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Abstract

"Mirror pain" or mirror-image pain (MP) is pain opposite to the side of injury. Mechanism and frequency in humans is not known. There is no consent on therapy. Here we report that unilaterally injected botulinum toxin type A (BT-A) has bilateral effect in experimental MP, thus deserves to be investigated as therapy for this condition. We examined the localization of BT-A's bilateral antinociceptive action in MP induced by 3% carrageenan intramuscular injection in Wistar rats. BT-A was applied peripherally (5 U/kg), into ipsilateral or contralateral hind paw pad (i.pl.) and centrally (1 U/kg), at spinal (intrathecally, i.t.) or supraspinal (intracisternally, i.c.) level. Additionally, we examined the involvement of central opioid and GABAergic systems, as well as the contribution of peripheral capsaicin-sensitive neurons to BT-A's bilateral antinociceptive effect. Ipsilateral ipl. and i.t. BT-A reduced the bilateral mechanical sensitivity to von Frey filaments, while contralateral i.pl. and i.c. treatments had no effect on either tested side. Bilateral antinociceptive effect of ipsilateral i.pl. BT-A was prevented by μ-opioid antagonist naloxonazine (1.5 μg/10 μl) and $GABA_A$ antagonist bicuculline (1 μg/10 μl) if applied at the spinal level, in contrast to supraspinal application of the same doses. Local treatment of sciatic nerve with 2% capsaicin 5 days following BT-A i.pl. injection caused desensitization of sciatic capsaicin-sensitive fibers, but did not affect bilateral antinociceptive effect of BT-A and the presence of cleaved SNAP-25 at the spinal cord slices. Present experiments suggest segmental actions of peripheral BT-A at spinal level, which are probably not solely dependent on capsaicin-sensitive neurons.

Keywords

Botulinum toxin A, mirror pain, μ-opioid antagonist, $GABA_A$ antagonist, capsaicin-sensitive neurons, cleaved SNAP-25

Abbreviations

BT-A – botulinum toxin type A, B – bicuculline, N – naloxonazine, SNAP-25 - Synaptosomal Associated Protein of 25kDa, clSNAP-25 – cleaved SNAP-25, MP – mirror pain, ASIC3 – acid-sensing ion channel 3, CGRP – calcitonin gene-related polypeptide, SP – substance P, GABA – γ-aminobutyric acid, TRPV1 – transient receptor potential vanilloid type 1, CFA – complete Freund's adjuvant, IoNC – infraorbital nerve constriction, CSF – cerebrospinal fluid, DRG – dorsal root ganglion, RVM – rostral ventromedial medulla, TNC

– trigeminal nucleus caudalis, TENS – transcutaneous electric nerve stimulation, i.m. – intramuscular, i.pl. – intraplantar, i.t. – intrathecal, i.c. – intracisternal, i.c.v. – intracerebroventricular, PBS - phosphate buffered saline, PBST - Triton X-100 in phosphate buffered saline, NGS - normal goat serum

Introduction

"Mirror pain" or "mirror-image pain" (MP) is a phenomenon in which unilateral injury results in bilateral pain sensation. Contralateral mirror-imaging has been experimentally demonstrated in a variety of injuries in laboratory animals: inflammatory cutaneous and joint (reviewed in Shenker et al. 2003), inflammatory and non-inflammatory muscle (Sluka et al. 2001, 2002; Radhakrishnan et al. 2003) and neuropathic (reviewed in Koltzenburg et al. 1999). Altered contralateral sensory perception after unilateral injury or in chronic pain syndromes is reported as well in clinics (Huge et al. 2008; de la Llave-Rincón et al. 2009; Fernández-de-las-Peñas et al. 2009; Konopka et al. 2012; Werner et al. 2013). However, the frequency of this disorder in humans is not known. Treatment varies from case to case and there is no consent. Based on experimental research, it is speculated that mechanisms of contralateral spread of pain involve yet unidentified spinal neural pathways (reviewed by Koltzenburg et al. 1999; Shenker et al. 2003; Huang and Yu 2010; Janalcek 2011) with critical contribution of deregulated supraspinal descending pathways (Tillu et al. 2008; Radhakrishnan and Sluka 2009). Antinociceptive effect of botulinum toxin type A (BT-A) was investigated in several MP models such as non-inflammatory muscle pain induced by repeated acidic saline intramuscular injection (Bach-Rojecky and Lacković 2009), trigeminal neuropathy induced by unilateral infraorbital nerve constriction injury (IoNC) (Filipović et al. 2012) and inflammatory pain induced by complete Freund's adjuvant (CFA) injection into temporomandubular joint (Lacković et al. 2016). Consistently, the bilateral reduction of pain was observed following BT-A's unilateral peripheral application. The same effect was demonstrated in poly-neuropathic states evoked by systemic paclitaxel (Favre-Guilmard et al. 2009) or streptozotocin (Bach-Rojecky et al. 2010), as well. As a molecule which comprises proteolytic activity against synaptosomal-associated protein 25 (SNAP-25), part of machinery necessary for neuroexocytosis (Jahn and Fasshauer 2012), BT-A inhibits neurotransmitter release and other possible presynaptic events mediated by SNAP-25 activity (Matak and Lacković 2014). In contrast to beneficial effects at hyperactive neuromuscular junctions and autonomous synapses, where locally applied BT-A cleaves SNAP-25, hyperalgesia and allodynia resulting from hyperactive nociceptive pathways is most probably reversed by SNAP-25 cleavage at central synapses (Matak and Lacković 2014), to where BT-A could be transported from the periphery by capsaicin-sensitive neurons, as recently demonstrated in trigeminal innervation area (Matak et al. 2014). At central synapses BT-A is proposed to

inhibit release of excitatory neurotransmitters from primary afferent terminals in dorsal horn of the spinal cord, such as glutamate, calcitonin gene-related polypeptide (CGRP) and substance P (SP) (Pavone and Luvisetto 2010). Our group was the first to show that antinociceptive action of peripheral BT-A can be blocked by intrathecally delivered opioid and $GABA_A$ antagonist (Drinovac et al. 2013, 2014), indicating that modulation of spinal inhibitory neurotransmitter systems might be an important component of central antinociceptive activity of BT-A. Still, the exact mechanism, as well as the localization of central antinociceptive effect is far from clear.

Hence, in attempt to further characterize BT-A's bilateral antinociceptive effect, in the present study using a model of MP induced by intramuscular 3% carrageenan injection, we investigated: 1) site of its action, 2) involvement of spinal or supraspinal opioid and GABAergic systems, and 3) if peripheral capsaicin-sensitive neurons contribute to its bilateral antinociceptive effect.

Materials and methods

Animals

A total number of 200 male Wistar rats (Department of Pharmacology, University of Zagreb School of Medicine, Croatia), housed in a 12 h light/dark cycle with free access to food and water, in groups of 3 rats per cage, were used in experiments. 3-month-old animals weighted average 350 g at the beginning of experiments. Experiments were performed according to 2010/63/EU Directive on the protection of animals used for scientific purposes and recommendations of the International Association for the Study of Pain (Zimmermann 1983). Animal treatment and experimental protocol involving animals is described according to the ARRIVE guidelines. Experiments were approved by the Ethical Committee of the University Of Zagreb School Of Medicine (permit No. 07-76/2005-43).

Substances

Following substances were used: BT-A (Botox®, Allergan, Inc., Irvine, USA); naloxonazine (Santa Cruz Biotechnology, Inc., CA, USA); ethanol (T.T.T., Zagreb, Croatia); λ-carrageenan, capsaicin, bicuculline, Tween80 and chloral hydrate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Applied doses and preparation of respective solutions are described in the text below.

For immunohistochemistry we used: Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA); normal goat serum (Sigma-Aldrich, St. Louis, MO, USA); rabbit polyclonal antibody to

cleaved SNAP-25 (produced by O.R.); rabbit polyclonal antibody to CGRP (Sigma-Aldrich, St. Louis, MO, USA); Fluorogel® (EMS, Hatfield, PA, USA); goat anti-rabbit Alexa Fluor 488 and 555 (Molecular Probes, Invitrogen, Carlsbad, CA, USA).

Induction of MP and behavioral testing

100 μL of 3% λ-carrageenan dissolved in saline (0.9% NaCl) was injected into the right gastrocnemius muscle (i.m.) of conscious, gently restrained rats (Radhakrishnan et al. 2003). Control animals were injected with saline.

Behavioral assessment of right and left hind paw sensitivity to mechanical stimulation (paw withdrawal threshold, PWT) was performed using a series of von Frey filaments (Stoelting Co., Wood Dale, IL, USA) ranging 0.4 – 26 g. Animals were habituated to the testing area (plastic cage with a wire mesh floor) for 30 min. Filaments were applied in Chaplan's up-down method (Chaplan et al. 1994). Each filament was applied 3 times, kept in bent position for 2 s, with an inter-stimulus interval of 5 – 10 s (Drinovac et al. 2013). The lowest filament that elicited a withdrawal response was considered the threshold stimulus. Defensive hind paw movement was regarded as a positive response.

Around 80% of initially treated animals developed bilateral secondary mechanical sensitivity (≤ 6 g threshold at both sides) 2 weeks following i.m. carrageenan treatment. Only those animals were included in further experiments. No mortality of animals occurred in the course of the study. Each experimental group contained 6 animals. In the following text, term ipsilateral denotes the right, pain induction side, while contralateral refers to the left side, opposite to pain induction.

Pharmacological treatments of animals with developed MP

Table 1 contains the summarized data of pharmacological treatments used in the present study. Doses and volumes of substances administered at the different sites and the time points at which behavioral measurement was done is chosen based on our previously published research or as described in the literature and shown in a table 1. Based on our previous experience, local application of BT-A in muscle causes muscle paralysis and complicates nociceptive measurement. Accordingly, in this study we have used subcutaneous route in hind paws as a peripheral site of application. As well, because of difficulties in nociceptive measurement due to different pharmacokinetic properties of bicuculline and naloxonazine, we have chosen different supraspinal routes of administration for the respective antagonists. Naloxonazine, owing to irreversible binding to μ-opioid receptors 24 hours following

application (Ling et al., 1986), was applied i.c.v. under deep general anesthesia and nociceptive measurements using von Frey filaments were performed the next day, allowing full recovery of animals. Bicuculline, due to very short in vivo half-life (<1 h) (Gale and Casu, 1981), could not be applied i.c.v., so i.c. administration was used which was performed under short inhalational anesthesia, allowing the nociceptive measurement to be done within 1 hour.

All drugs except capsaicin were dissolved in 0.9% saline. Capsaicin was dissolved in 10% Tween80 and 10% ethanol in saline. 1 unit (1 U) of BT-A corresponds to 48 pg of purified C. botulinum neurotoxin A complex.

Table 1 Pharmacological treatments of animals that developed MP 2 weeks following i.m. 3% carrageenan injection

Substance	Administration	Dose	Volume	Nociceptive measurement (days after treatment)	References
BT-A	i.pl. ipsilateral	5 U/kg	20 µL	5 days	Bach-Rojecky and Lacković 2009; Drinovac et al. 2014
BT-A	i.pl. contralateral	5 U/kg	20 µL	5 days	
BT-A	i.t.	1 U/kg	10 µL	1 day	
BT-A	i.c.	1 U/kg	10 µL	1 day	
Capsaicin	perisciatic	2%	/	7 days	Pertovaara 1988.; Matak et al. 2014
Naloxonazine	i.t.	1.5 µg	10 µL	1 day	Drinovac et al. 2013
Naloxonazine	i.c.v.	1.5 µg	5 µL	1 day	
Bicuculline	i.t.	1 µg	10 µL	30 min	Drinovac et al. 2014
Bicuculline	i.c.	1 µg	10 µL	30 min	

Legend: i.pl. – intraplantar; i.t. – intrathecal; i.c. – intracisternal; i.c.v. - intracerebroventricular

For each type of pharmacological treatment, animals in respective control groups received 0.9% saline or vehicle (if treated with capsaicin). Substance or saline/vehicle administration is performed as described:

a) Intraplantarly (i.pl.): subcutaneously into the plantar surface of the right or left hind paw to conscious, gently restrained animals.

b) Intrathecally (i.t.): at the lumbar L4/L5 level to anesthetized animals**.** A small skin incision (2 cm) was made at the lumbar L4/L5 level, substance or saline was injected (27G Tuberculin syringe) between the vertebrae and the skin was sutured (Drinovac et al. 2013). Correctness of application was verified by the animal's tail or hind limb brisk move.

c) Intracisternally (i.c.): into the cisterna magna, through a needle (27G Tuberculin syringe) carefully advanced between the occipital protuberance and the spine of the atlas, to anesthetized animals (Drinovac et al. 2014). Correctness of application was verified by extraction of a small amount of cerebrospinal fluid (CSF).

d) Intracerebroventricular (i.c.v.): into lateral cerebral ventricles, according to previously described procedure (Noble et al. 1967) in anesthetized animals. Small skin incision (1cm) was performed at the top of the animal's head and 1 mm opening in both parietal bones, 1.5 - 2 mm diagonal from sagital and coronal suture intersection, was drilled (NSK Ultimate XL, Hoffman Estates, IL, USA). Total dose of applied substances was divided and in equal volumes applied with Hamilton syringe (MICROLITER syiringe 10 µL, Hamilton, Höchst, Germany), set on 4 mm depth, in both ventricles.

e) Perisciatic: The right sciatic nerve of anesthetized animals was exposed in the thigh. A cotton ball soaked in 2% capsaicin or vehicle was placed on the exposed right sciatic nerve for 15 min. The skin was sutured and animals were allowed to recover (Pertovaara 1988; Matak et al. 2014).

Experimental protocol

Experiment 1: Investigation of localization of BT-A's antinociceptive effect

We tested four different ways of BT-A application in animals with chronic MP:

a) i.pl. to ipsilateral hind paw; b) i.pl. to contralateral hind paw; c) i.t. and d) i.c. For each BT-A treatment, a group of animals was injected with the same volume of saline to test possible effects of the respective way of application on mechanical thresholds.

Experiment 2: Examination of involvement of central opioid and GABAergic neurotransmitter systems in BT-A' antinociceptive effect

In this experiment, the effects of spinally or supraspinally applied selective μ-opioid antagonist naloxonazine (N) and selective $GABA_A$ antagonist bicuculline (B) were assessed in animals with MP treated with ipsilateral i.pl. BT-A or saline. 5 days following BT-A or saline application, the same dose of naloxonazine was applied i.t. or i.c.v., and sensitivity to von Frey filaments was assessed the next day (Ling et al. 1986), while mechanosensitivity after i.t. or i.c. bicuculline was tested after 30 min (Drinovac et al. 2014).

Experiment 3: Investigation of contribution of peripheral capsaicin-sensitive neurons to BT-A's effect

Ipsilateral sciatic nerve was locally treated either with 2% capsaicin or with vehicle, as described in previous section, 5 days following ipsilateral i.pl. BT-A (5 U/kg) application to animals with MP (the time point in which antinociceptive effect of BT-A, as well as the cleaved SNAP-25 in the CNS is expected to occur) (Matak et al. 2011). To verify if the described perisciatic treatment desensitized capsaicin-sensitive afferent neurons, in a preliminary experiment we injected pro-algesic 0.1% capsaicin i.pl. into the right hind paw pad.

The experimenter was unaware of the treatment groups in all experiments.

Immunohistochemical analysis

Deeply anesthetized animals from the experiment 3 (chloral hydrate 300 mg/kg intraperitoneally) were transcardially perfused with saline and 4% paraformaldehyde in phosphate-buffered saline (PBS). Spinal cord tissue was excised and placed in paraformaldehyde fixative containing 15% sucrose, followed by 30% sucrose in PBS. The samples were stored at -80 ºC until further use (Drinovac et al. 2013). 40 μm thick lumbar spinal cord sections (L3 – L5), cryostat-cut (Leica, Germany), 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 1:1700 rabbit polyclonal antibody to cleaved SNAP-25 (clSNAP-25) or rabbit polyclonal antibody to CGRP. The antibody to clSNAP-25 is previously characterized and recognizes specifically the BT-A-cleaved SNAP-25 (Antonucci et al. 2008; Matak et al. 2011). The next day, sections were incubated 2h with fluorescent secondary antibody 1:300 goat anti-rabbit Alexa Fluor 555 (for clSNAP-25) or 1:400 goat anti-rabbit Alexa Fluor 488 (for CGRP),

washed, mounted on microscope slides and covered with anti-fading agent Fluorogel®. Sections from 4 animals per group (8 sections per animal) were examined. Immunostained sections were visualized with Olympus BX-51 fluorescent microscope coupled to DP-70 digital camera (Olympus, Tokyo, Japan). Images were processed for brightness and contrast with Adobe Photoshop.

Statistical analysis

Results, presented as mean ± SEM, were analyzed by ANOVA one-way analysis of variance followed by Tukey's post-hoc test (StatSoft 10 Statistica Enterprise). $P<0.05$ was considered significant.

Results

Peripheral and spinal, but not supraspinal BT-A reduces MP induced by i.m. carrageenan injection

Two weeks following i.m. carrageenan injection animals developed bilateral hypersensitivity, measured as decreased mechanical threshold on both hind paws ($p<0.01$). BT-A (5 U/kg) applied i.pl., ipsilateraly to i.m. carrageenan, after 5 days increased mechanical threshold on both sides ($p<0.01$). In contrast, BT-A applied in the same dose opposite to pain induction had no effect on pain on either side (Fig. 1). Bilateral reduction of mechanical hypersensitivity to von Frey filaments ($p<0.01$) was also observed 1 day following BT-A (1 U/kg) i.t. administration. However, when applied in the cerebellomedullary cistern, BT-A had no effect on either tested side.

Bilateral antinociceptive effect of BT-A involves interaction with opioid and GABAergic system at the spinal level

Intrathecal low dose of selective μ-opioid antagonist naloxonazine (1.5 μg) abolished the bilateral antinociceptive effect of BT-A ($p<0.01$ on both sides) (Fig. 2A). In contrast, the same dose of naloxonazine injected i.c.v. had no effect on BT-A's bilateral action. Tested dose of naloxonazine alone, either i.t. or i.c.v., had no effect on carrageenan induced bilateral mechanical hypersensitivity to von Frey filaments.

Similarly to naloxonazine, intrathecal bicuculline (1 μg) applied 30 min prior to nociceptive testing abolished the bilateral antinociceptive action of peripheral BT-A (5 U/kg). However, bicuculline applied into the cisterna magna in the same dose did not affect BT-A's bilateral

antinociceptive effect (Fig. 2B). The respective doses of bicuculline alone, applied i.t. or i.c., did not affect pain behavior measured with von Frey filaments (results not shown).

Chemical desensitization of sciatic nerve with 2% capsaicin has no effect on BT-A's antinociceptive activity

Direct application of 2% capsaicin to the sciatic nerve selectively desensitized a subset of transient receptor potential vanilloid type 1 (TRPV1) expressing neurons, which was confirmed in preliminary experiment as an absence of acute nocifensive behavior evoked by i.pl. 0.1% capsaicin injection (results not shown). Acute mechanical sensitivity measured with von Frey filaments was not altered, compared to the opposite side or to vehicle treated animals (results not shown). Qualitative analysis of lumbar L4 segments of the spinal cord revealed no visible difference in CGRP immunoreactive fibers between capsaicin and vehicle perisciatic treatments, indicating that the 2% capsaicin treatment most probably did not destroy capsaicin-sensitive neurons (Fig. 3).

In rats which developed MP 14 days after i.m. carrageenan injection, capsaicin application on ipsilateral sciatic nerve produced significant antinociceptive effect at that side ($p<0.01$) but, contrary to BT-A, had no effect on contralateral mechanical hypersensitivity to von Frey filaments (Fig. 1; Fig. 4). When applied 5 days after ipsilateral i.pl. BT-A treatment, we observed no synergistic or additive effects on ipsilateral mechanical threshold (Fig. 4). Moreover, capsaicin treatment did not affect BT-A's reduction of contralateral pain (Fig. 4).

Cleaved SNAP-25 in dorsal spinal cord as an evidence of spinal site of BT-A action

BT-A-cleaved-SNAP-25 immunoreactivity (clSNAP-25) appeared in ipsilateral lumbar ventral horns (results not shown), with localization similar as in previous study by Matak et al. (2012), which examined the occurrence of clSNAP-25 after i.pl. BT-A (15 U/kg) injection. In the present study, we observed at least 2 small individual fibers in ipsilateral dorsal horn of the L4 lumbar spinal cord after the i.pl. 5 U/kg BT-A (Fig. 5). The fibers were in most sections observed at the deeper laminae III and IV. We did not observe clSNAP-25 immunoreactive fibers at contralateral dorsal horn (Fig 5). Seven days following perisciatic 2% capsaicin treatment truncated SNAP-25 immunoreactivity was still present in ipsilateral dorsal spinal cord of BT-A treated animals, in the similar localization (Fig. 5).

Discussion

"Mirror pain" or mirror-image pain phenomenon is described in many case reports in humans. However, the precise frequency of the disorder was never investigated and the mechanism of its development is not known. Accordingly, the treatments vary from case report to case report, producing only a short lasting pain relief. One of the main features of BT-A, which distinguishes it from other known analgesics is its long-lasting antinociceptive effect after local application (Bach-Rojecky et al., 2005), which is not associated only with site of application, but spreads on distant sites as well (Bach-Rojecky and Lacković, 2009; Favre-Guilmard et al., 2009; Bach-Rojecky et al., 2010; Filipović et al. 2012; Lacković et al. 2016). In the present study we aimed to further investigate the bilateral antinociceptive effect of BT-A in less investigated MP model. The results of the present study on experimental MP model indicate that these distant antinociceptive effects might occur at the level of dorsal horn of the spinal dorsal horn, but apparently not at the higher levels of CNS, interfering with spinal opioid and GABAergic systems.

In carrageenan-induced MP model employed in the present study BT-A exerts bilateral antinociceptive effect 5 days after its unilateral i.pl. (5 U/kg) and 1 day following its i.t. injection (1 U/kg) (Fig. 1), similarly as previously demonstrated in repeated acidic saline-induced MP (Bach-Rojecky and Lacković 2009). Additionally, using immunohistochemistry of cleaved SNAP25 we show that bilateral effect of BT-A occurs after only unilateral enzymatic activity of the toxin in ipsilateral dorsal horn (Fig. 5). Moreover, here we show that BT-A's application in cisterna magna has no effect on bilateral pain, thus suggesting spinal site of its antinociceptive action. However, at present we cannot exclude the possibility that BT-A may not reach deep supraspinal sensory centers after i.c. application. Investigation of clSNAP-25 in brain regions following supraspinal application might provide insight into more firm conclusions about segmental antinociceptive effect of BT-A.

Both models, acidic saline- and carrageenan-induced MP, are developed to study chronic, widespread and neuronally mediated musculoskeletal pain (Radhakrishnan et al. 2004) and share some common characteristics: 1. muscle hyperalgesia lasts for up to 4 – 8 weeks (Sluka et al. 2001; Radhakrishnan et al. 2003); 2. in addition to primary hyperalgesia at the site of injury, secondary hyperalgesia at distant sites develops (Radhakrishnan et al. 2004); 3. contralateral spread of pain occurs (Sluka et al., 2001; Radhakrishnan et al. 2003); and, in contrast to other chronic pain models, 4. no glial activation is observed and glial inhibitors are not effective in reversing bilateral pain (Ledeboer et al. 2006). Acidic saline and λ-

carrageenan activate acid-sensing ion channel 3 (ASIC3) in primary afferent fibers innervating muscle, which is responsible for the development of secondary mechanical hyperalgesia (Sluka et al. 2003; Sluka et al. 2007). However, ASIC3 apparently does not participate in maintenance of MP, since ASIC antagonists are ineffective in reducing MP once it is developed (Gautam et al. 2012). In contrast to e.g. referred pain from viscera to belonging dermatome, the anatomical background for specific pain at homonymous areas in contralateral limb is not clear, but it favors neurally mediated spinal signaling mechanisms, probably through commissural interneurons, which make synapses between the two sides of spinal cord (Koltzenburg 1999; Jancalek 2011). In addition to spinal mechanisms (Skyba et al. 2002, 2005), sensitization and enhancement of facilitatory descending pathways from the rostral ventrolateral medulla (RVM) was demonstrated to contribute to development, contralateral spread and chronicity of muscle hyperalgesia after acidic saline injections (Ren and Dubner 1996; Radhakrishnan and Sluka 2009; Tillu et al., 2008; Da Silva et al. 2010). MP induced by i.m. carrageenan is assumed to be centrally mediated as well, since the myositis occurs on ipsilateral side only (Radhakrishnan et al. 2003). Additionally, gastrocnemius transcutaneous electric nerve stimulation (TENS) alleviates bilateral pain independently of side of application (Ainsworth et al. 2006). This effect is assumed to be mediated through RVM modulation (Kalra et al. 2001; Desantana et al. 2009). Lack of the effect of BT-A applied into the cisterna magna (Fig. 1) might suggest that, in contrast to TENS, the antinociceptive action of BT-A may involve segmental spinal circuits only.

In the present study, BT-A applied contralateral to pain induction side failed to reduce hypersensitivity on either side (Fig. 1). This is different from our previous finding in acidic saline model where contralateral BT-A reduced hypersensitivity, but on that side only (Bach-Rojecky and Lacković 2009). This might be explained by differences between the employed models. First, the protocol of induction and the time pattern in which contralateral pain develops is different. In acidic saline model, MP develops 24 hours after second injection, while in carrageenan model MP develops 14 days after single injection (Sluka et al. 2001; Radhakrishnan et al. 2003). Second, in contrast to acidic saline which induces MP without any peripheral tissue damage (Sluka et al., 2001; Radhakrishnan et al. 2004), λ-carrageenan causes acute non-immune mediated inflammation in muscle which progresses to chronic one, in time-course that parallels the contralateral development of hyperalgesia (Radhakrishnan et al. 2003). However, in the model where λ-carrageenan was injected into the rat's paw pad, local inflammatory reaction was not affected by BT-A (Bach-Rojecky et al., 2008).

Altogether, those differences emphasize the need to investigate different models of MP because of the complex mechanisms.

As demonstrated in our previous studies, the activation of endogenous opioid and GABAergic systems at spinal level, possibly through changes in the respective receptors and/or neurotransmitters, is involved in the central antinociceptive effect of peripherally administered BT-A (Drinovac et al. 2013, 2014). Systemically or intrathecally applied μ-opioid antagonist naloxonazine and $GABA_A$ antagonist bicuculline decreased the antinociceptive effects of i.pl. BT-A on formalin-induced second phase spontaneous pain, as well as on partial sciatic nerve transection-induced mechanical and cold allodynia (Drinovac et al. 2013, 2014). In line with these results, in the present study we showed that i.t. naloxonazine (1.5 μg/10 μl), as well as bicuculline (1 μg/10 μl), in doses which had no effect on mechanosensitivity if applied alone, abolished the antinociceptive effect of peripheral BT-A (5 U/kg i.pl.) ipsilaterally and contralaterally to carrageenan injection (Fig. 2). On the contrary, supraspinally applied opioid (i.c.v.) and $GABA_A$ antagonist (i.c.) failed to affect the i.pl. BT-A's bilateral action, thus suggesting possibility that BT-A interferes with the opioid and GABAergic neurotransmission at the spinal level.

The axonal transport of BT-A from periphery to CNS was demonstrated in behavioral and immunohistochemical experiments using an axonal transport blocker colhicine, which prevented the antinociceptive effect of BT-A along with the occurrence of BT-A-truncated-SNAP-25 in dorsal horn of the spinal cord or trigeminal nucleus caudalis (TNC) (reviewed in Matak and Lacković 2014). More specifically, it is hypothesized that the axonal transport of BT-A might occur through specific subtype of neurons (Matak et al. 2014). In the trigeminal innervation area was demonstrated that destruction of capsaicin-sensitive neurons abolishes the beneficial effect of BT-A in the second phase of the formalin test, as well as the occurrence of cleaved SNAP-25 in TNC (Matak et al. 2014). Thus, it was proposed that axonal transport of BT-A involves capsaicin-sensitive sensory neurons, at least in trigeminal area. Therefore, in the present study we examined if sciatic capsaicin-sensitive neurons contribute to the bilateral antinociceptive effect of i.pl. BT-A. Application of 2% capsaicin solution locally on sciatic nerve 5 days following BT-A i.pl. injection did not affect bilateral antinociceptive effect of BT-A (Fig. 4). Additionally, cleaved SNAP-25 was still present at the spinal cord slices after the capsaicin treatment (Fig. 5). Contrary to experiments in trigeminal area, where intraganglionic 2% capsaicin destroyed capsaicin-sensitive neurons (Matak et al., 2014), the same concentration of capsaicin applied locally to the sciatic nerve, produced antinociceptive effect but did not destroy neurons, which was verified qualitatively

using immunohistochemistry, where between vehicle and 2% capsaicin treated animals was not visible difference in spinal lumbar L4 CGRP immunoreactivity (Fig 3). However, we observed that, in contrast to bilateral effect of i.pl. BT-A (Fig. 4) on MP, the perisciatic capsaicin exerts effect only on the side of application.

Owing to the different effects of i.pl. BT-A (bilateral antinocicetion) and perisciatic capsaicin (only ipsilateral antinociception) on MP, most likely explanation is that in sciatic innervation area BT-A, in addition to capsaicin-sensitive neurons, may require other types of neurons to achieve bilateral antinociceptive effect. Alternatively, presented results might indicate differences between transport of toxin in trigeminal and sciatic area, however further studies are needed to confirm this speculation.

Conclusion

In addition to acidic saline induced MP, we found bilateral antinociceptive effect of BT-A in a less investigated model of unilateral carrageenan intramuscular injection. BT-A's bilateral antinociceptive effect can be achieved after peripheral ipsilateral and intrathecal, but not intracisternal application. This might indicate that its effects on MP, as well as the interaction with opioid and GABAergic systems, occur at segmental level of the spinal cord. BT-A is the only substance known so far with bilateral effect in different types of experimental MP after unilateral application. Possible benefit in cases of human bilateral pain might be important to investigate.

Conflict of interest

We declare no conflict of interest.

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Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. Experiments were approved by the Ethical Committee of the University Of Zagreb School Of Medicine (permit No. 07-76/2005-43).

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Figure legends:

Figure 1. The effect of BT-A on MP caused by i.m. carrageenan. BT-A (5 U/kg) was injected peripherally, i.pl. into right (ipsilateral to carrageenan) or left hind paw (contralateral to carrageenan) and centrally, i.t. or i.c. (1 U/kg) 14 days after unilateral i.m. carrageenan injection. Mechanical sensitivity was measured at both hind paws using von Frey filaments 5 days following BT-A peripheral and 1 day following BT-A central treatments. Control = i.m. saline treated animals; all other groups = i.m. carrageenan treated animals. Results are presented as mean ± SEM (N=6). * P<0.01 compared to control; + P<0.01 compared to saline i.pl. One way ANOVA followed by Tukey *post hoc*.

Figure 1

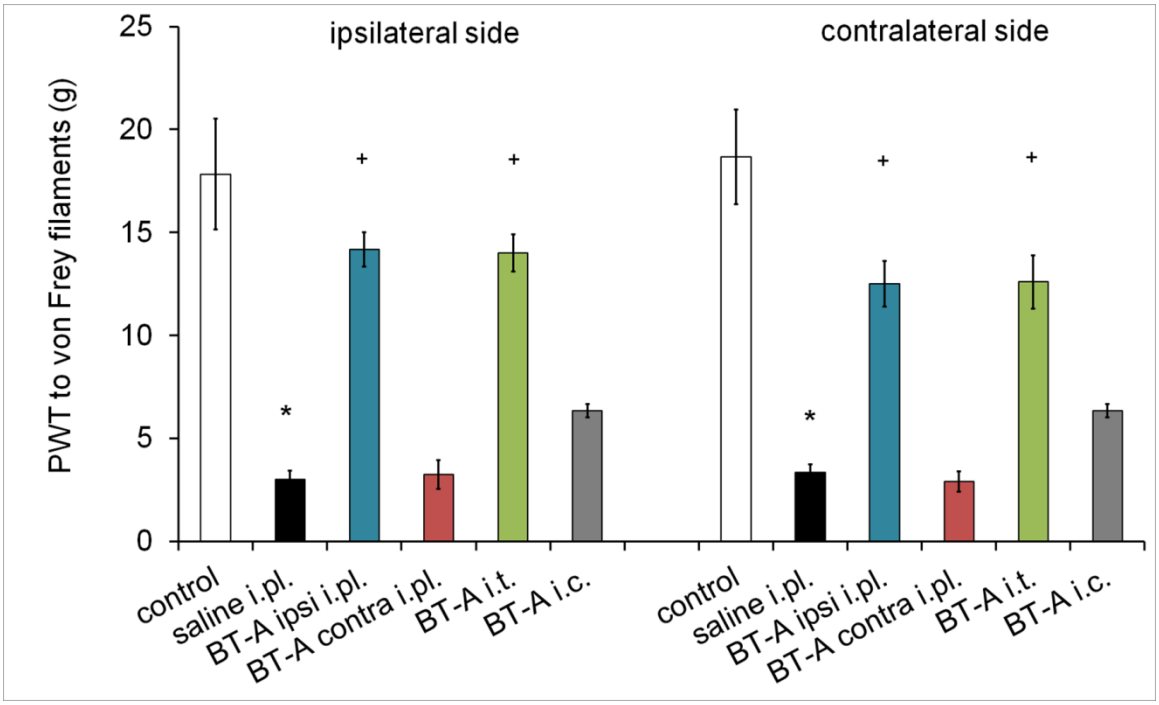
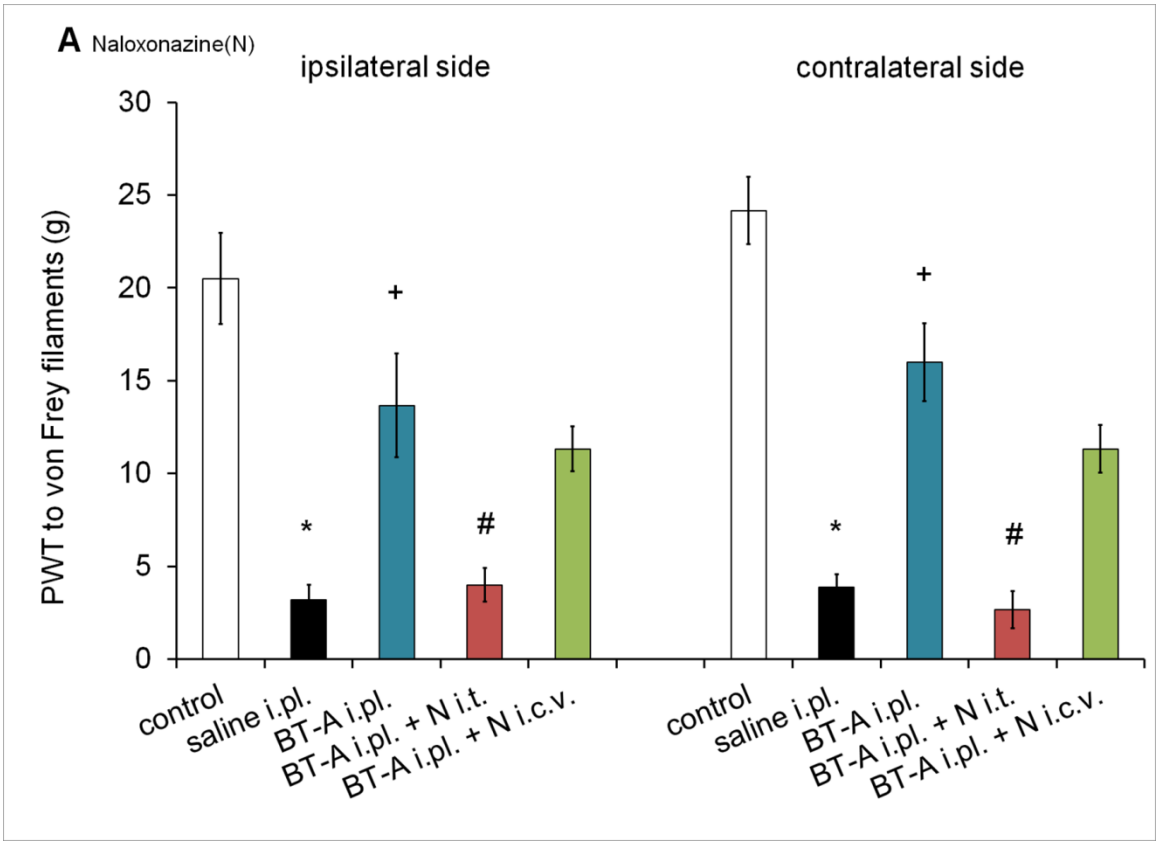
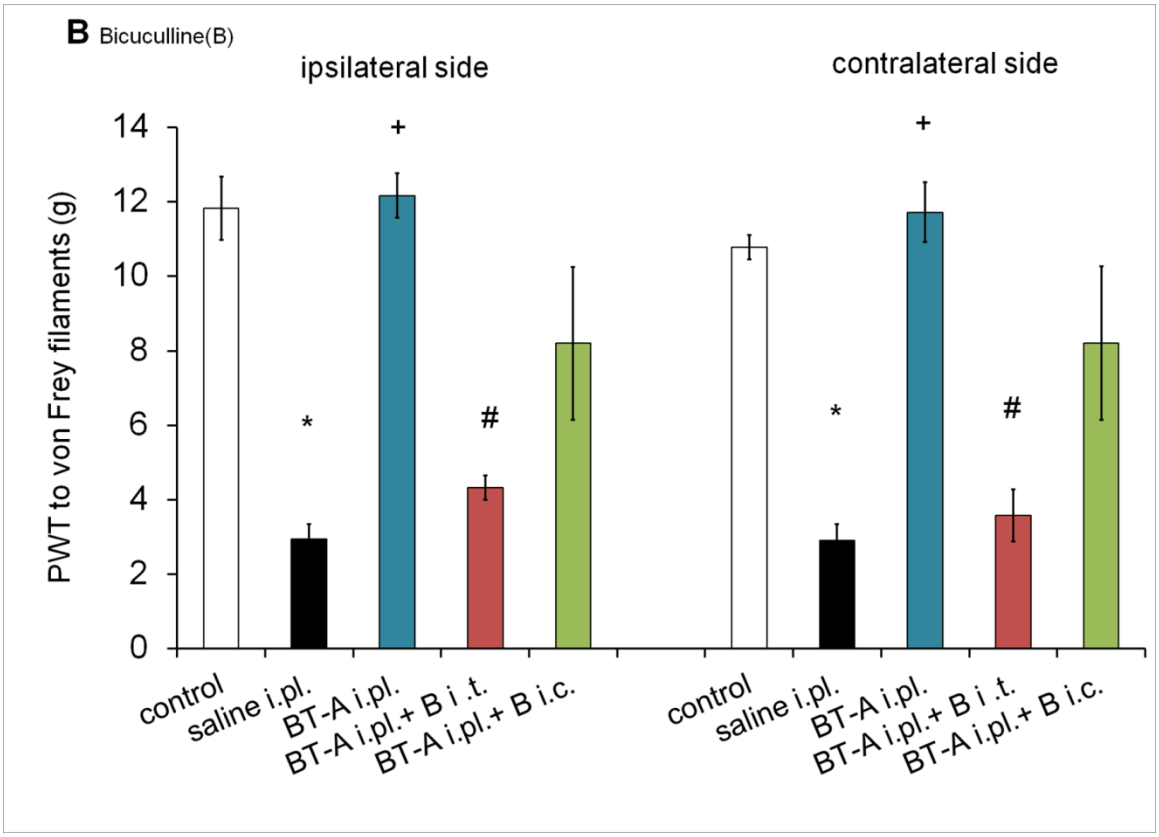


Figure 2. Bilateral antinociceptive effect of BT-A in i.m. carrageenan induced MP involves interaction with spinal opioid and GABAergic system. Measurement of mechanical sensitivity to von Frey filaments was performed 5 days following BT-A (5 U/kg, ipsilateral i.pl.) and: A) 24 hours following naloxonazine (1.5 μg) i.t. or i.c.v. application; B) 30 min following bicuculline (1 μg) i.t. or i.c. application in animals with developed bilateral pain. Control = i.m. saline treated animals; all other groups = i.m. carrageenan treated animals. Results are expressed as mean ± SEM (N=6). * P<0.01 compared to control, + P<0.01 compared to saline i.pl., # P<0.01 compared to BT-A i.pl. One way ANOVA followed by Tukey *post hoc.*

Figure 2




B Bicuculline(B)
ipsilateral side
contralateral side
PWT to von Frey filaments (g)
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control
saline i.pl.
BT-A i.pl.
BT-A i.pl.+ B i .t.
BT-A i.pl.+ B i.c.
control
saline i.pl.
BT-A i.pl.
BT-A i.pl.+ B i .t.
BT-A i.pl.+ B i.c.

Figure 3. The effect of ipsilateral perisciatic 2% capsaicin application on the CGRP content in the L4 lumbar dorsal horn of the spinal cord. Fluorescent images of CGRP (green immunoreactivity). N (animals per group) = 4 (8 sections were examined per each animal). Scale bar = 50 μm.

Figure 3

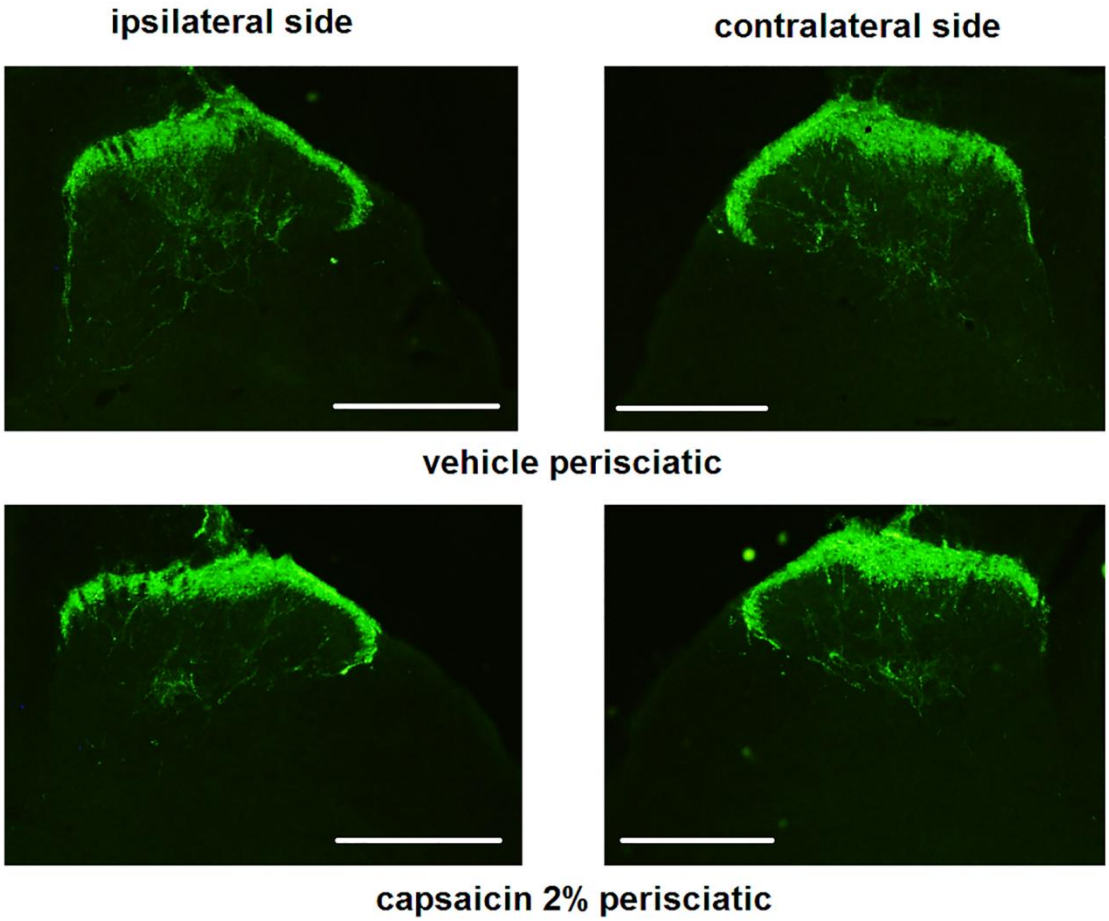

Figure 4. Functional impairment of TRPV-1 expressing sciatic nerve neurons with 2% capsaicin has no effect on BT-A's antinociceptive activity in MP. Ipsilateral treatment of sciatic nerve with capsaicin results in unilateral antinociceptive effect, while ipsilateral BT-A application has bilateral antinociceptive effect. Five days after i.pl. BT-A (5 U/kg) or saline application, ipsilateral sciatic nerve of rats was treated with 2% capsaicin or vehicle. PWT to von Frey filaments was examined 7 days after perisciatic treatments at ipsilateral and contralateral hind paw. Control = i.m. saline treated animals; all other groups = i.m. carrageenan treated animals. Results are represented as mean ± SEM (n=6). ** P<0.01 in comparison to control; + P<0.05 in comparison to saline i.pl. + vehicle; ++ P<0.01 in comparison to saline i.pl. + vehicle. One way ANOVA followed by Tukey *post hoc*.

Figure 4

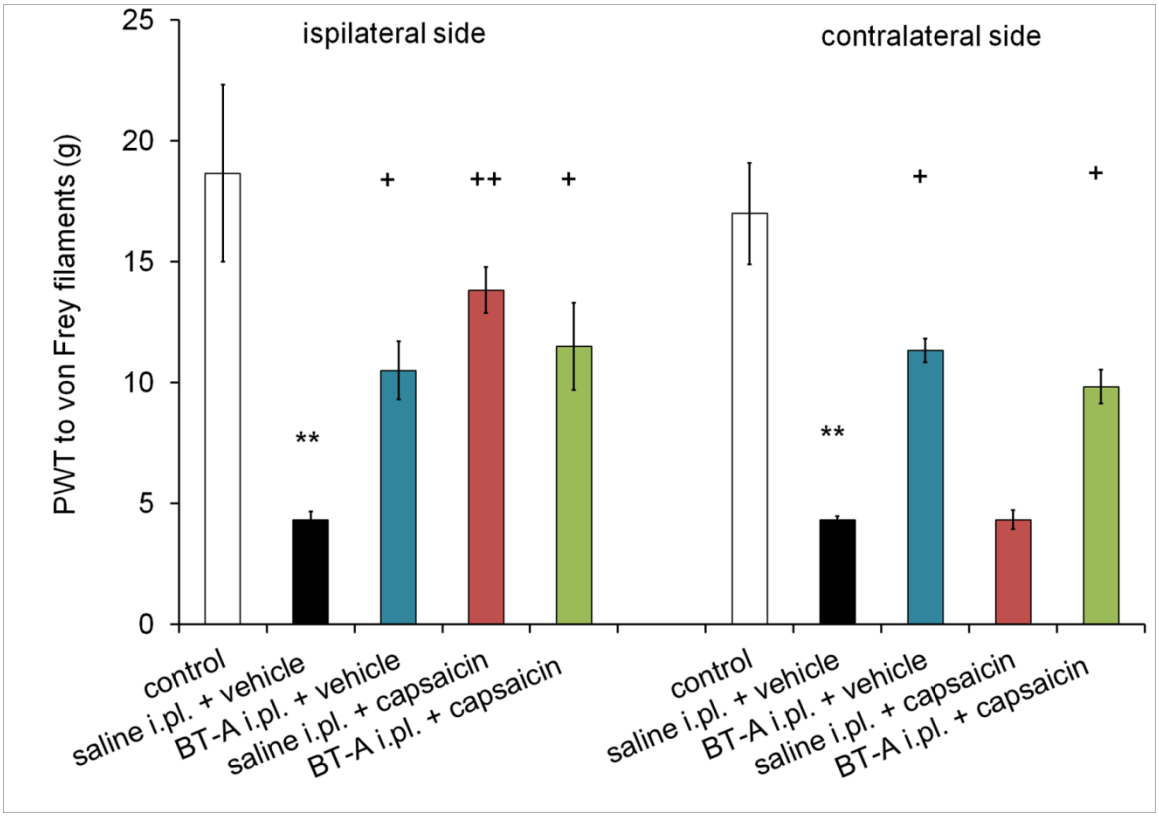

Figure 5. Proteolytic activity of BT-A in ipsilateral L4 dorsal horn of the spinal cord is present after the desensitization of sciatic capsaicin-sensitive neurons with high concentration of capsaicin (2%). Fluorescent images of clSNAP-25 12 days after ipsilateral i.pl. injection of BT-A (5 U/kg) and 7 days following ipsilateral perisciatic 2% capsaicin. 2% capsaicin treatment of sciatic nerve 5 days after administration of peripheral BT-A (5 U/kg) did not affect the occurrence of clSNAP-25. Red immunostaining represents clSNAP-25 (arrows) and typically is found in lamina III. N (animals per group) = 4 (8 sections were examined per each animal). Scale bar = 20 μm.

Figure 5