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Association of antinociceptive action of botulinum toxin type A with GABA-A receptor

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Abstract

The mechanism of botulinum toxin type A (BTX-A) antinociceptive action in the central nervous system is little known. The potential interaction between BTX-A and GABAergic system has not been investigated previously. In the present study we demonstrate prevention of BTX-A antinociceptive effect on formalin-induced inflammatory pain and partial sciatic nerve transection-induced mechanical allodynia by GABA-A antagonist bicuculline, thus suggesting association of the GABA-A receptors and antinociceptive action of BTX-A.

Introduction

Botulinum toxin type A (BTX-A) is an endopeptidase which cleaves SNAP25 (Synaptosomal Associated Protein of 25 kDa) and consequently prevents acetylcholine and other neurotransmitter release (Aoki, 2008). More recently antinociceptive activity of BTX-A was found in preclinical (reviewed by Pavone and Luvisetto, 2010) and clinical research (reviewed by Jabbari and Machado, 2011). During the last decades behavioral (Bach-Rojecky and Lacković, 2009; Bach-Rojecky et al., 2010; Favre-Guilmard et al., 2009; Filipović et al., 2012) and immunobiochemical data revealed that BTX-A antinociceptive action takes place primarily in the central nervous system (CNS) after its retrograde axonal transport from periphery (Antonucci et al., 2008; Matak et al., 2011, 2012). However, the mechanism of central antinociceptive action is not clear. It could be associated with μ-opioid receptor (Drinovac et al., 2013), while SNAP-25 cleavage might be only the first step in the overall mechanism of BTX-A antinociceptive action. Sensory information transmitted to the dorsal horn is under strong inhibitory control by gamma-aminobutyric acid (GABA), released by local interneurons and inhibitory descending fibers (Antal et al., 1994; Bardoni et al., 2013). Using bicuculline, a GABA-A receptor antagonist, here we report that the antinociceptive action of BTX-A is associated with GABA-A receptor activity at the spinal level.

Materials and Methods

Animals

Male Wistar rats (University of Zagreb School of Medicine) 3 months old and weighing 300-400 g were kept under standard conditions with free access to food and water. Experiments were conducted according to the European Communities Council Directive (86/609/EEC) and recommendations of the International Association for the Study of Pain. 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).

Drug administration

Animals were injected subcutaneously into the plantar surface of the hind paw (intraplantarly, i.pl.) with BTX-A (Botox®, Allergan, Inc., Irvine, USA) diluted in 0.9% saline. BTX-A was injected in a dose of 5 U/kg or 7 U/kg and in a volume of 20 µl.

Bicuculline hydrochloride (Sigma, St.Louis, MO, USA) was injected: 1. intraperitoneally (i.p., 2 mg/kg, in a volume of 1 ml); 2. intrathecally (i.t.,1 µg/10 µl) at the lumbar L3-L4 level; and 3. intracisternally (i.c., 1 µg/10 µl). Bicuculline was dissolved in 0.9% saline by addition of one drop of 0.01% HCl. Doses of bicuculline were chosen according to the literature (Micov et al., 2010; Yamamoto and Yaksh, 1993).

For i.p. injections, rats were conscious and gently restrained. For bicuculline i.t. and i.c. application, animals were briefly anesthetized with shortly acting diethyl ether (Sigma, St.Louis, MO, USA). For i.t. injection, animal's hair was shaved at the lumbar L3-L4 level. After skin incision bicuculline or saline were injected between the vertebrae using a Hamilton syringe. During i.c. application, animals were placed in a position in which the posterior neck area was easy to reach. The needle of the Hamilton syringe was carefully advanced between the occipital protuberance and the spine of the atlas to the cisterna magna (Ueda et al. 1979).

Behavioral testing

Formalin-induced pain. Conscious, gently restrained rats were s.c. injected with 5% formalin (50 µl) into the plantar surface of the hindpaw pad and immediately returned to the transparent cage for 1 h observation period. The number of licking, flinching and shaking of the injected paw was measured (Tjolsen et al., 1992). Each experimental group contained 5-6 animals.

BTX-A was injected 5 days and bicuculline 40 min prior to induction of pain, respectively. Control animals received 0.9% saline in the appropriate volumes and underwent the same procedure as animals treated with bicuculline.

Sciatic nerve transection-induced mechanical allodynia. A total number of 45 rats underwent sciatic nerve partial transection, as previously described (Lindenlaub and Sommer, 2000; Drinovac et al., 2013). Five rats were subjected to sham procedure; sciatic nerve was exposed, but not transected and five naive rats served as control. Two weeks following the peripheral nerve injury, animals which developed ipsilateral mechanical allodynia (at least 20% changes from the mean of the sham-operated group) were included into the further

experiment. Nociceptive measurements of mechanical allodynia with von Frey filaments were conducted as previously described (Drinovac et al., 2013.). Neuropathic animals were divided in four groups (6 animals per group) as follows: (1) 0.9% saline (i.pl.), (2) BTX-A (7 U/kg; i.pl.), (3) bicuculline (2 mg/kg, i.p.), (4) BTX-A + bicuculline. Nociceptive measurements were preformed 5 days after BTX-A i.pl. injection and 40 min prior to bicuculline i.p. injection.

During all behavioral measurements the experimenter was unaware of the treatment groups.

Immunohistochemistry

Immunohystochemical analysis of c-Fos expression in the rat spinal cord was done as previously described (Drinovac et al., 2013) in samples collected from formalin test experiment.

Statistical analysis

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

Results

Systemic bicuculline (2 mg/kg, i.p.) application 40 min prior to the formalin test abolished the antinociceptive action of peripheral BTX-A (5 U/kg) injection in the second phase of the test ($p<0.01$, Fig. 1). To examine the possible central site of bicuculline action on BTX-A induced antinociception, low dose-bicuculline (1 µg/10 µl) was injected intrathecally and intracisternally, respectively. Similarly to several hundred times higher systemic dose, intrathecally applied bicuculline abolished the antinociceptive effect of peripherally applied BTX-A ($p<0.01$; Fig. 1). However, bicuculline injection into the cerebellomedullary cistern did not affect BTX-A induced antinociceptive effect. The respective doses of bicuculline alone, applied i.p., i.t. or i.c. did not affect pain behavior (data not shown). None of the tested drugs or their combinations affected pain in the first phase of the formalin test (data not shown).

In addition to inflammatory pain, GABA-A receptor antagonist prevented the antiallodynic effect of BTX-A in partial sciatic nerve transection-induced mechanical allodynia as well ($p<0.01$, Fig. 1b).

Immunohistochemical data showed reduction of c-Fos positive neurons in BTX-A pretreated animals in comparison to formalin group ($p<0.01$, Fig. 2). Bicuculline (2 mg/kg, i.p.) prevented the effect of BTX-A on c-Fos expression ($p<0.05$, Fig. 2).

Discussion

Contrary to usual assumption (Aoki and Francis, 2011), numerous recent behavioral data suggest that antinociceptive action of BTX-A occurs within CNS. Discovery of BTX-A axonal transport provides physiological background for these behavioral observations (Antonucci et al., 2008; Restani et al., 2011). Moreover, truncated SNAP25 was found in the CNS sensory nuclei after toxin's peripheral application (Matak et al., 2011, 2012). Recently we demonstrated prevention of BTX-A effects on pain and c-Fos expression by opioid antagonists thus suggesting the association between antinociceptive action of BTX-A and central μ-opioid receptor (Drinovac et al., 2013). However, based on complex neurotransmitter network involved in central modulation of nociception, it seems unlikely that opioid system is the only one affected by the BTX-A.

The superficial laminae of the spinal cord dorsal horn are under strong inhibitory control, primarily by inhibitory transmitter GABA, released by both local interneurons and inhibitory descending fibers. Acting through ionotropic GABA-A and metabotropic GABA-B receptors, GABA controls the flow of sensory information by regulating the excitability of dorsal horn neurons and by modulation of transmitter release from primary afferent terminals (Bardoni et al., 2013; Melin et al., 2013). It is assumed that damaged dorsal horn inhibition contributes to persistent pain hypersensitivity (Moore et al., 2002).

We hypothesized that the site of BTX-A interaction with GABA-A receptors occurs in the spinal cord, although we couldn't exclude a supraspinal structures. Therefore, bicuculline was applied in three ways: intraperitoneally, intrathecally – in the spinal canal, and into the cistern magna. When injected intrathecally in a dose as low as 1 μg, as well as after intraperitoneal application in several hundred times higher dose (0.6-0.8 mg), bicuculline abolished the antinociceptive activity of peripherally applied BTX-A, which confirms the central site of BTX-A action. In support to that, bicuculline (2 mg/kg, i.p.) prevented the effect of BTX-A on c-Fos expression ($p<0.05$, Fig. 2). However, bicuculline application into the cisterna magna did not affect the antinociceptive action of BTX-A, excluding supraspinal level as a possible site of interaction between GABA and BTX-A action. Additionally, prevention of antinociceptive effect of BTX-A was demonstrated in another type of pain, as well. Mechanical allodynia is common feature of neuropathic pain. I.pl. injection of BTX-A reduced mechanical sensitivity to von Frey filaments ($p<0.01$, Fig. 1b) in neuropathic animals, but i.p. injection of GABA-A antagonist abolished the antinociceptive effect in BTX-A pretreated animals ($p<0.01$, Fig. 1b).

We speculate that antinociceptive activity of BTX-A is prevented by GABA-A antagonist bicuculline due to reduction of GABAergic synaptic inhibition in the spinal cord, which is believed to be important factor contributing to the generation and maintenance of chronic pain (Bardoni et al., 2013). In our previous work

(Drinovac et al., 2013.), opioid antagonists prevented the antinociceptive effect of BTX-A. Since the mechanism of BTX-A action is inhibition of neurotransmitter release by cleavage of SNAP-25, we hypothesized that in some neuronal loop BTX-A should decrease inhibitory GABA activity. This could in turn disinhibit / increase the stimulation of μ-opioid receptor. However, observed result did not confirm our hypothesis. Apparently the picture is more complex and activation of two spinal inhibitory neurotransmitter systems, GABA and endogenous opioids, is involved. We still assume that existence of some complex neuronal loop could be the best explanation for BTX-A effects on opioid and GABA transmission. Since mature GABAergic nerve terminals probably lack SNAP-25 (reviewed by Matteoli et al., 2009), the effect of BTX-A cannot be other than indirect. However, how GABA and opioids are stimulated with a drug that blocks neurotransmitters release remains a puzzle for further investigation.

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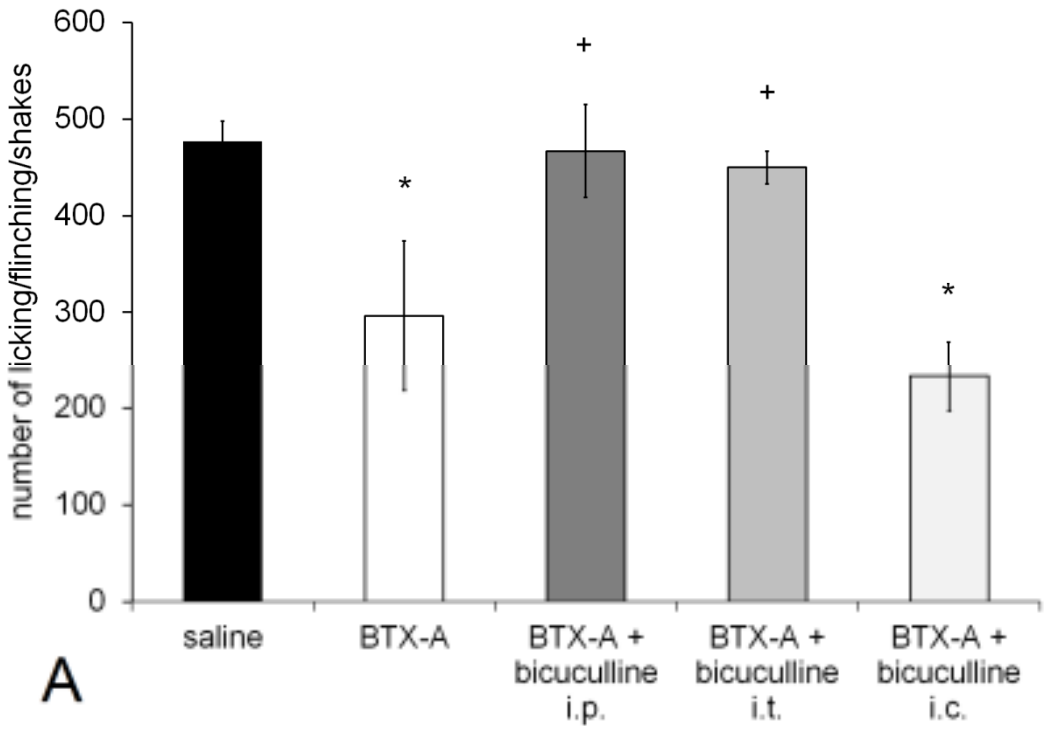
FIGURE CAPTIONS

Figure 1. Influence of bicuculline on the antinociceptive action of BTX-A in: A.) the second phase of the formalin test (BTX-A 5 U/kg was applied 5 days prior formalin injection into the plantar surface of the rat's hind-paw; bicuculline was injected: intraperitoneally (i.p., 2 mg/kg); intrathecally (i.t., 1μg/10μl), and intracisternally (i.c., 1μg/10μl) 40 min prior nociceptive testing; results are presented as mean±SD; n=5-6; *$p<0.01$ compared to saline (control); $^{+}p<0.01$ compared to BTX-A treated animals, Tukey *post hoc*); and B.) partial sciatic nerve transection-induced mechanical allodynia (measurements were conduced 5 days following i.pl. BTX-A 7 U/kg injection and 40 min following i.p. bicuculline 2 mg/kg injection; results presented as mean ± SD; n=5-6. *$p<0.01$ compared to neuropathic control, bicuculline and BTX-A + bicuculline; Tukey *post hoc*).



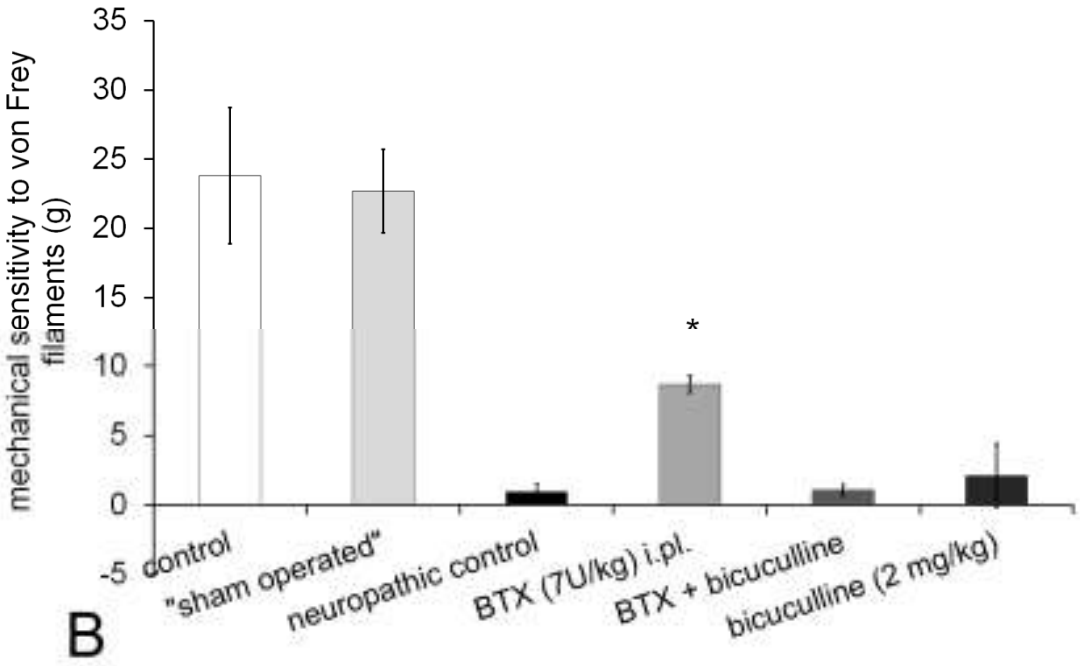
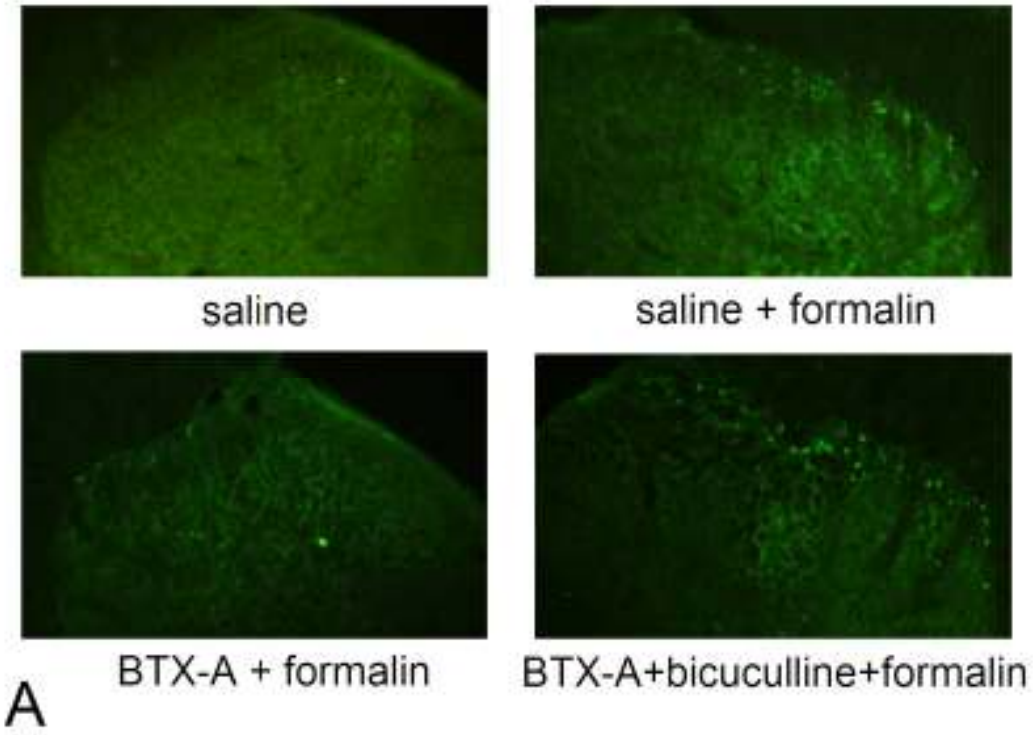
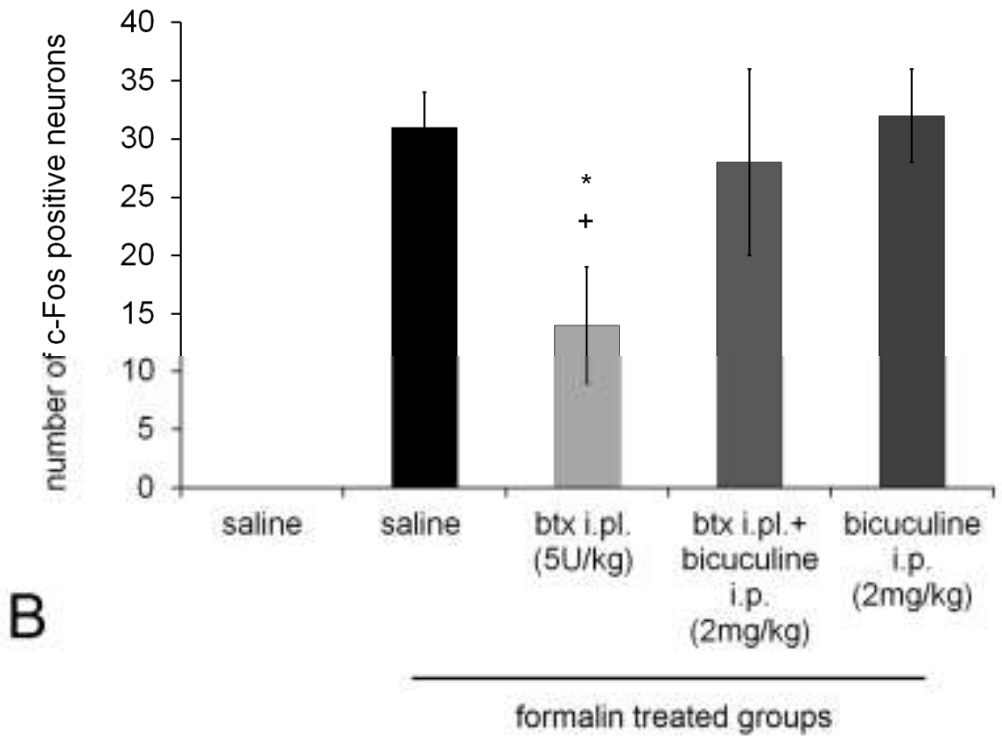

mechanical sensitivity to von Frey filaments (g)
35
30
25
20
15
10
5
0
-5
*
control
"sham operated"
neuropathic control
BTX (7U/kg) i.pl.
BTX + bicuculline
bicuculline (2 mg/kg)
B

Fig 2. A) Expression of immunfluorescently labelled c-Fos (green punctate immunoreactivity) in the ipsilateral (to the site of formalin-injection) superficial laminae of the L4 spinal cord sections. Representative examples of 10x magnification images. Scale bar: 200 μm. B) Quantitative analysis of c-Fos expression in laminae I and II 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 + bicuculline; bicuculline. Average number of c-Fos positive neurons for each animal was calculated from three spinal cord sections. Mean ± SD, n=4; * - $p<0.01$ compared to control (saline); + - $p<0.05$ compared to BTX-A + bicuculline (Tukey *post hoc*).




number of c-Fos positive neurons
40
35
30
25
20
15
10
5
0
*
+
saline
saline
btx i.pl. (5U/kg)
btx i.pl.+ bicuculine i.p. (2mg/kg)
bicuculine i.p. (2mg/kg)
formalin treated groups

B