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Abstract

Botulinum toxin A (BTX-A) is approved for treatment of chronic migraine and has been investigated in various other painful conditions. Recent evidence demonstrated retrograde axonal transport and suggested the involvement of CNS in antinociceptive effect of BTX-A. However, the mechanism of BTX-A central antinociceptive action is unknown. In this study we investigated the potential role of opioid receptors in BTX-A's antinociceptive activity.

In formalin-induced inflammatory pain we assessed the effect of opioid antagonists on antinociceptive activity of BTX-A. Naltrexone was injected subcutaneously (0.02 - 2 mg/kg) or intrathecally (0.07 ng/ 10µl – 350 µg/10 µl), while selective µ-antagonist naloxonazine was administered intraperitoneally (5 mg/kg) prior to nociceptive testing. The influence of naltrexone (2 mg/kg s.c.) on BTX-A antinociceptive activity was examined additionally in an experimental neuropathy induced by partial sciatic nerve transection. To investigate the effects of naltrexone and BTX-A on neuronal activation in spinal cord, c-Fos expression was immunohistochemically examined in a model of formalin-induced pain.

Antinociceptive effects of BTX-A in formalin and sciatic nerve transection-induced pain were prevented by non-selective opioid antagonist naltrexone. Similarly, BTX-A-induced pain reduction was abolished by low dose of intrathecal naltrexone and by selective µ-antagonist naloxonazine. BTX-A-induced decrease in dorsal horn c-Fos expression was prevented by naltrexone.

Prevention of BTX-A effects on pain and c-Fos expression by opioid antagonists suggest that the central antinociceptive action of BTX-A might be associated with the activity of endogenous opioid system (involving µ-opioid receptor). These results provide first insights into the mechanism of BTX-A's central antinociceptive activity.

Keywords: botulinum toxin A, opioid antagonist, μ-opioid receptors, formalin, peripheral neuropathy, c-Fos

Highlights:

- Opioid antagonists prevent the antinociceptive activity of peripheral BTX-A
- This effect occurs on spinal cord level
- BTX-A-induced pain reduction is mediated by μ-opioid receptor
- The effect is present in different types of pain
- Behavioral effect is accompanied by changes in dorsal horn c-Fos activation

Abbreviations: BTX-A – botulinum toxin type A, SNAP-25 - Synaptosomal Associated Protein of 25kDa, i.pl. – intraplantarly, s.c. – subcutaneously, i.t. – intrathecally, i.p. – intraperitoneally, PBS – phosphate-buffered saline, PBST - Triton X-100 in phosphate-buffered saline, NGS - normal goat serum

1. Introduction

Botulinum toxin type A (BTX-A), an endopeptidase derived from *Clostridium botulinum*, cleaves SNAP-25 (Synaptosomal Associated Protein of 25kDa), one of the proteins essential for neuroexocytosis (Blasi et al., 1993). Prevention of acetylcholine release in neuromuscular junction and autonomous synapses is the main feature of BTX-A poisoning. The same mechanism enables local application of BTX-A in low picomolar doses to be used in treatment of neuromuscular and autonomous disorders (Lim and Seet, 2010).

Apart from its effect on neuromuscular junction, recent preclinical and clinical studies reported the efficacy of BTX-A in reduction of allodynia and hyperalgesia in pain of different origins (Jabbari and Machado 2011; Pavone and Luvisetto, 2010). Moreover, BTX-A was recently registered for treatment of chronic migraine (Dodick et al., 2010) and several controlled clinical studies in other painful conditions are in progress (Jabbari and Machado, 2011; Singh, 2010). Importance of BTX-A application in clinical practice results from its unique ability to reduce pain in a long lasting manner (up to 6 months in humans).

It was suggested that antinociceptive effect of BTX-A results from inhibition of neurotransmitter release from peripheral sensory nerve endings (Aoki, 2005; Cui et al., 2004), similarly as in neuromuscular junction. On the other hand, recent behavioral (Bach-Rojecky and Lackovic, 2009; Bach-Rojecky et al., 2010; Favre-Guilmard et al., 2009; Filipović et al., 2012) and immunohistochemical studies (Matak et al., 2012, 2011) indicate that the antinociceptive action occurs primarily in the central nervous system where BTX-A is axonally transported (Antonucci et al., 2008; Matak et al., 2012, 2011). However, there are no *in vivo* data regarding central molecular mechanism, receptors and possible neurotransmitters involved. Here we report that the antinociceptive action of BTX-A is associated with central μ-opioid receptor activity.

2. Materials and methods

2.1 Animals

Male Wistar rats (University of Zagreb School of Medicine, Croatia) weighing 300-400 g, kept in temperature-regulated environment (23℃) under 12 h light-dark cycle, with free access to food and water (except during testing), were used in all experiments. Experiments were conducted according to the European Communities Council Directive (86/609/EEC) and recommendations of the International Association for the Study of Pain (Zimmerman, 1983). All efforts were made to reduce the number of animals used and to reduce their suffering. Experiments were approved by the Ethical Committee of the University of Zagreb, School of Medicine (permit No. 07-76/2005-43).

2.2 Drugs

The following drugs and chemicals were used: BTX-A (Botox®, Allergan, Inc., Irvine, USA); non-selective opioid antagonist naltrexone (Sigma, St.Louis, MO, USA); selective μ-opioid antagonist naloxonazine (Santa Cruz Biotechnology, Inc., CA, USA); chloral-hydrate (Sigma, St.Louis, MO, USA); diethyl ether (Sigma, St.Louis, MO, USA); acetone (Sigma, St.Louis, MO, USA). To obtain the doses needed, BTX-A and opioid antagonists were dissolved in 0.9% saline. Each vial of Botox® contains 100U (~4,8ng) of purified *Clostridium botulinum* type A neurotoxin complex.

For immunohistochemical experiment the following chemicals are used: paraformaldehyde (Sigma-Aldrich, St.Louis, MO, USA), Triton X-100 (Sigma-Aldrich, St.Louis, MO, USA), normal goat serum (Vector, Inc., Burlingame, CA, USA), c-Fos rabbit polyclonal antibody (Santa Cruz Biotechnology, Inc., CA, USA), goat anti-rabbit Alexa Fluor-448 (Invitrogen,

Carlsbad, CA, USA), anti-fading agent (Fluorogel, Electron Microscopy Sciences, Hatfield, PA, USA).

2.3 Animal treatment

BTX-A was injected subcutaneously into the plantar surface of the hind paw (intraplantarly, i.pl.), ipsilateral to formalin injection/ nerve injury, to conscious, gently restrained rats, in a volume of 20 µl with a 27 ½ gauge needle. To test the effect on formalin-induced pain, BTX-A was injected in a dose of 5 U/kg, while 7 U/kg BTX-A was used in the model of neuropathic pain. Doses were chosen based on previous experiments from our laboratory (Bach-Rojecky et al., 2005; Bach-Rojecky and Lacković, 2005).

Naltrexone was injected: 1. subcutaneously (s.c., 0.02 mg/kg - 2 mg/kg) into the abdominal area in a volume of 250 µl; and 2. intrathecally (i.t., 0.07 µg/10 µl – 350 µg/10 µl) at the lumbar L3-L4 level . Dose of s.c. injected naltrexone was chosen based on literature (Correa et al., 2010), while dose of i.t. naltrexone (100-fold lower than s.c. dose) was based on preliminary experiment. 1 ml of naloxonazine was injected intraperitoneally (i.p.) in a dose of 5 mg/kg, as used by other authors (de Freitas et al., 2011).

2.4 Nociceptive assessment

Nociceptive experiments were performed in a quiet laboratory, between 10 a.m. and 4 p.m. Animals were allowed to accommodate to the testing environment for 10 min. Evaluation of nociceptive testing was performed by observer unaware of the animal treatment. Animal treatment was known to other experimenter who treated and marked the animals. However, the main observer could recognize the injured hind-limb either in formalin-induced pain (paw edema) or nerve injury (limping and characteristic posture of injured paw).

Formalin test. Conscious, gently restrained rats were s.c. injected with saline-diluted 5% formalin solution (50 µl) into the plantar side of the right hind paw pad and immediately returned to the transparent cage for 1 h observation period. Pain was measured as the number of nocifensive behaviors (licking, flinching and shaking of the injected paw). Recording time was divided in two phases: acute phase I (0-15 min) response caused by direct stimulation of peripheral sensory nerve endings with formalin, and inflammatory phase II (15-60 min) characterized by peripheral sensitization (Tjolsen et al., 1992). Each experimental group contained 5-6 animals.

BTX-A (5 U/kg i.pl.) was injected 5 days before the formalin testing, while naltrexone (0.02 mg/kg - 2 mg/kg s.c.; 0.07 µg/ µl - 350 µg/ µl i.t.) and naloxonazine (5 mg/kg i.p.) were injected 40 min and 24 h prior to the formalin test, respectively. Control animals received 0.9% saline in the appropriate volumes. For intrathecal application of naltrexone, animals were briefly anesthetized with diethyl ether until no reflexive response to paw pinch was elicited. Animal's hair was shaved at the lumbar L3-L4 level. Small skin incision (1 cm) was performed. Naltrexone or saline were injected between the vertebrae. Animals recovered from diethyl ether anesthesia in approx. 10 min. Shortly acting diethyl ether was used to achieve fast recovery prior to nociceptive testing (40 min following i.t. injection).

To investigate whether the effect of naltrexone on BTX-A- induced antinociception is short-lasting or long-lasting, we performed an additional experiment where naltrexone was administered 24 h prior to formalin testing. 24 h point was chosen based on the time required for complete elimination of naltrexone from the organism (Verebey et al., 1976.).

Peripheral neuropathy. A total number of 38 rats underwent sciatic nerve partial transection, as previously described (Bach-Rojecky et al., 2005; Lindenlaub and Sommer, 2000). In brief, right sciatic nerve was exposed in rats under general anesthesia (chloral hydrate 300 mg/kg)

and the middle of the nerve trunk was pierced using a thin surgical needle. Half of the nerve diameter was transected by the scalpel in the needle direction. Six rats were subjected to sham procedure; sciatic nerve was exposed, but not transected. Six naive rats served as control.

Two weeks following the peripheral nerve injury, animals which developed mechanical sensitivity to pressure/ von Frey filaments and cold allodynia (at least 20% changes from the mean of the sham-operated group) were included into the further experiment. Animals were divided in four groups (5-6 animals per group) as follows: (1) 0.9% saline (i.pl.), (2) BTX-A (7 U/kg; i.pl.), (3) naltrexone (2 mg/kg, s.c.), (4) BTX-A + naltrexone.

Nociceptive measurements were performed 5 days following BTX-A i.pl. injection, and 40 min following naltrexone s.c. injection. The assessment of each animal started with mechanical sensitivity to pressure, followed by mechanical sensitivity to von Frey filaments and cold allodynia measurements, with 30 min period between each type of measurement.

2.4.2.1. Mechanical sensitivity to paw pressure

Mechanical sensitivity was measured by the modified paw pressure test, originally described by Randall and Selitto (1957), on both hind paws. Average mechanical nociceptive threshold expressed in grams was calculated from 3 measurements. Measurements were repeated in 10 min intervals by applying increased pressure to the dorsal surface until paw withdrawal or struggling of the animal occurred (Bach-Rojecky et al., 2010).

2.4.2.2. Mechanical sensitivity to von Frey filaments

Paw withdrawal threshold in response to a mechanical stimulus was determined using a series of von Frey filaments (Stoelting Co, Wood Dale, IL, USA) ranging from 0.6 g to 26 g. Animals were placed in a plastic cage with a metal mesh floor 10 min prior to testing. Von Frey filaments were applied to the mid-plantar surface of the hind paw through the mesh floor.

Each filament was applied 3 times, kept in bent position on the rat's hind paw for 4 s. Filaments were applied in ascending order, and the lowest filament that elicited a foot withdrawal response was considered the threshold stimulus (Wei et al., 1998).

2.4.2.3. Cold allodynia

Cold allodynia was measured as the number of foot withdrawal responses after an application of cold stimuli (a drop of 100% acetone) to the plantar surface of the hind paw. Testing was repeated five times with an interval of approximately 5 min between each test. Response frequency to acetone was expressed as a percent withdrawal frequency [(number of paw withdrawals/number of trials) × 100] (Park et al., 2006).

2.5 Immunohistochemistry

Immunohistochemical analysis was performed on samples collected from the formalin test experiment with s.c. applied naltrexone.

Two hours following the i.pl. formalin injection rats were deeply anesthetized using chloral-hydrate (300 mg/kg) and transcardially perfused with 250 ml of 0,9% saline, followed by 250 ml of fixative (4% paraformaldehyde in 0,01M phosphate-buffered saline (PBS), pH 7.4). Spinal cord was removed and cryoprotected at 4^{o}C overnight in 15% sucrose-fixative solution, followed by 30% sucrose in PBS the next day, until the tissue sank. Lumbar spinal cord (L4/L5 segment) sections of 4 rats belonging to each experimental group were processed for immunohistochemical analysis. Frozen sections (30 µm), cut on cryostat (Leica, Germany), were taken for free floating in wells with PBS. Sections were washed 3x5min in 0,25% PBST (PBS + 0.25% TritonX-100), blocked in 10% normal goat serum (NGS) for 1 h and incubated overnight at room temperature with rabbit anti-c-Fos polyclonal antibody diluted in 1% NGS. Sections were washed in PBST and incubated for 2h at room temperature

with 1:400 goat anti-rabbit Alexa Fluor-448 fluorescent secondary antibody in the dark, diluted in 1% NGS. Sections were washed and mounted on glass slides with anti-fading agent. Sections were visualized with fluorescent microscope (Olympus BX51, Olympus, Tokyo, Japan) connected to digital camera (Olympus DP-70, Olympus, Tokyo, Japan) and photographed using 10x and 40x magnification. C-Fos-positive neurons were counted in sensory laminae of the spinal cord dorsal horn (I to VI) by experimenter unaware of the treatment groups. Average number of c-Fos-positive neurons for each animal was calculated from three randomly selected sections. Figures were assembled using Microsoft Paint and processed for brightness and contrast using Adobe Photoshop.

2.6 Statistical analysis

Results, presented as mean ± SEM, were analyzed by one-way analysis of variance (ANOVA) followed by the Newman-Keuls post hoc test. $P<0.05$ was considered significant.

3. Results

3.1 Opioid antagonists abolish the antinociceptive effect of BTX-A during phase II of the formalin test

3.1.1. Systemically applied naltrexone inhibits the antinociceptive effect of BTX-A during phase II of the formalin test

Peripheral BTX-A pre-treatment (5 U/kg, i.pl.) significantly reduced the number of licking/flinching during second phase of formalin-induced pain ($p<0.001$). Naltrexone (2 mg/kg s.c.), applied 40 min prior to formalin test, abolished the antinociceptive effect of

BTX-A ($p<0.001$, Fig. 1A). Additionally, formalin test was performed 24 h after naltrexone (2 mg/kg) s.c. injection to investigate whether naltrexone interferes with antinociceptive effect of BTX-A only shortly, until eliminated from the organism, or it has long-lasting reducing effect on BTX-A antinociceptive action. Naltrexone had no effect on BTX-A induced antinociception in this experiment (Fig. 1A). Naltrexone alone did not influence formalin-induced pain (Fig. 1A).

3.1.2 Centrally applied low dose naltrexone reduces antinociceptive effect of peripheral BTX-A

To examine the possible central site of naltrexone action on BTX-A induced antinociception, low dose-naltrexone (7μg/10 μl) was injected intrathecally. Similarly to 100-fold higher systemic dose (2mg/kg), it abolished the antinociceptive effect of peripherally applied BTX-A ($p<0.01$; Fig. 1B). However, the same low dose of naltrexone applied s.c. (7μg/ 10 μl) did not influence BTX-A antinociceptive effect (Fig. 1B).

3.1.3 Antinociceptive action of BTX-A is dependent on μ-opioid receptor

Single i.p. injection of selective μ-opioid antagonist naloxonazine (5 mg/kg) applied 24 h before the test abolished the antinociceptive effect of BTX-A in phase II of formalin test ($p<0.01$; Fig. 1C). Naloxonazine alone did not alter formalin-evoked licking and flinching of the injected paw (Fig. 1C).

3.2 Systemic and central naltrexone dose-dependently prevents the antinociceptive effect of BTX-A

Naltrexone dose-dependently decreased the antinociceptive effect of BTX-A (5 U/kg i.pl.) in the second phase of the formalin test (Fig. 2). The highest tested doses of naltrexone (2 mg/kg

s.c. or 350 µg/10 µl i.t.) had maximal effect on prevention of BTX-A antinociceptive effect ($p<0.001$). A decrease in preventing the antinociceptive effect of BTX-A was found with lowering naltrexone dose (Fig. 2).

The highest tested doses of naltrexone alone (10 mg/kg s.c., and 350 µg/10 µl i.t.) had no significant influence on formalin-induced pain (number of nocifensive behaviors: 454.6 ± 20.7 vs. saline s.c.: 489.8 ± 15.2 and 485.8 ± 16.4 vs. saline i.t.: 494 ± 22.7). Accordingly, lower doses of naltrexone alone (2 mg/kg s.c.; 0.7 µg/10 µl and 0.07 µg/10 µl i.t.) had no significant influence on formalin-induced pain, as well (results not shown).

3.3 Opioid antagonist reduces the antinociceptive effect of BTX-A in experimental neuropathic pain

Two weeks following the partial sciatic nerve transection 60% of rats developed mechanical sensitivity and cold allodynia ipsilateral to the nerve injury. A single i.pl. BTX-A (7 U/kg) injection significantly decreased mechanical sensitivity to pressure and von Frey filaments, as well as cold allodynia (Fig. 3).

Naltrexone (2 mg/kg, s.c.) applied 5 days after BTX-A injection reversed the antinociceptive effect of BTX-A on mechanical sensitivity to pressure (Fig. 3A), and reduced its effects on mechanical sensitivity to von Frey filaments and cold allodynia (Fig. 3B and C). All measurements were conducted starting from 40 min after naltrexone injection. Naltrexone alone did not alter the pain in all nociceptive tests.

3.4 Naltrexone prevents the effect of BTX-A on c- Fos expression in dorsal horn of the spinal cord

Behavioral changes in previously described experiment were accompanied by changes of c-Fos protein expression (Fig. 4) in the dorsal horn in a similar fashion. The number of c-Fos

positive neurons was significantly reduced ($p<0.05$, Fig. 4B) in BTX-A treated animals compared to control (saline + formalin) group. Naltrexone prevented the effect of BTX-A on c-Fos expression in formalin test ($p<0.05$, Fig. 4B).

4. Discussion

According to textbook description, BTX-A does not enter CNS and its therapeutic, as well as toxic effects, are associated with peripheral nerve endings only. However, the antinociceptive effect of BTX-A cannot be explained without assumption that it is centrally mediated. This is demonstrated by numerous behavioral evidence such as: bilateral effect following unilateral toxin injection in different bilateral or polyneuropathic pain models, prevention of antinociceptive effect by axonal transport blocker colchicine, effect on contralateral side after injection to distally cut sciatic nerve (Bach-Rojecky and Lacković, 2005, 2009; Bach-Rojecky et al., 2010; Favre-Guilmard et al., 2009; Filipovic et al., 2012; Matak et al., 2011). Moreover, using antibody specific for BTX-A-cleaved SNAP-25, Caleo and co-workers discovered axonal and transynaptic transport of BTX-A inside the brain, and from periphery to the facial motor nucleus (Antonucci et al., 2008; Restani et al., 2011), while our group discovered the axonal transport of BTX-A within sensory neurons projecting from periphery to the brainstem (trigeminal sensory nuclei) or spinal cord (corresponding segment of dorsal horn). In our experiments, axonal transport blocker colchicine prevented the antinociceptive activity of BTX-A, as well as central SNAP-25 cleavage (Matak et al., 2011, 2012), suggesting that toxin's retrograde axonal transport to CNS is necessary for the antinociceptive activity.

Up to now, the mechanism of BTX-A antinociceptive action in the CNS is unknown. Since SNAP-25 is believed to be the main target of BTX-A, it could be assumed that, in sensory areas of CNS, BTX-A directly or indirectly affects central neurotransmitters involved in pain processing. But, up to now, there are no studies specifically examining this issue *in vivo*. Few

previous observations (Auguet et al., 2008; Vacca et al. 2012.) discovered synergistic effect of BTX-A and morphine, thus, indirectly suggested the association between opioid system and BTX-A. Therefore, we investigated if the central antinociceptive activity of BTX-A is associated with the endogenous opioid system, since opioids play a pivotal role in control of nociception and they are one of the most studied innate pain-relieving systems (Holden et al., 2005).

We tested the effects of selective and non-selective opioid antagonists on antinociceptive action of BTX-A in two different types of pain: formalin-induced inflammatory pain and partial sciatic nerve transection-induced neuropathic pain.

In the present study a non-selective opioid receptor antagonist naltrexone (2 mg/kg s.c.), as well as selective μ-opioid antagonist naloxonazine (5 mg/kg i.p.), prevented the antinociceptive effect of peripheral BTX-A on second inflammatory phase of the formalin test (Fig 1). To exclude the possibility that opioid antagonists increase pain by a mechanism independent from the BTX-A effect, dose-response experiment was preformed (Fig. 2). Moreover, lower doses of naltrexone alone tested, as well as high doses, had no effect on formalin-induced pain (results not shown), while the same doses injected to BTX-A pretreated animals affected differently BTX-A induced antinociception (Fig. 2). This result indicates the possible involvement of μ-opioid receptor in antinociceptive effect of BTX-A. Although selective μ-opioid antagonist reversed BTX-A effect similarly as non-selective opioid antagonist (Fig. 1A and C), the involvement of other than μ-opioid receptor cannot be completely excluded, since experiments with selective κ- and δ-opioid antagonists were not preformed.

Since previous studies (Bach-Rojecky and Lacković, 2009; Filipovic et al., 2012; Matak et al., 2011) indicated that BTX-A antinociceptive action is of central origin, we hypothesized that the site of BTX-A interaction with opioid receptors in our experiments occurs in the spinal

cord. Therefore, to eliminate possible systemic effects of naltrexone, the low, systemically ineffective dose of naltrexone (7 μg/10 μl) was administered intrathecally. Centrally applied naltrexone abolished the antinociceptive activity of peripherally applied BTX-A (Fig. 1B), which confirms the central site of BTX-A action.

To examine whether the association of BTX-A action with opioid receptor is common in different types of pain, we additionally investigated the effect of naltrexone and BTX-A in a model of neuropathic pain induced by partial sciatic nerve transection. Naltrexone (2 mg/kg, s.c.) significantly reduced the antinociceptive effect of BTX-A on mechanical sensitivity to pressure and von Frey filaments, as well as cold allodynia (Fig. 2) in neuropathic animals. This further demonstrates that the mechanism of BTX-A activity in acute inflammatory pain and chronic neuropathic pain, and possibly in other types of pain, is connected with opioid receptor.

Further support for involvement of μ-opioid receptors in antinociceptive effect of BTX-A was demonstrated by examining neuronal activation in spinal cord dorsal horn (Fig. 4). Using c-Fos expression as a measure of neuronal activation in pain pathways (Coggeshall et al., 2005) after formalin-induced pain, we showed that BTX-A (5 U/kg i.pl.) significantly reduced the number of c-Fos positive neurons in sensory laminae of spinal cord (I-VI) (Fig. 4 A and B). The effect of BTX-A on c-Fos expression in the spinal cord was shown previously in the formalin test (Aoki, 2005), as well as in chronic bladder inflammation model (Vemulakonda et al., 2005). However, in our experiments, effect of BTX-A on nociceptive neuronal activation was prevented by naltrexone (Fig. 4A and B), which parallels the behavioral effect of naltrexone on BTX-A-induced antinociception.

Our results, which suggest the interaction of BTX-A with opioid system, are in accordance with previous studies which examined antinociception of combined application of BTX-A and morphine in low, non-active doses. Auguet et al. (2008) observed significant antinociceptive

effect of ineffective dose of morphine (0.03 mg/kg i.p.) applied 3 days after ineffective dose of BTX-A (Dysport) (10 U/kg) in carrageenan-induced inflammatory pain in rats. More recently, significant antinociceptive effect was shown in another study (Vacca et al., 2012) also combining ineffective doses of BTX-A (2 pg/paw) and morphine (1 mg/kg s.c.) in formalin-induced inflammatory pain in mice.

Major finding of present study points to association of BTX-A antinociceptive activity with μ-opioid receptors. The nature of this association remains to be elucidated. The possibility that BTX-A has direct agonistic effect on μ-opioid receptors seems unlikely, primarily since the analgesic properties and mechanism of actions of BTX-A and opioid agonists are different (the only known mechanism of BTX-A action is the prevention of neuroexocytosis by SNAP-25 cleavage). Most likely explanation for the prevention of BTX-A analgesic effects by opioid antagonists might be that BTX-A, during pain, indirectly increases the antinociceptive activity of endogenous opioid system. This could be achieved either by (1) enhanced synthesis/release of opioid peptides or (2) enhanced opioid receptor function.

(1) Modulation of pain by endogenous opioid peptides as a consequence of their enhanced synthesis/release has been observed in some conditions like stress (Parikh et al., 2011), placebo administration (Eippert et al., 2009; Zubieta et al., 2005), infections (Glattard et al., 2010) and exercise (Goldfarb and Jamurtas, 1997). Possibility that BTX-A enhances spinal opioid peptide synthesis may be supported by studies which reported that intramuscular injection of BTX-A induces the enkephalin mRNA synthesis in the spinal cord ventral horn (Humm et al., 2000; Jung et al., 1997). These studies examined ventral horn enkephalin mRNA levels, while dorsal horn was not investigated. Effect was visible also in the contralateral side of spinal cord, and at more rostral and caudal spinal cord levels (Humm et al., 2000; Jung et al., 1997).

Recently, Mika et al. (2011) reported that BTX-A lowers the expression of pronociceptin, prodynorphin and proenkephalin mRNA within the dorsal root ganglia, but not in the spinal cord of neuropathic animals. The authors examined the synthesis of endogenous opioids involved in development of neuropathic pain, whose effects can also be mediated by non-neuronal inflammatory cells. Down-regulation of opioid synthesis only at the level of dorsal root ganglia is difficult to associate with present observations, where BTX-A-mediated increase in spinal cord endogenous opioid expression might be expected. There is also a possibility that BTX-A effects in our study are mediated by some other endogenous opioids which were not examined in study of Mika et al. (2011).

Since the only known mechanism of BTX-A action is the prevention of SNARE-mediated neurotransmitter release, to explain how the release of opioids might be enhanced, we speculate that BTX-A indirectly increases the opioid system activation, via yet unknown mechanism. This indirect action may involve several neurons or neurotransmitters. Additionally, participation of non-neuronal cells, such as glia, cannot be excluded (Mika et al., 2011; Vacca et al., 2012).

If, hypothetically, the antinociceptive effect of BTX-A is associated with increased release of opioid polypeptides, it might be expected that the effect of BTX-A and opioid analgesics, such as morphine, are similar. However, preclinical data suggested that the actions of BTX-A and morphine are quite different. While morphine reduces both phases of the formalin test (Hunskaar and Hole, 1987), BTX-A acts only in the second, inflammatory phase (Cui et al, 2004; Fig 1). Additionally, BTX-A, does not increase normal nociceptive thresholds (Bach-Rojecky et al., 2005; Cui et. al, 2004) unlike morphine. Reasons for this are not clear, but may be associated with selectivity of the BTX-A effect, in contrast to morphine, for more limited subsets of neurons or synapses targeted by BTX-A.

(2) Another theoretical option is that BTX-A enhances the expression or function of μ-opioid receptors. At present there is no direct evidence for that option, but it might be supported by the recent observation that BTX-A antagonizes the morphine-induced tolerance. Morphine-induced tolerance is suggested to be associated with decreased μ-opioid receptor functional binding sites (Vacca et al., 2012).

Reversal of antinociceptive activity of BTX-A by shortly acting opioid antagonists is difficult to reconciliate with the known mechanism of BTX-A long term action on SNARE complexes and neurotransmitter release. Cleaved SNAP-25 in our experiment is already present in CNS 5 days following the peripheral administration, prior to antagonist administration. However, the possibility that naltrexone, by unknown mechanism, permanently interferes with BTX-A effect on SNAP-25 could not be ruled out. Therefore, we examined if the effect of naltrexone is short-lasting. In contrast to its effect 40 min prior to formalin test, naltrexone administered 24 h prior to nociceptive testing did not influence BTX-A-induced antinociception. This experiment demonstrated that the effect of naltrexone is short-lasting (in line with its pharmacokinetic properties), and that it does not directly prevent the BTX-A activity by reversal of SNAP-25 cleavage.

In summary, SNAP-25 cleavage in CNS is probably only the first step in the overall mechanism of BTX-A action, which leads to activation of endogenous opioid system.

5. Conclusion

Present results demonstrate for the first time that the BTX-A activity in acute inflammatory pain and chronic neuropathic pain, and possibly in other types of pain, is associated with μ-opioid receptor.

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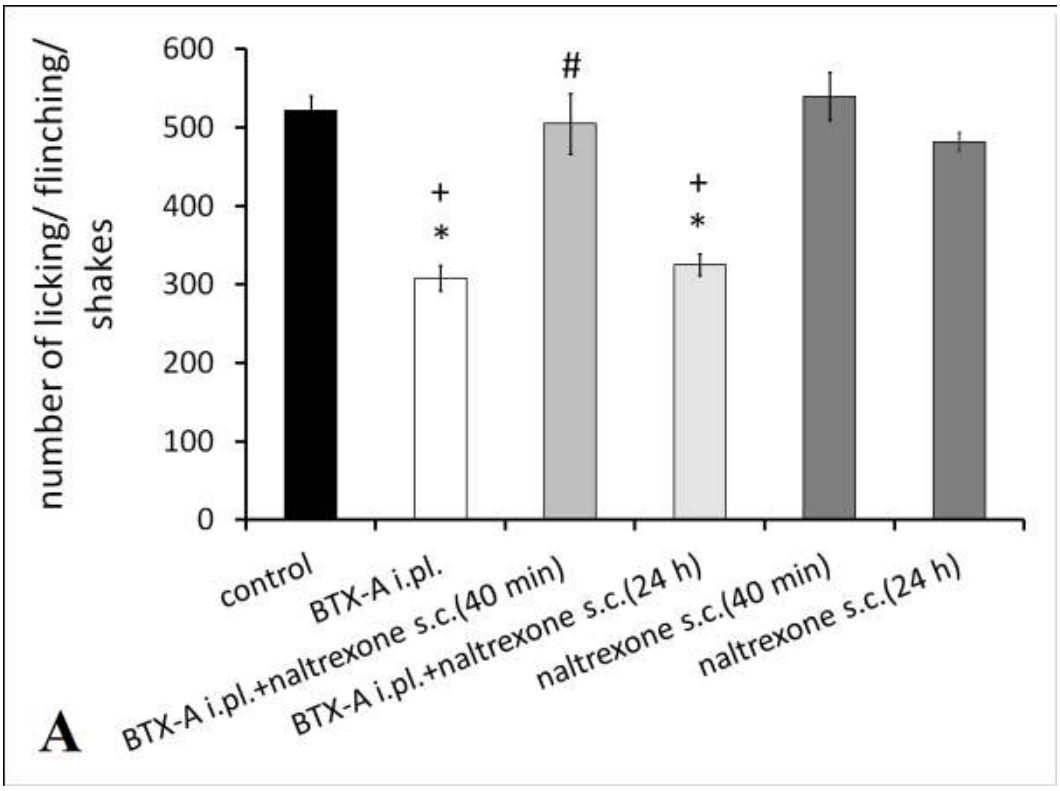
Legends

Fig. 1 Effects of opioid antagonists, naltrexone and naloxonazine, on antinociceptive effect of BTX-A in the second phase of the formalin test. Measurements were performed 5 days after BTX-A (5 U/kg) i.pl. injection and: A) 40 min or 24 h after naltrexone (2 mg/kg) s.c. injection; B) 40 min after naltrexone (7 μg/ 10 μl i.t.) i.t. injection (control group intrathecally injected with saline); C) 24 h after naloxonazine (5 mg/kg) i.p. injection. Animals were treated with naltrexone or naloxonazine 5 days after BTX-A (5 U/kg) i.pl injection. A) Naltrexone administered 40 min prior to formalin test blocks antinociceptive effect of BTX-A, while naltrexone administered 24 h prior to formalin test has no effect on antinociceptive action of BTX-A. Mean ± SEM, n=5-6, * - $p<0.001$ compared to control, + - $p<0.001$ compared to naltrexone (40 min) and naltrexone (24 h), # - $p<0.01$ compared to BTX; B) Low dose of intrathecal naltrexone prevents BTX-A antinociceptive action in formalin-induced pain. Mean ± SEM, n=5, * - $p<0.01$ compared to control; + - $p<0.001$ compared to naltrexone; # - $p<0.01$ compared to BTX;

C) Selective μ-opioid antagonist naloxonazine reverses the antinociceptive action of BTX-A. Mean ± SEM, n=6; * - $p< 0.005$ compared to control; + - $p< 0.01$ compared to naloxonazine; # - $p< 0.01$ compared to BTX-A (Newman-Keuls post hoc)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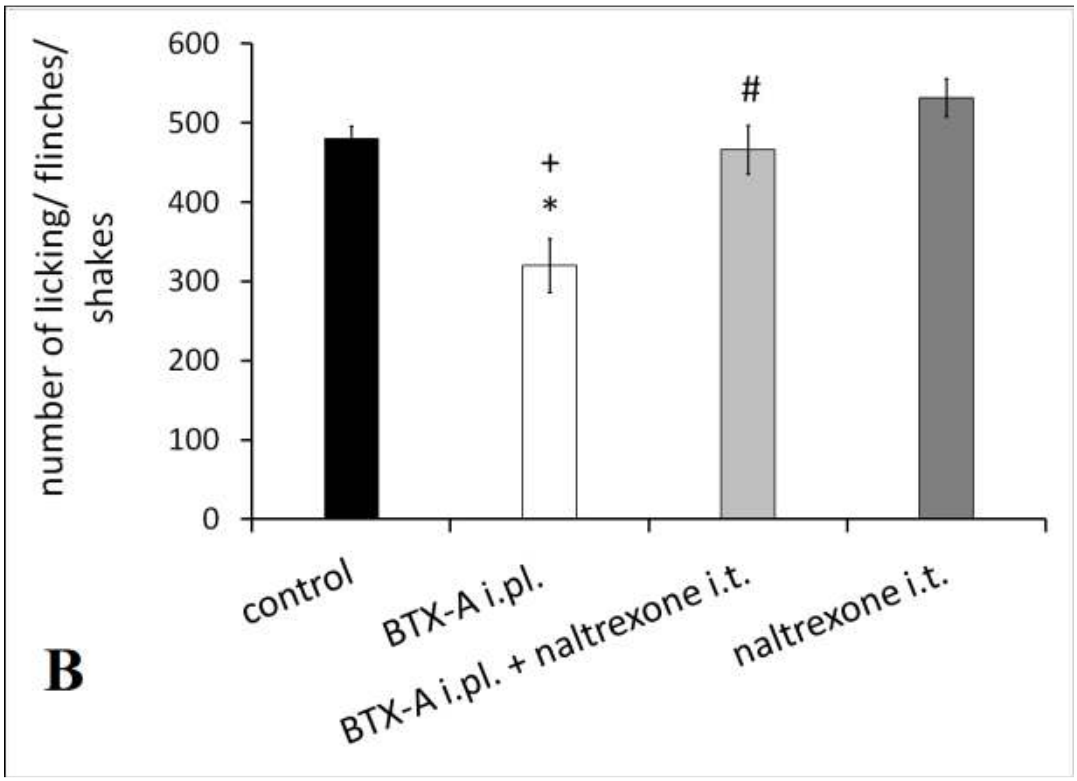
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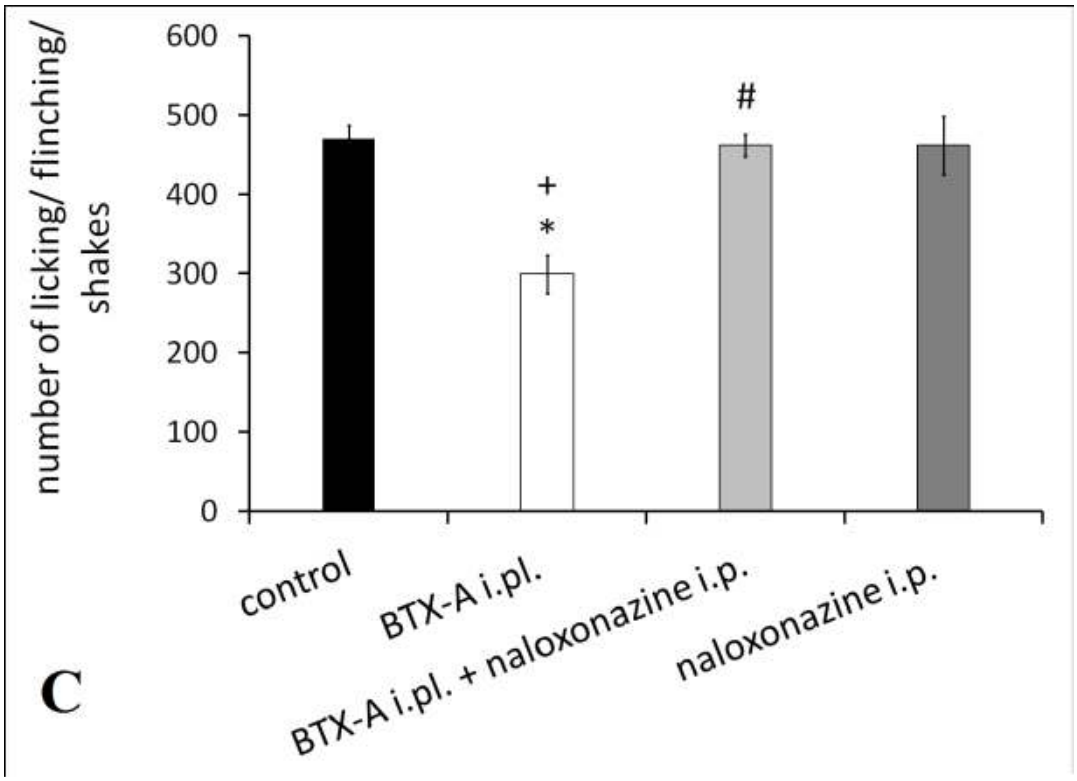
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naloxonazine i.p.
C

Fig. 2 Naltrexone dose-response: the effect of different doses of naltrexone injected systemically or intrathecally on antinociceptive effect of peripheral BTX-A in the second phase of the formalin test. Measurements were performed 5 days after BTX-A (5 U/kg) i.pl. injection and 40 min after naltrexone 2 mg/kg, 1 mg/kg, 0.2 mg/kg and 0.02 mg/kg s.c. injection or 350 µg/10 µl, 7 µg/10 µl, 0.7 µg/10 µl and 0.07 µg/ 10 µl i.t. injection (in graph, all doses are shown as (µg/kg)). Zero value represents BTX-A alone. Note the log-scale on x-axis. Mean ± SEM, n=5; ○ Naltrexone subcutaneous: ** - $p<0.01$ compared to control, *** - $p<0.001$ compared to control, ++ - $p<0.01$ compared to BTX-A, +++ -$p<0.001$ compared to BTX-A ; ● Naltrexone intrathecal: * - $p<0.05$ compared to control, ** -$p<0.01$ compared to control, *** -$p<0.001$ compared to control; ++ -$p<0.01$ compared to BTX-A, +++ -$p< 0.001$ compared to BTX-A (Newman-Keuls post hoc)

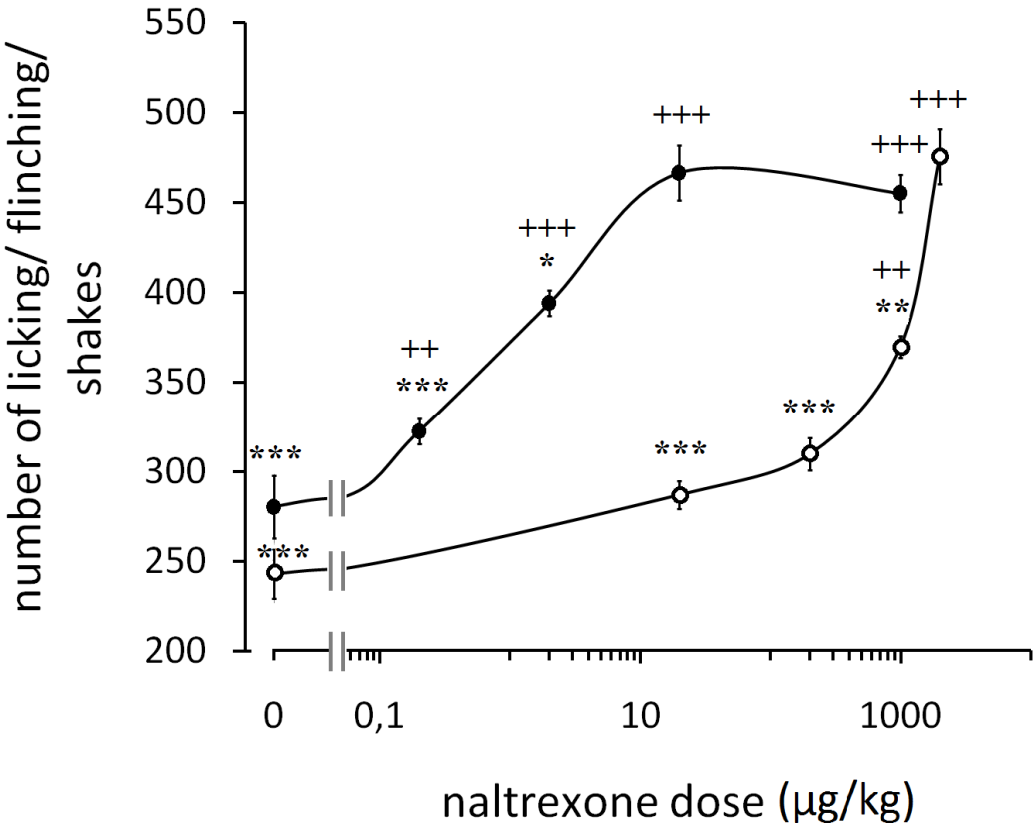
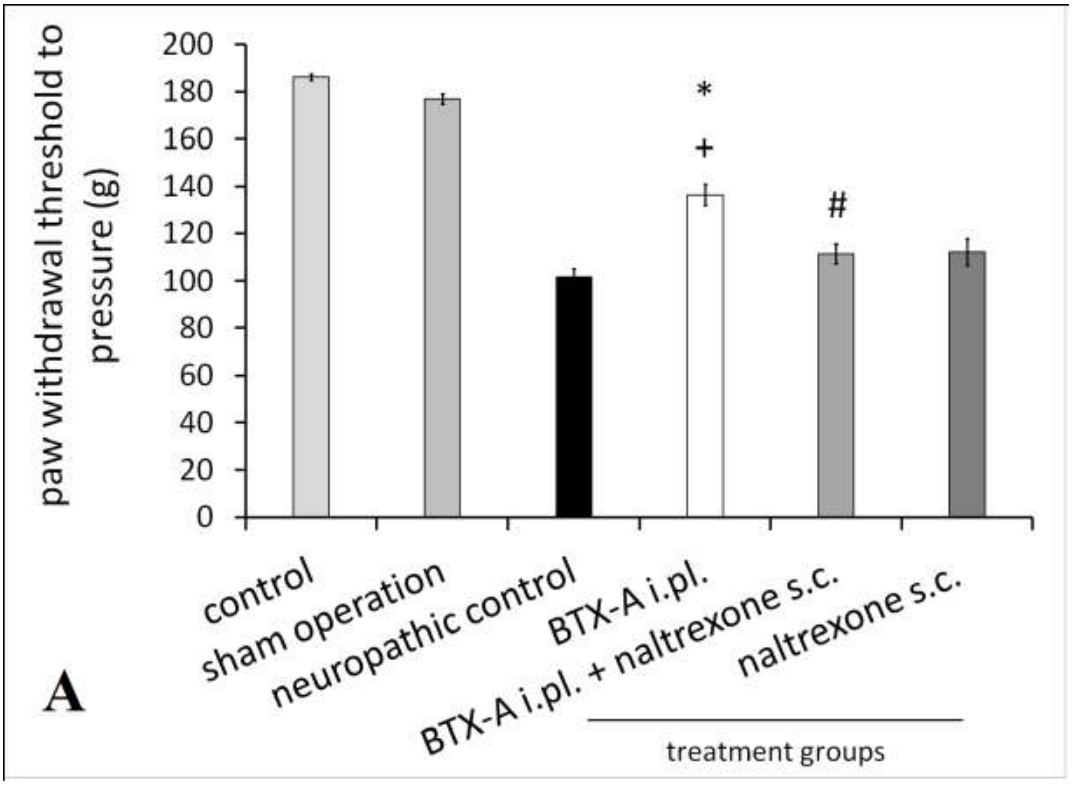
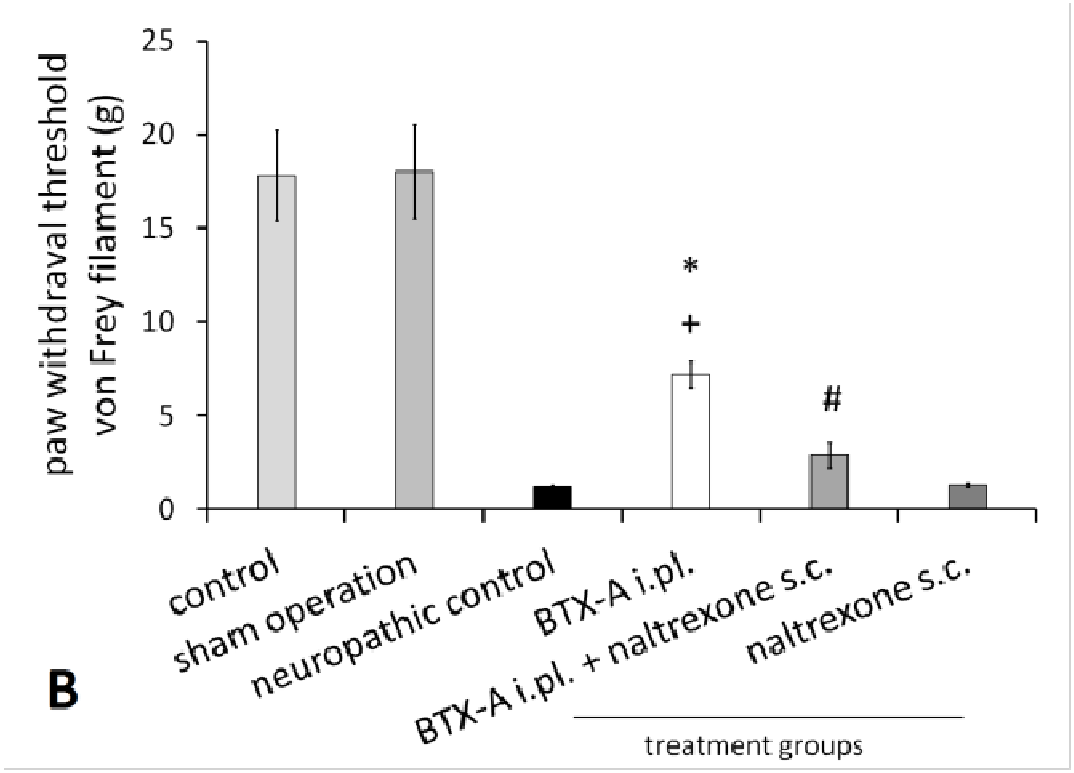


Fig. 3 Systemic application of naltrexone reduces the antinociceptive effect of BTX-A in the experimental neuropathic model induced by partial sciatic nerve transection. Nociceptive tests were performed 5 days following BTX-A (7 U/kg, i.pl.) and 40 min following naltrexone (2 mg/kg, s.c.) application in animals with developed neuropathy. Neuropathy developed ipsilaterally (at the site of the transected nerve). A) Mechanical sensitivity measured with paw pressure test; results expressed in grams. Mean± SEM, n=5-6; * - p< 0.0001 compared to neuropathic control; + - p<0.005 compared to naltrexone; # - p< 0.001 compared to BTX-A (Newman-Keuls post hoc). B) Mechanical sensitivity measured with von Frey filaments; results expressed in grams. Mean ± SEM, n=5-6; * - p< 0.05 compared to neuropathic control; + - p<0.05 compared to naltrexone; # - p<0.05 compared to BTX-A (Newman-Keuls post hoc). C) Cold allodynia; results expressed as percentage of paw withdrawals caused by drop of 100% acetone. Mean ± SEM, n=5-6; * - p<0.0005 compared to neuropathic control and naltrexone; # - p<0.0005 compared to BTX-A (Newman-Keuls post hoc)

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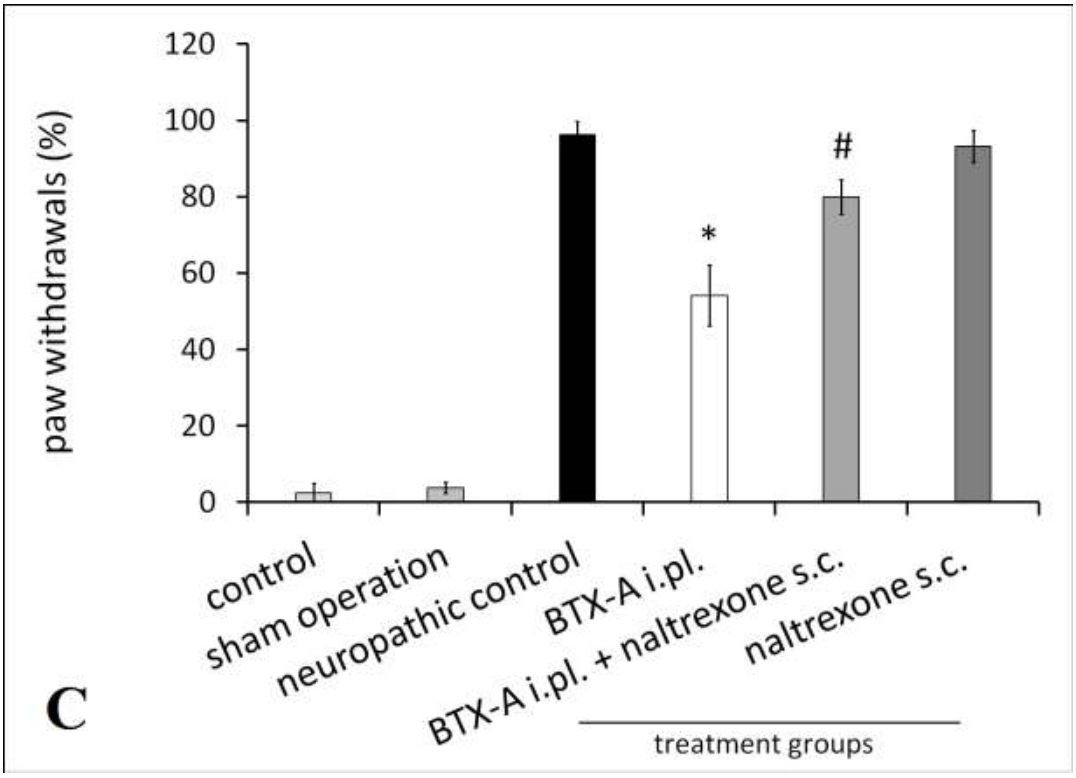
treatment groups
B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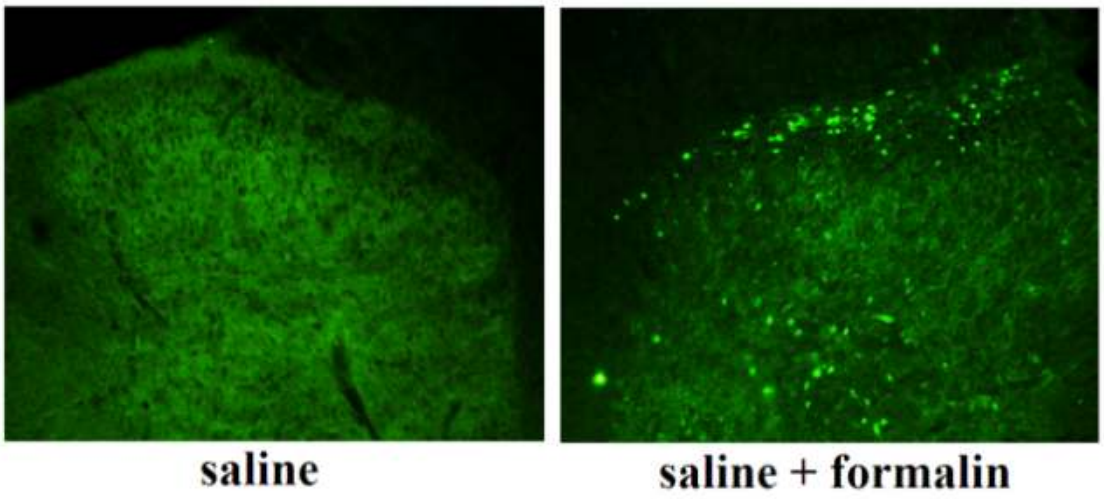
BTX-A i.pl.
BTX-A i.pl. + naltrexone s.c.
naltrexone s.c.
treatment groups
C

Fig. 4 A) Expression of immunfluorescently labelled c-Fos (green punctate immunoreactivity) in the ipsilateral (to the site of formalin-injection) superficial laminae of the L4/L5 spinal cord sections. Representative examples of 10x magnification images. Experimental groups: saline; saline+formalin; BTX-A (5 U/kg, s.c.) + formalin; BTX-A (5 U/kg s.c.) + naltrexone (2 mg/kg, s.c.) + formalin. Scale bar: 200 μm. B) Quantitative analysis of c-Fos expression in sensory laminae of the spinal cord sections from 10x magnification images. Total number of c-Fos positive neurons in sensory laminae in different formalin-treated experimental groups: saline; BTX-A; BTX-A + naltrexone; naltrexone. Average number of c-Fos positive neurons for each animal was calculated from three spinal cord sections. Mean ± SEM, n=4; * - $p<0.05$ compared to control (saline); + - $p<0.05$ compared to naltrexone; # - $p<0.05$ compared to BTX-A (Newman-Keuls post hoc)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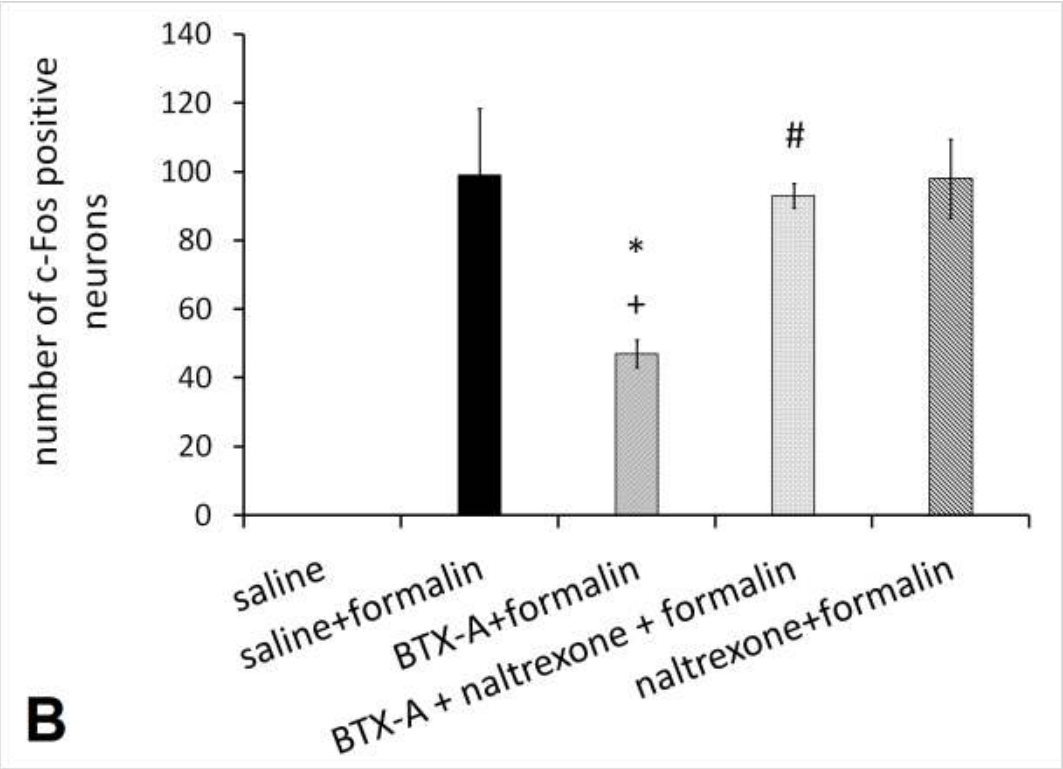
number of c-Fos positive neurons
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saline
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BTX-A+formalin
BTX-A + naltrexone + formalin
naltrexone+formalin
B

Conflict of interest

The authors declare no conflict of interest.