK1R antagonists prevent pain and hyperalgesia in rodent pain models (Lee and Kim, 2007; Cumberbatch et al., 1998; King et al., 2000). However, in our study we found that deletion of SP/NKA and NK1R did not change sensitivity to pain in comparison to control animals (Figs. 1, 2 and 6). The lack of effect of *tac1* and *tac1r* knockout is in line with previous behavioral observations. In partial sciatic nerve ligation neuropathy, knockout and wild-type mice exhibited similar spontaneous pain-related behavior, mechanical and cold allodynia, and mechanical hyperalgesia (Cao et al., 1998; Martinez-Caro and Laird, 2000; Botz et al., 2013). Lack of the effect of *tac1* gene knockout was shown in model of CFA-induced pain inflammation, as well as formalin test (Cao et al., 1998). Those observations suggest that, at least in knockout animals, SP and NK1R are dispensable for the development of mechanical hyperalgesia. On the other hand, in normal wild-type animals, SP-NK1R system is actively participating in the pain control and can be targeted pharmacologically with BoNT/A or NK1R antagonists. Key difference between pharmacological manipulation and gene knockouts is that pharmacological treatment is limited in duration. Thus, we speculate that the permanent deletion of *tac1* and *tac1r* genes leads to change in neuronal plasticity and/or development of alternative pain transmission mechanisms insensitive to BoNT/A.

As discussed previously, the effect of *tac1* and *tac1r* knockout on nociceptive thresholds and development of mechanical hypersensitivity is very similar in examined experimental types of pain. This could be due to the fact that NK1R is the dominant receptor which mediates SP effects on pain, thus, the effects exerted by knockout of either the neurotransmitter, or its receptor, are similar. However, in a model of chronic systemic arthritis evoked by repeated CFA challenge, some subtle

differences in mechanical pain thresholds occur with a delay (Borbély et al., 2013). Eleven days following the pain induction, the mechanical hyperalgesia becomes smaller in *tac1r* knockout animals while in *tac1* knockouts it is not significantly reduced in comparison to wild type (Borbély et al., 2013).

CONCLUSION

BoNT/A antinociceptive activity is dependent on integrity of SP/NKA and NK1R.

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