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Antinociceptive effect of botulinum toxin type A on experimental abdominal pain

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## Abstract

Visceral pain, especially in the abdominal region, represents one of the most common types of pain. Its chronic form is usually very hard to treat by conventional analgesic agents and adjuvants. We investigated the antinociceptive effect of botulinum toxin type A (BTX-A) in male Wistar rats in two models of visceral pain: peritonitis induced by intraperitoneal injection of 1% acetic acid and colitis induced by intracolonic instillation of 0.1% capsaicin. Pain was measured as the number of abdominal writhes. Additionally, referred mechanical sensitivity in the ventral abdominal area was evaluated by von Frey test and the extent of spinal c-Fos expression was immunohistochemically examined. BTX-A significantly reduced the number of abdominal writhes in both models of visceral pain after intrathecal application in a dose of 2 U/kg. In the experimental colitis model, BTX-A (2 U/kg) reduced both referred mechanical allodynia and c-Fos expression in the dorsal horn of the spinal cord (S2/S3 segments). In contrast to intrathecal administration, BTX-A (2 U/kg) administered into the cisterna magna had no effect on pain suggesting that the primary site of its action is a spinal cord.

**Keywords:** botulinum toxin type A, visceral pain, abdominal pain, capsaicin, acetic acid, rat

## 1. Introduction

Botulinum toxin type A (BTX-A) inhibits neurotransmitter release due to its endopeptidic activity against SNAP-25 (Synaptosomal Associated Protein of 25 kDa). This enables its therapeutic use in disorders characterized by overactive muscles, overactive exocrine glands and, most recently, non-muscular pain conditions (reviewed by Dressler, 2013, Matak and Lacković, 2014). Antinociceptive activity of BTX-A was demonstrated in various preclinical (reviewed by Pavone and Luvisetto, 2010) and clinical studies (reviewed by Jabbari and Machado, 2011) during the last decade. It was shown that a single injection reduces pain over a prolonged period of time (several months), which represents the unique feature of BTX-A among available analgesics.

Visceral pain, defined as pain arising from the internal organs of the body, is one of the most common forms of pain. In contrast to somatic pain, key features of visceral pain are diffuse localization typically referred to somatic sites (e.g. muscle and skin), stronger emotional and autonomic reactions, an unreliable association with pathology, and intense referred hyperalgesia (Ness, 1999, Sanoja et al., 2010). Despite of these differences, treatment has been the same as for somatic pain: based on non-steroidal anti-inflammatory drugs (NSAIDs), opioids and adjuvant analgesics like tricyclic antidepressants, antiepileptic drugs etc.

Unlike acute forms of visceral pain, chronic visceral pain might be refractory to the listed drugs because of short duration of action, variable effectiveness, and unwanted side effects (Davis, 2012). Thus, there is a need for new therapeutic options with better effectiveness, longer duration of action and acceptable tolerability.

Up to now, visceral effects of BTX-A were clinically investigated mostly in painful bladder and pelvic syndromes (reviewed by Russell et al., 2013, Jhang et al., 2014). In addition, a few case reports pointed toward the beneficial effect of BTX-A in non-cardiac chest pain

(Maradey-Romero and Fass, 2014) and perineal pain (Lim et al., 2010). Experimentally, the antinociceptive effect of BTX-A was investigated only in cystitis (Chuang et al., 2004, Coelho et al., 2014) and prostatitis (Chuang et al., 2008). In all listed reports and studies, the antinociceptive effect of primarily local BTX-A injections was examined.

Here we report that intrathecal, but not local and systemic, BTX-A application reduces pain as well as referred hyperalgesia and spinal c-Fos expression in experimental models of acetic acid induced peritonitis and capsaicin induced colitis.

## 2. Materials and Methods

### 2.1 Animals

Experiments were carried out on male Wistar rats (300-400 g; University of Zagreb School of Medicine, Croatia). Animals were housed in a 12 h light/dark cycle with food and water available ad libitum, except during behavioral testing. Experiments were conducted according to the European Communities Council Directive (2010/63/EU). Care and handling of the animals were in accordance with the recommendations of the International Association for the Study of Pain (Zimmermann, 1983). Efforts were made to minimize the number of animals used. Study design, experimental protocols, and descriptions of animal treatment are closely followed by ARRIVE guidelines. 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); acetic acid (Kemika, Croatia); capsaicin (Capsaicin®, Sigma, St. Louis, MO, USA); chloral

hydrate (Chloral hydrate®, Merck KGaA, Germany); ethanol (Kemika, Zagreb, Croatia); Tween 80 (Sigma, St. Louis, MO, USA); petroleum jelly (Kemig, Zagreb, Croatia).

Each vial of Botox® contains 100 U (~4.8 ng) of purified *Clostridium botulinum* type A neurotoxin complex. To attain the needed doses, all drugs were diluted or dissolved in 0.9% saline, except capsaicin, which was dissolved in 0.9 % saline containing 10% Tween and 10% ethanol.

Doses selected to test antinociceptive effect were chosen based on preliminary experiments and published data in models of somatic pain (Cui et al., 2004, Bach-Rojecky et al., 2010).

The following chemicals were used for the immunohistochemistry: 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 Drug administration

*Intrathecal (i.t.) injection.* A small skin incision (2 cm) was performed at the lumbar L4/L5 level. BTX-A (2 U/kg) or 0.9% saline was injected between the vertebrae in a volume of 20 µl and the skin was sutured; correctness of application was verified by the animal's tail or hind limb brisk move.

*Intracisternal (i.c.) injection.* The animals were placed in a position in which the posterior neck area was easy to reach. The needle was carefully advanced between the occipital protuberance and the spine of the atlas to the cisterna magna, and BTX-A (2 U/kg) or 0.9% saline was injected in a volume of 20 µl; correctness of application was verified by extraction of a small amount of cerebrospinal fluid (CSF).

*Intracolonic (i.col.) instillation.* BTX-A (10 U/kg) or 0.9% saline in a volume of 1 mL were guided 7 cm proximally from the anocutaneous line, via the anus with a transparent 1 mm diameter cannula.

The animals were anesthetized with i.p. injection of chloral hydrate (300 mg/kg) during the i.t., i.c. and i.col. administration.

*Intraperitoneal (i.p.) injection.* BTX-A (15 U/kg) or 0.9% saline were injected in a volume of 10 mL/kg to conscious, gently restrained animals.

## 2.4 Behavioral testing

Before testing, the animals were habituated to the testing area (plastic cage/plastic cage with a wire mesh floor) for 30 min. Each experimental group contained 5-6 animals.

*Acetic acid induced visceral pain model ("The writhing test").* The animals were i.p. injected with 10 ml/kg of 1% acetic acid and immediately returned to the transparent cage for a 1 h observation period, as described by Koster et al., 1959. Pain was measured as the number of abdominal writhes. A writhe is defined as arching of the back, extension of hind limbs and contraction of abdominal musculature. Measurements were conducted 5 days after i.c. and i.p. BTX-A or 0.9% saline injection and 2 days after the i.t. injections.

*Capsaicin induced visceral pain model.* The model was originally developed for mice (Laird et al., 2001.), and later adapted for use in rats (Sanoja et al., 2010.). In brief, 200 µl of 0.1% capsaicin was administered into the colon, 7 cm proximally from the anocutaneous line, via the anus, with a transparent 1 mm diameter cannula. Before i.col. capsaicin instillation, petroleum jelly (Vaseline) was applied on the perianal area to avoid direct contact with the irritant. Control animals were subjected to the same treatment, but instead of capsaicin, received 0.9% saline. Animals injected with i.t. BTX-A or 0.9% saline were tested after 2 days, while those i.col. instilled, after 5 days.

Immediately after i.col. instillation of 0.1% capsaicin or 0.9% saline, the animals were returned to the transparent cage for a 20 min observation period where spontaneous behavior was observed and counted. Visceral pain behavior was considered licking and contraction of the abdomen, stretching, hump-backed position and hunching (Sanoja et al., 2010). After 20 min, referred mechanical hyperalgesia was tested by using a series of von Frey filaments (Stoelting Co, Wood Dale, IL, USA) ranging from 1 g to 26 g, in ascending order, to the abdominal area with the exception of genital area. Repeated stimulation of the same area was avoided to prevent sensitization. The lowest filament that elicited a withdrawal response was considered the threshold stimulus. Each filament was applied 3 times, kept in bent position for 2 s and with an inter-stimulus interval of 5-10 s.

## 2.5 Immunohistochemical analysis

An immunohistochemical analysis was performed as previously described (Drinovac et al., 2013) on samples collected from the capsaicin induced visceral pain model. The number of c-Fos was counted in the superficial sensory laminae of the spinal cord dorsal horn (I and II) at the sacral S3/S2 section.

## 2.6 Statistical analysis

Results, presented as mean  $\pm$  S.D., were analyzed by one-way analysis of variance followed by the Tukey's post hoc test.  $P < 0.05$  was considered significant.

### 3. Results

#### 3.1 Intrathecal BTX-A reduces pain induced by intraperitoneal acetic acid injection

I.p. acetic acid induced abdominal writhes, a stereotypic response pattern in a form of abdominal contractions, twisting and turning of the trunk, and extension of the hind limbs (Koster et al., 1959). BTX-A injected i.t. reduced the number of abdominal writhes ( $P < 0.05$ ). However, BTX-A application into the cisterna magna failed to affect pain induced by acetic acid injection (Fig. 1).

A large volume (10 mL/kg) and dose (15 U/kg) of BTX-A administered into the peritoneal area had no significant antinociceptive effect on experimental peritonitis (results not shown). BTX-A applied in this dose caused significant muscle weakness which may have affected the results.

#### 3.2. Intrathecal BTX-A reduces spontaneous pain and referred hyperalgesia induced by intracolonic capsaicin injection

I.col. instillation of capsaicin evoked a significant increase in visceral spontaneous pain-related behavior during a 20 min observation period compared to i.col. instillation of saline.

Abdominal contractions induced by saline instillation were related to the injected volume and colonic distention. BTX-A pretreatment did not reduce abdominal contractions induced by saline instillation per se (Fig. 2A). However, i.t. BTX-A pretreatment alleviated spontaneous pain behavior caused by capsaicin instillation ( $P < 0.01$ ).

I.col. capsaicin, but not saline, produced referred secondary mechanical hyperalgesia to the abdomen (Fig. 2B). I.t. BTX-A pretreatment reduced the pain referred to the abdominal area ( $P < 0.01$ ).

Local, i.col., administration of BTX-A (10 U/kg) did not affect spontaneous pain nor referred hyperalgesia in the colitis model as well (results not shown).

### 3.3. Intrathecal BTX-A reduces c-Fos immunoreactivity in capsaicin induced visceral pain

C-Fos immunoreactivity was investigated in the lumbosacral segment (L6-S2) of the spinal cord of rats with capsaicin-induced colitis (Fig. 3). I.t. BTX-A significantly reduced the number of c-Fos positive cells in the sacral S2/S3 region ( $P < 0.05$ ). The majority of capsaicin-evoked c-Fos-positive neurons were located in the superficial dorsal horns (Fig. 3B).

## 4. Discussion

In contrast to different types of somatic pain (reviewed by Pavone and Luvisetto, 2010, Matak and Lacković, 2014) the information related to the beneficial action of BTX-A on pain arising from the viscera is mostly limited to the pelvic region. It was found that local BTX-A injections efficiently decrease pain in patients suffering from bladder pain syndrome/interstitial cystitis, prostatitis and pelvic-floor muscle disorder (Abbott et al., 2006., Chung et al., 2012, El-Khawand et al., 2013, Gottsch et al., 2011, Kuo, 2013, Pinto et al., 2013, Smith et al., 2004, Zermann et al., 2000). In addition, there are isolated case reports on the beneficial effect of BTX-A on non-cardiac chest pain (Maradey-Romero and Fass, 2014) and perineal pain (Lim et al., 2010).

Since preclinical investigations on visceral pain are sparse, limited just to rat models of cystitis and prostatitis (Chuang et al., 2004, 2008, 2009), the aim of the present study was to investigate the effect of BTX-A after its central (i.t. and i.c.) application on two pathophysiologically different types of visceral pain. Central application was used because it was shown in behavioral studies (Bach-Rojecky and Lacković 2005, 2009; Bach-Rojecky et

al., 2010; Favre-Guilmard et al., 2009; Filipović et al., 2012), and by immunohistochemistry, that the antinociceptive action of BTX-A is associated with its axonal transport from the periphery to central sensory centers (Matak et al., 2011, 2012), in contrast to classical assumption that BTX-A inhibits neurotransmitter release only peripherally (Aoki, 2005). Although the mechanism of central BTX-A's action is unknown, we recently demonstrated an interaction with spinal endogenous opioid peptides and  $\gamma$ -aminobutyric acid (GABA) (Drinovac et al., 2013, 2014).

Accordingly, in our experiments BTX-A was injected peripherally (i.p., i.col.) or centrally (i.t., i.c.) to investigate the effect of BTX-A injections on spontaneous pain, referred hyperalgesia and neuronal activation induced by i.col. capsaicin instillation, as well as i.p. acetic acid induced pain behaviors.

These two models of visceral pain mutually differ:

1. Capsaicin induced colonic pain represents visceral pain, associated with the activation of visceral nociceptors (Sanoja et al., 2010), while acetic acid induced peritoneal pain is considered to be mixed somato-visceral pain, i.e. the somatic component cannot be excluded due to possible sensitization of somatic peritoneal nociceptors by acetic acid (Ness, 1999).
2. In addition to the splanchnic and pelvic nerve, visceromotor response to i.p. acetic acid is mediated by the vagus nerve as well (De Groat, 1986; Sengupta, 2009).
3. I.col. capsaicin instillation produces referred hyperalgesia, a characteristic feature of visceral pain, due to the convergence of somatic afferents on viscerosensitive spinal neurons (Cervero, 2000, Traub 2003). This is not observed in acetic acid induced peritonitis.

In the present study we showed that i.t. BTX-A injection significantly reduced acetic-acid induced pain behaviors (Fig. 1). To investigate whether the effect of BTX-A is restricted to the spinal cord, we applied the toxin into the cisterna magna, in the same volume and dose as into the spinal canal. After i.c. application, BTX-A failed to affect pain induced by acetic acid,

thus indicating that BTX-A's antinociceptive action is predominantly of spinal origin. Since studies on different models of somatic pain demonstrated the antinociceptive effect of BTX-A after peripheral, subcutaneous application (reviewed by Pavone and Luvisetto, 2010, Matak and Lacković, 2014), here we investigated the effect of i.p. applied BTX-A. Injection of BTX-A in peritoneal area also had no antinociceptive effect, but rather caused visible abdominal muscle weakness which made evaluation of nociceptive behavior unreliable.

I.t. BTX-A injection in a dose as low as 2 U/kg decreases spontaneous pain as well as referred hyperalgesia induced by i.col. capsaicin instillation (Fig. 2). Reduction of secondary referred hyperalgesia cannot be explained without the assumption that BTX-A interferes with central sensitization at the spinal level. Local application of BTX-A (10 U/kg) into the lumen of the colon had no significant antinociceptive effect, even though the applied dose was five times higher than the i.t. dose (2 U/kg) (results not shown). The reasons might be a short retention of BTX-A in the colon, insufficient absorption of BTX-A, as well as an inability of BTX-A to enter the nerves from the lumen of the colon. The latter seems likely since it was demonstrated that after instillation into the lumen of the bladder in guinea pigs, BTX-A does not enter the nerve fibers (Coelho et al., 2012).

Visceral primary afferents, contrary to somatic primary afferents, enter the spinal cord and spread largely to enter multiple spinal segments above and below the segment of entry.

Additionally, they form synaptic contacts with both superficial and deep dorsal horn neurons ipsilateral and contralateral to the side of entry (Sugiura and Tonosaki, 1995). The result is extensive, diffuse, central nervous system activation which counteracts the somatotopic organization that provides the precise localization of somatic stimuli. Capsaicin instillation into the colon induced neuronal activity in the lumbosacral spinal cord which appears to mediate reflex responses to acute and inflammatory colonic pain (Pitcher et al., 2013). Here,

we showed that i.t. BTX-A significantly reduced c-Fos expression in the sacral S2/S3 segments of the spinal cord (Fig. 3).

An important finding of this study is the reduction of referred mechanical sensitivity by i.t. BTX-A in experimental colitis (Fig. 2B). The mechanism of transfer of visceral hypersensitivity to somatic structures is poorly understood and investigated. It is considered to be centrally mediated by mechanisms of central sensitization, similar to somatic pain (Gebhart and Bielefeldt, 2008). Recent experimental findings suggest that the development of somatic and visceral hyperalgesia and allodynia is paralleled by an increase in the responsiveness of RVM On-like cells (Rostral Ventromedial Medulla On-like cells) (Sanoja et al., 2010). This was demonstrated in clinics as well, in healthy volunteers (Brock et al., 2010), in patients with irritable bowel syndrome (Wilder-Smith and Robert-Yap, 2007) and functional dyspepsia (Wilder-Smith et al., 2014).

Up until recently, data about BTX-A's antinociceptive effect in the pelvic region were limited to investigating the effects of local injections on spontaneous visceral pain behavior (Chuang et al., 2004, 2008, 2009). In contrast to that, Coelho et al. (2014) most recently demonstrated, in a model of cyclophosphamide-induced cystitis, the efficacy of i.t BTX-A injections in reducing mechanical hypersensitivity to the abdominal and hind paw area. Together with our findings, these data reinforce the link between BTX-A and central sensitization, since central BTX-A injections were effective towards referred pain in different models of visceral pain. Considering that the only known target molecule for BTX-A is SNAP-25, we speculate that the inhibition of excitatory neurotransmitter release from the central endings of primary afferent terminals may be responsible for the antinociceptive action of BTX-A. Another possibility is that BTX-A, administered centrally, also inhibits neurotransmitter release from descending fibers, responsible for facilitation of pain transmission and generation of referred somatic and visceral hyperalgesia (Sanoja et al., 2010). Coelho et al., 2014,

immunohistochemically found that BTX-A applied i.t. may be anterogradely transported to peripheral nerve endings in the urinary bladder. The transport from central site to periphery could also contribute to the multifaceted antinociceptive effect of BTX-A in visceral pain. In conclusion, the presented results provide evidence of antinociceptive effects of intrathecal BTX-A in experimental colitis and peritonitis, including reduction of referred hyperalgesia, one of the most important features of visceral pain, thus supporting predominantly central site of BTX-A antinociceptive action. Furthermore, these results designate BTX-A's potential in treatment of patients with long lasting visceral pain.

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#### Conflict of interest

Authors declare no conflict of interest.

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## Figure captions

Fig. 1. I.t. BTX-A (2 U/kg) injection reduces pain behavior induced by 1% acetic acid i.p. injection. Mean  $\pm$  S.D.; n=6;  $P<0.05$  compared to saline (ANOVA followed by Tukey's post hoc)

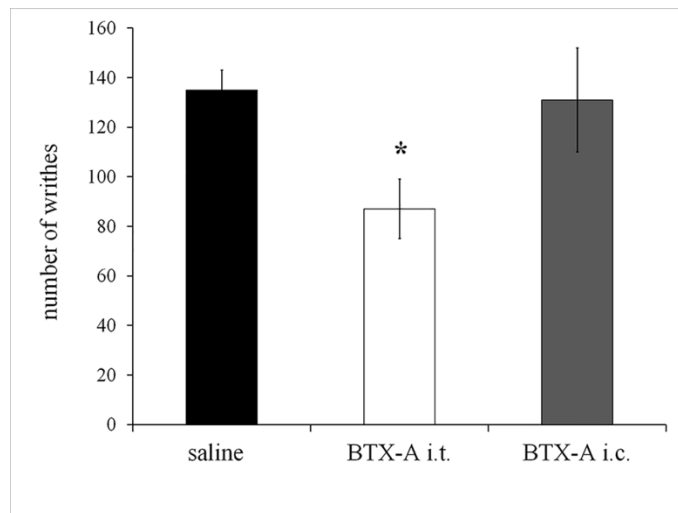


Fig. 2. I.t. BTX-A (2 U/kg) pretreatment reduces: A) spontaneous pain behavior induced by capsaicin (0.1%) i.col. instillation. Mean  $\pm$  S.D., n=6; \*\* P<0.01 compared to saline i.t. + saline i.col., BTX-A i.t. + saline i.col. and BTX-A i.t. + capsaicin i.col.; B) referred mechanical hyperalgesia 20 min after i.col. capsaicin instillation. Mean  $\pm$  S.D., n=6; \* P<0.05 compared to saline i.t. + saline i.col. and BTX-A i.t. + saline i.col.; \*\* P<0.01 compared to saline i.t. + saline i.col., BTX-A i.t. + saline i.col. and BTX-A i.t. + capsaicin i.col. (ANOVA followed by Tukey's post hoc)

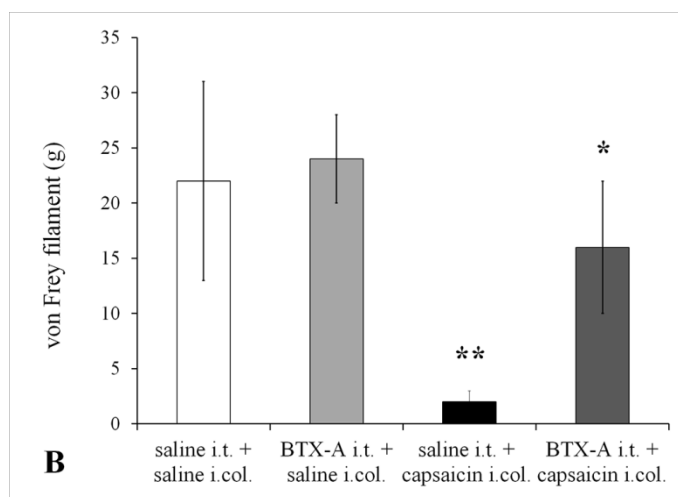
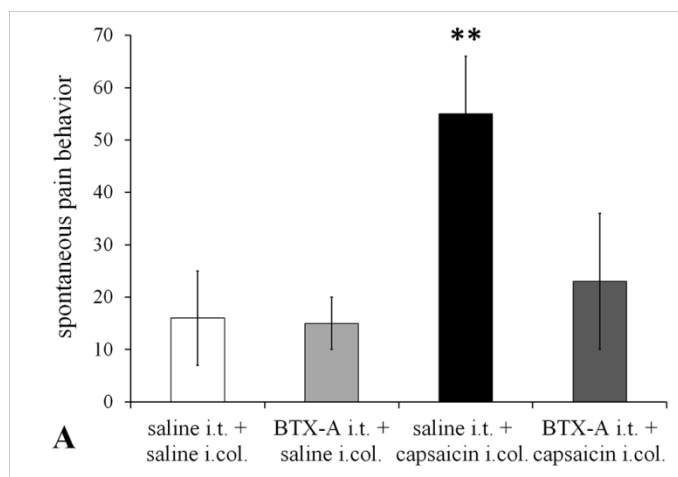
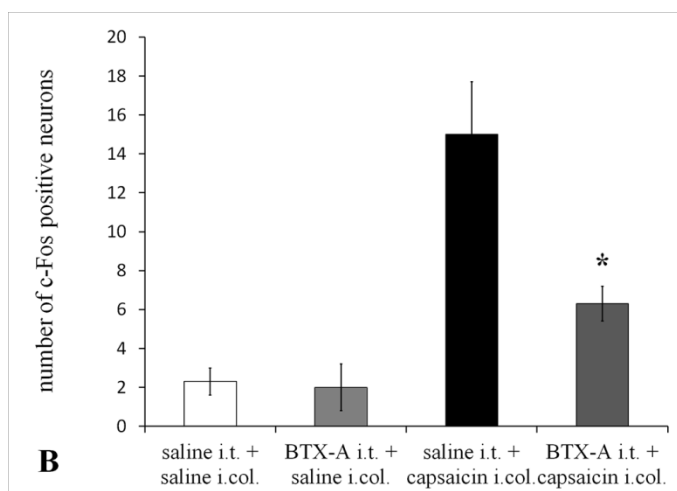
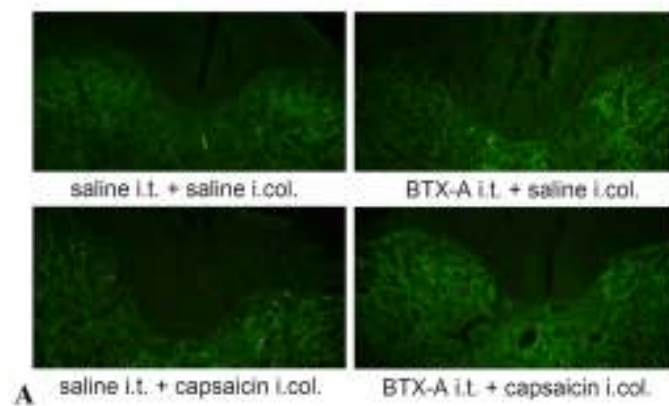


Fig. 3. A) Expression of immunfluorescently labeled c-Fos (green punctate immunoreactivity) in the superficial laminae of the S2/S3 spinal cord sections. Representative examples of 10x magnification images. Scale bar: 200  $\mu$ m. B) Quantitative analysis of c-Fos expression in laminae I and II from 10x magnification images. Total number of c-Fos positive neurons in superficial sensory laminae of spinal cord dorsal horn. The average number of c-Fos positive neurons for each animal was calculated from three spinal cord sections. Mean  $\pm$  S.D., n=4; \*  $P < 0.01$  compared to saline i.t. + capsaicin i.col. (ANOVA followed by Tukey's post hoc)



List of abbreviations:

BTX-A – botulinum toxin type A

CNS – central nervous system

CSF – cerebrospinal fluid

GABA –  $\gamma$ -aminobutyric acid

i.c. – intracisternal

i.col. – intracolonic

i.p. – intraperitoneal

i.t. – intrathecal

NGS – normal goat serum

NSAIDs – Non-steroidal anti-inflammatory drugs

PBS – phosphate buffered saline

PBST – PBS + 0.25% TritonX-100

RVM – rostral ventromedial medulla

SNAP-25 – SyNaptosomal Associated Protein of 25 kiloDaltons