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Central origin of the antinociceptive action of botulinum toxin type A

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Abstract

Here we provide behavioural evidence for an axonal transport and the central origin of the antinociceptive effect of botulinum toxin type A (BTX-A). In rats we investigated the effectiveness of BTX-A on “mirror pain” induced by unilateral repeated intramuscular acidic saline injections (pH 4.0). Since experimental evidence suggest that bilateral pain induced by acidic saline is of central origin, peripheral application of BTX-A should have no effect on this type of pain. However, here we demonstrated that the unilateral subcutaneous BTX-A (5 U/kg) application diminished pain on the ipsilateral, but on the contralateral side too. When injected into the proximal part of a distally cut sciatic nerve, BTX-A still reduced pain on the contralateral side. Colchicine, an axonal transport blocker, when injected into the ipsilateral sciatic nerve, prevented the effect of the peripheral BTX-A injection on both sides. Additionally, when BTX-A (1 U/kg) was applied intrathecally in the lumbar cerebrospinal fluid, the bilateral hyperalgesia was also reduced. The results demonstrate the necessity of retrograde axonal transport and involvement of the central nervous system for the antinociceptive activity of BTX-A.

Key words: botulinum toxin, antinociception, axonal transport, mirror pain, acidic saline, rat

1. Introduction

Botulinum toxin type A (BTX-A) is used in therapy of several neuromuscular and autonomic disorders (Dressler et al., 2005; Truong and Jost, 2006). Additionally, it was observed that BTX-A reduces pain in some conditions with concomitant muscle contraction, like in painful dystonias (Tsui et al., 1986) but also in pain states not associated with muscle hypercontraction such as migraine (Gobel, 2004), trigeminal neuralgia (Allam et al., 2005), neuropathic pain (Ranoux et al., 2008), refractory joint pain (Mahowald et al., 2006) and low-back pain (Jabbari, 2008).

It is generally believed that the molecular mechanism of BTX-A action on the neuromuscular junction and other cholinergic nerve endings is the cleavage of SNAP-25 (synaptosomal associated protein of 25 kDa), one of the SNARE proteins essential for neurotransmitter release (Aoki, 2005; Grumelli et al., 2005).

Based on several in vitro experiments, it was assumed that the mechanism of BTX-A-induced antinociception might be the prevention of the release of neuropeptide transmitters, like substance P, calcitonin gene-related peptide from the primary sensory neurons (Welch et al., 2000; Durham et al., 2004). Cui et al. (2004) were the first to demonstrate that a subcutaneous BTX-A injection into the rat hindpaw decreases formalin-induced inflammatory pain. Additionally, reduced formalin-induced glutamate release in the dialysate of the hindpaw, reduced number of the Fos-like immunoreactive cells in the dorsal horn of the spinal cord, and inhibited excitation of wide dynamic range neurons of the dorsal horn were demonstrated after the BTX-A peripheral application (Cui et al., 2004; Aoki, 2005). Kitamura et al. (2009) have recently demonstrated that intradermal BTX-A injection in the area of infraorbital branch of the trigeminal nerve decreases exaggerated CGRP release from trigeminal ganglion neurons in vitro and relieves neuropathy induced behavior by infraorbital nerve constriction in rats. Consequently, it was logical to propose that BTX-A inhibits release of neurotransmitters

from the peripheral nerve endings and peripheral sensitization, which leads to an indirect reduction in central sensitization of the dorsal horn neurons.

However, we have recently reported that BTX-A (5 U/kg) reduces inflammatory hyperalgesia, but not local edema or protein extravasation induced by the carrageenan and capsaicin injections into the rat hindpaw pad (Bach-Rojecky and Lackovic, 2005; Bach-Rojecky et al., 2008). Since inflammation is a peripheral phenomenon, the observed lack of the effect on inflammation brings into question the importance of peripheral exocytosis for the antinociceptive action of BTX-A. Furthermore, Antonucci et al. (2008) recently provided the first biochemical evidence that the BTX-A cleaves SNAP-25 distinct from the site of injection, thus suggesting an axonal transport of BTX-A within central neurons and motoneurons.

Based on these observations, we hypothesized that the antinociceptive activity of BTX-A might be centrally mediated. In the present study we investigated the effectiveness of BTX-A on a specific “mirror pain”, i.e. bilateral pain after unilateral intramuscular acidic saline injections in rats (Sluka et al., 2001). The bilateral secondary hyperalgesia was proposed to be centrally mediated (Tillu et al., 2008).

In the present paper investigating the acidic saline-induced pain, we found bilateral antinociceptive effect of the unilateral BTX-A injection. Employing colchicine and sciatic nerve transection, we found that axonal transport is a prerequisite for the toxin antinociceptive action. The results demonstrate involvement of the central nervous system (CNS) in the antinociceptive activity of BTX-A.

2. Materials and methods

2.1. Animals

Male Wistar rats weighing 250-300 g were used in all experiments (5-8 rats per experimental group). Animals were kept under a constant 12 h/12 h light/dark cycle with free access to food and water. The experiments were conducted according to the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985) and recommendations of the International Association for the Study of Pain (Zimmerman, 1983). The 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 were used: botulinum toxin type A (BOTOX®, Allergan, Inc., Irvine, USA); colchicine (Sigma, St. Louis, MO, USA); chloral hydrate (Sigma, St. Louis, MO, USA), formalin (Sigma, St. Louis, MO, USA).

Each vial of BOTOX® contains 100 U of purified *Clostridium botulinum* toxin type A neurotoxin complex. One unit (U) of Botox® contains approximately 48 pg of whole molecule of BTX-A, with molecular weight of approximately 900 kDa. To obtain respective doses, BTX-A was reconstituted in adequate volume of 0.9% saline.

2.3. BTX-A injections

BTX-A was injected in three ways: 1. subcutaneously (5 U/kg or 65 pg of the toxin/animal weighing 250-285 g) into the plantar surface of the hindpaw to conscious rats in a volume of 20 μl (concentration 3.6 pM) with a 27 ½ gauge syringe; 2. intraneuronally (0.5 U/kg or 6.7 pg of the toxin/animal weighing 275-285 g) into the femoral segment of sciatic nerve of

anaesthetised rat (chloral hydrate 300 mg/kg, intraperitoneally) in a volume 2 µl (concentration 3.7 pM) using a Hamilton syringe, and 3. intrathecally (approximately 1 U/kg or 14.4 pg of the toxin/animal weighing 290-300g) at L3-L4 level to anaesthetised rat in a volume 10 µl (concentration 1.6 pM) using a Hamilton syringe.

Because inter-group differences in body weight between animals injected with BTX-A were 5% (intrathecally) -15% (subcutaneously), each animal in these experimental groups received the same respective dose of BTX-A.

Doses of BTX-A were chosen based on several preliminary experiments on a small number of animals.

In the text below, central application denotes an intrathecal BTX-A application in the lumbar (L3-L4) cerebrospinal fluid, peripheral means a s.c. injection into the hinpaw pad and intraneuronal denotes a direct injection into the sciatic nerve.

2.4. Induction of bilateral muscle hyperalgesia

Two acidic saline injections (pH 4.0±0.1 adjusted with HCl) in a volume 100 µl were applied into the right gastrocnemius muscle 4 days apart (Sluka et al, 2001). The control animals were subjected to the same injection protocol with normal saline. Mechanical sensitivity was measured 24 h after the second injection. Around 60% of animals developed mechanical hyperalgesia, defined in present experiments as paw-withdrawal threshold reduced for at least 25% compared to the control - saline injected animals on both sides and were included in further experiments. The remaining animals developed either unilateral hyperalgesia or demonstrated paw-withdrawal threshold reduction of less than 25% compared to the control animals.

In the text below, ipsilateral means the right – pain induction side and contralateral means the left side, opposite to the pain induction (i.m. acidic saline injections).

2.5. Measurement of mechanical sensitivity

The sensitivity to mechanical stimuli was measured by the paw-pressure test as described by Randall and Selitto (1957). Mechanical nociceptive thresholds expressed in grams were measured by applying increased pressure to the dorsal surface of the hind paw until paw-withdrawal or overt struggling was elicited. The measurements were performed bilaterally 3 times alternating ipsilateral and contralateral paw in 10-min intervals. The experimenter was unaware of the treatment groups.

2.6. Inhibition of axonal transport

2.6.1. Inhibition of axonal transport by colchicine

The rats were anesthetized with chloral hydrate (300 mg/kg, i.p.) and bilateral sciatic nerves were exposed. The nerves were elevated slightly, such that a thin strip of Parafilm could be placed underneath to prevent accidental systemic absorption of the drug. Colchicine (5 mM, 2 µl) was injected slowly into the femoral segment of the sciatic nerve with a Hamilton syringe. On the opposite side, a sham operation was performed, i.e. the femoral segment of the sciatic nerve was injected with saline (Murphy et al., 1999). The wounds were sutured with 5-0 nylon and the animals were left to recover to the next day when BTX-A or saline s.c. injections were administered.

2.6.2. Inhibition of anterograde axonal transport by nerve transection

The rats were anesthetized with chloral hydrate (300 mg/kg, i.p.). BTX-A (0.5 U/kg, 2µl) was injected into the right sciatic nerve. One minute after the toxin injection, sciatic nerve was cut

1 cm distally to the toxin injection site. The wounds were sutured with 5-0 nylon and the animals were left to recover for 3 days.

2.7. Experimental design

The experimental design was as follows:

1. BTX-A was injected s.c. into the right rat hindpaw pad and the effect on mechanical hypersensitivity on the ipsilateral and contralateral side was tested on day 1 and day 5 following the toxin application;

2. In one experiment BTX-A was not injected on the ipsilateral side, but on the contralateral left hindpaw pad and mechanical sensitivity on both sides was measured on day 5;

3. To exclude anterograde axonal transport, as described earlier, sciatic nerve was distally transected after the BTX-A nerve injection and mechanical sensitivity was measured on the contralateral side;

4. To prevent retrograde axonal transport, colchicine was injected: a) into the ipsilateral right n. ischiadicus or b) into the left contralateral sciatic nerve 1 day before the BTX-A s.c. injection into the ipsilateral right hindpaw and 5 days later sensitivity to mechanical stimuli was measured bilaterally;

5. Influence of the intrathecal injection of BTX-A (at the level of L3-L4) on bilateral mechanical hyperalgesia was investigated;

2.8. Statistical analysis

The results, presented as mean±S.E.M., were analyzed by one-way ANOVA followed by the Newman-Keuls's post hoc test. A $P<0.05$ was considered significant.

3. Results

3.1. Bilateral antinociceptive effect of BTX-A after unilateral injection

In the animals which developed bilateral mechanical hyperalgesia after two i.m. acidic saline injections (see Material and Methods), the application of BTX-A (5 U/kg, s.c.) into the right hindpaw pad increased paw withdrawal threshold on day 5 ipsilaterally and contralaterally as well (Fig. 1). BTX-A had no direct antinociceptive action, i.e. 5 U/kg BTX-A did not modify mechanical pain threshold in control animals without acidic saline induced hyperalgesia (paw-withdrawal thresholds were: 152.7±13 g for BTX-A- treated vs. 147.8±14.4 g for BTX-A untreated animals).

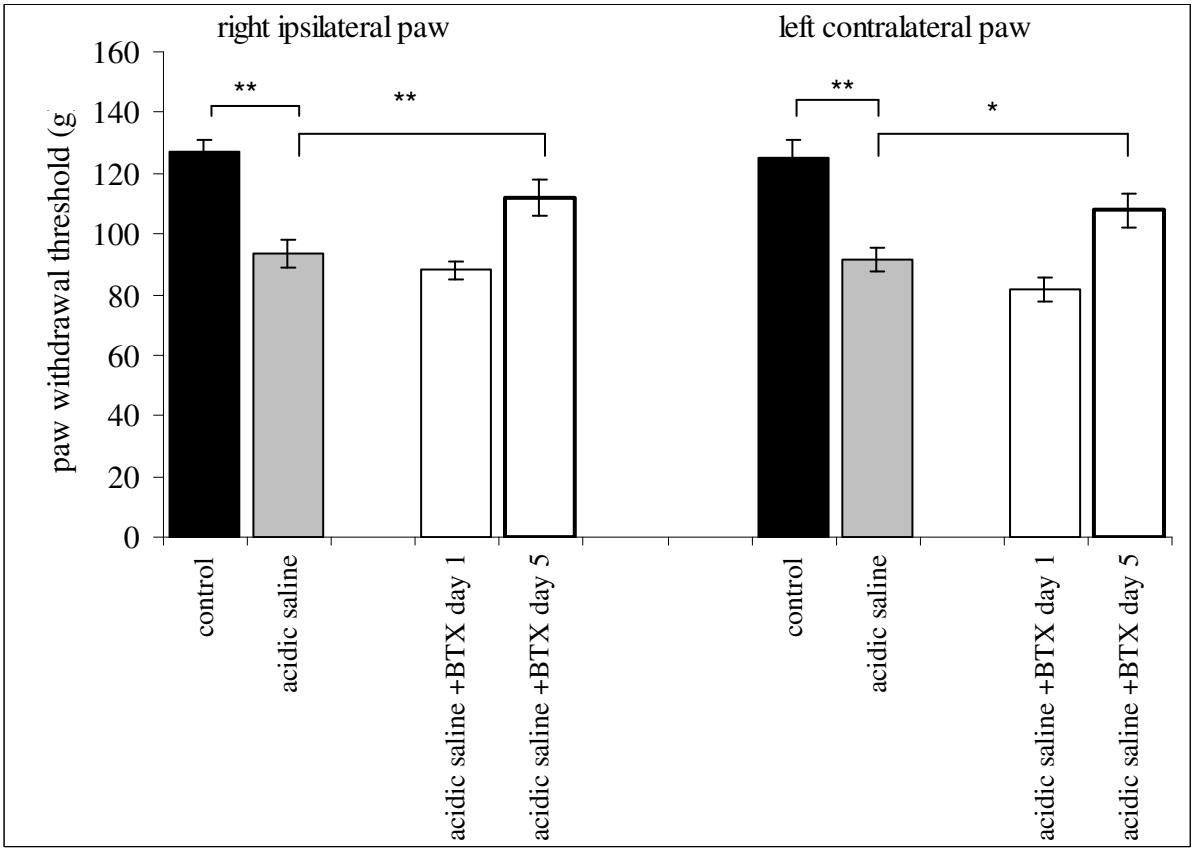

Fig 1. Appearance of the antinociceptive effect of BTX-A (5 U/kg s.c.) on the ipsilateral and the contralateral sides on day 1 and day 5 following the toxin application. Bilateral mechanical hyperalgesia was induced by two acidic saline injections into the right

gastrocnemius muscle. Results are presented as mean ± SEM, $n = 5–8$. *$p < 0.05$; **$p < 0.01$ (Newman–Keuls' post hoc test).

3.2. The antinociceptive effect is bilateral only if BTX-A is injected on the pain induction side

When BTX-A (5 U/kg) was injected s.c. into the left hindpaw pad contralaterally to the acidic saline injections, it reduced the mechanical hyperalgesia on that side only (Fig. 2).

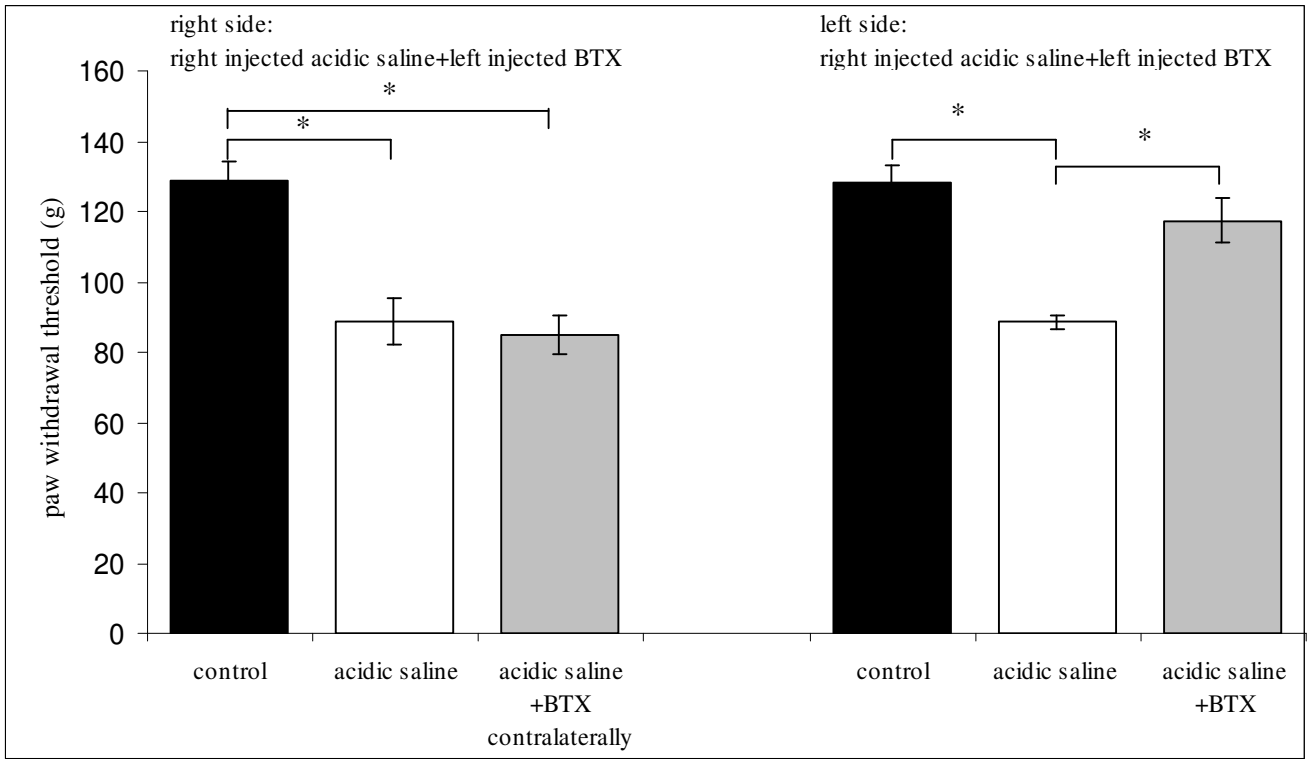

Fig. 2. Influence of BTX-A (5 U/kg) applied contralaterally to the pain induction side on bilateral mechanical hyperalgesia. Results are presented as mean ± SEM, $n = 5–6$. *$p < 0.01$ (Newman–Keuls' post hoc test).

3.3. The antinociceptive effect of BTX-A is independent of the peripheral nerve endings

BTX-A in a dose as low as 0.5 U/kg, injected into the proximal part of a distally cut sciatic nerve reduced mechanical hypersensitivity on the contralateral side on day 3 (on the ipsilateral side flaccid paresis of the hindlimb occurred) (Fig. 3).

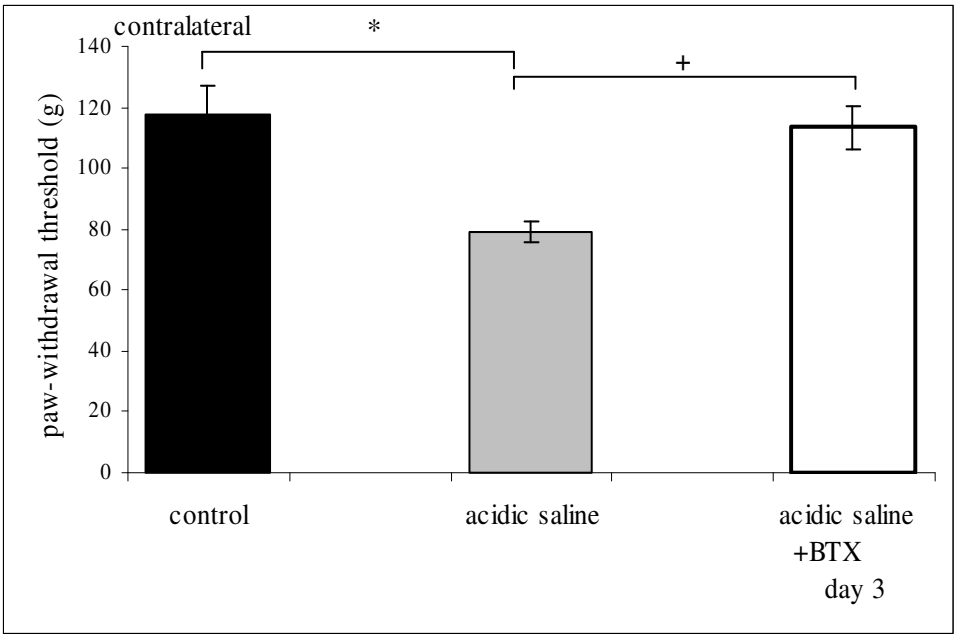

Fig. 3. Antinociceptive effect of BTX-A 0.5 U/kg, injected into the right sciatic nerve followed by a distal transection of the nerve, on the left contralateral side. Results are presented as mean ± SEM; $n = 7$. $^{*}p < 0.001$; $^{+}p < 0.05$ (Newman–Keuls' post hoc test).

3.4. Ipsilateral colchicine prevents the antinociceptive action of peripheral BTX-A

When colchicine, an axonal transport inhibitor, was injected into the ipsilateral sciatic nerve 1 day before the BTX-A (5 U/kg, s.c.) injection into the hindpaw pad, it prevented antinociceptive activity of the toxin on the ipsilateral as well as on the contralateral side (measured on day 5) (Tab.1). However, when colchicine was injected into the sciatic nerve opposite to the site of pain induction and BTX-A injection (1 day before the BTX-A 5 U/kg,

s.c.) it did not prevent the BTX-A antinociceptive effect on either side (Tab. 1). Mechanical hyperalgesia was not affected by colchicine per se.

Tab. 1.

Influence of intraneuronal colchicine (injected ipsilaterally and contralaterally to acidic saline and BTX-A) on the bilateral antinociceptive effect of BTX-A.

Treatment groups [a]	**Paw-withdrawal threshold (g)** [b]	
	Right ipsilateral paw	**Left contralateral paw**
Control	134 ± 15	146.6 ± 12.3
Acidic saline	101 ± 4.4*	105.5 ± 2.8*
Acidic saline + BTX-A	142.5 ± 3.6^{+}	138.5 ± 4.1^{+}
Acidic saline + ipsilateral colchicine	90.7 ± 4.5*	89 ± 4.6*
Acidic saline + BTX-A + ipsilateral colchicine	89.1 ± 3.8*	83 ± 7.7*
Acidic saline + contralateral colchicine	99.5 ± 4.4*	98.5 ± 5.9*
Acidic saline + BTX-A + contralateral colchicine	138.8 ± 3.9$^{+\#}$	133.5 ± 3.3$^{+\#}$

*$p < 0.001$ compared to control; $^{+}p < 0.001$ compared to acidic saline; $^{\#}p < 0.01$ compared to acidic saline + contralateral colchicine (Newman–Keuls' post hoc test).

[a] BTX-A (5 U/kg, s.c.) was applied into the rat right ipsilateral hindpaw on the pain induction side (ipsilaterally) one day after the colchicine (5 mM) injection into the right (ipsilateral) or left (contralateral) sciatic nerve. The measurements of mechanical sensitivity were done on day 5 following the toxin application.

[b] Results are presented as mean ± SEM; $n = 4–7$.

3.5. The effect of the intrathecal BTX-A application on mechanical hypersensitivity

Two days following the intrathecal injection in the lumbar cerebrospinal fluid, BTX-A in a dose 1 U/kg significantly reduced bilateral pain hypersensitivity induced by the acidic saline injections (Fig. 4).

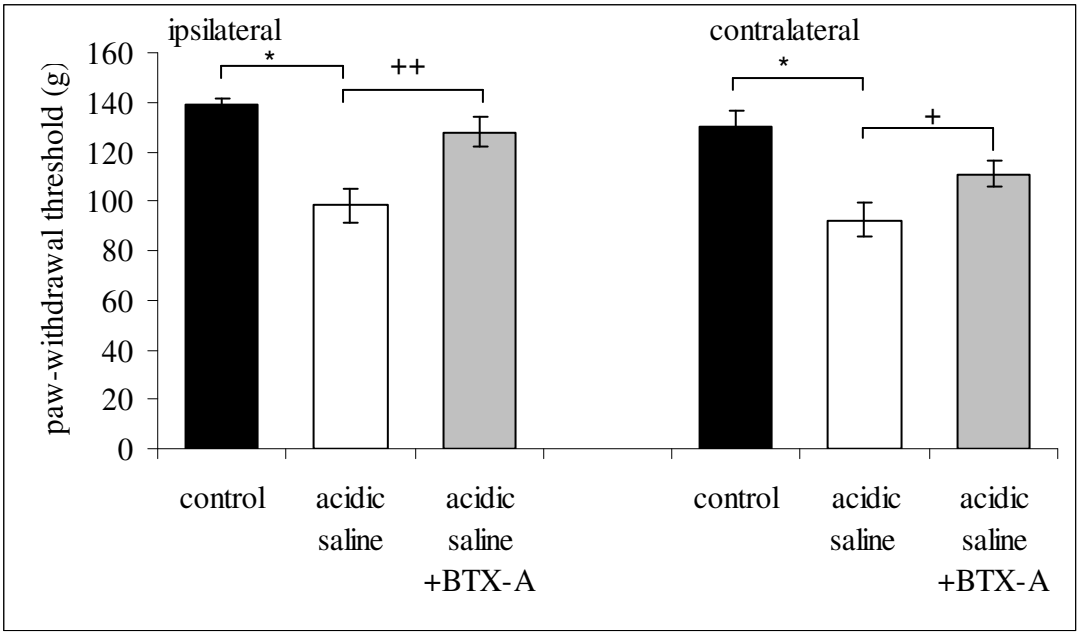

Fig. 4. Influence of BTX-A (1 U/kg), injected intrathecally at L3–L4 level, on mechanical hyperalgesia on the ipsilateral and the contralateral sides. Measurements were done 2 days after the BTX-A intrathecal injection. Results are presented as mean ± SEM, $n = 7–8$. $^{*}p < 0.001$; $^{+}p < 0.05$, $^{++}p < 0.01$ (Newman–Keuls' post hoc test).

4. DISCUSSION

4.1. Bilateral antinociceptive effect of BTX-A after unilateral application

Repeated intramuscular acidic saline injections (pH 4.0) produce a long-lasting mechanical hyperalgesia in rat (Sluka et al., 2001). Mechanical hyperalgesia from the muscles spreads to the adjacent tissue (paw) and to the contralateral side, i.e. a secondary hyperalgesia develops. Bilateral hyperalgesia was not abolished by a lidocaine injection into the same gastrocnemius muscle nor was it affected by a unilateral dorsal rhizotomy. It was assumed that the peripheral nervous system has negligible if any effect in the bilateral pain induced by acidic saline injections (Sluka et al., 2001). Bilateral effects of a unilateral injury have been reported in other pain models like bee venom, capsaicin and carrageenan (Chen et al., 2000; Sluka, 2002; Radhakrishnan et al., 2003). It is widely accepted that contralateral spread of hyperalgesia (mirror pain) depends most likely on the plastic changes in the CNS (central sensitization) and that it might be maintained by spinal and supraspinal mechanism (Koltzenburg et al., 1999; Graven-Nielsen and Arendt-Nielsen, 2002). An increase in the release of glutamate in the spinal cord was demonstrated after the second acidic saline injection (Skyba et al., 2005). Furthermore, spinal neurons show increased excitability after the acidic saline injections characterized by bilateral spread of the receptive field (Sluka et al., 2003) and bilateral increase in phosphorylation of the transcription factor CREB (Hoeger-Bement and Sluka, 2003). Recent experiments have shown that a descending facilitator input from the rostral ventromedial medulla (RVM) is involved in the initiation and maintenance of cutaneous and muscle hyperalgesia associated with chronic muscle pain (Tillu et al., 2008).

It is generally accepted that BTX-A acts peripherally and, consequently, it is difficult to imagine how BTX-A could affect hyperalgesia on the side contralateral to its peripheral injection. In spite of that, in the present study BTX-A injected into the rat hindpaw pad on the same side as i.m. acidic saline in a dose 5 U/kg not only reduced secondary mechanical

hyperalgesia on that side but, surprisingly, on the contralateral side as well. The effect on both sides was evident on day 5 and was of similar intensity (Fig. 1). At the same time, BTX-A did not affect the normal pain threshold on either side. This is in line with the previous observation by us and other authors that BTX-A effectively reduces only pain hypersensitivity but not the acute normal pain threshold (Bach-Rojecky and Lacković, 2005; Cui et al., 2004). Antinociceptive effect in present experiments could not be due to the possible locomotor deficits induced with BTX-A. Peripheral BTX-A injection into the hindpaw pad in a dose 5 U/kg did not affect the locomotion in our experiments (data not shown), nor in experiment done by Cui et al. (2004). Obviously, the antinociceptive effect of BTX-A in this model cannot be explained only by the common assumption about the peripheral origin of BTX-A action and a local inhibition of neuropeptide release from the sensory nerve endings. Bilateral effect of the unilateral peripheral BTX-A injection suggests the central action of BTX-A after its peripheral application.

When BTX-A was injected into the hindpaw pad contralateral to the pain induction side, it reduced mechanical hypersensitivity on that side only (Fig. 2). The observation that BTX-A is effective independently whether injected in the side with repeated tissue damage or in the contralateral side without any local damage deserves further investigation. However, this result is an exception because in all other presented experiments, the effect of BTX-A was bilateral as well as the biochemical and physiological changes associated with this model of mirror pain seem to be bilateral (Hoeger-Bement and Sluka, 2003). Obviously the mechanism of the BTX-A antinociceptive action injected on the side of pain induction and injected in the contralateral side are not equal. For now there is no answer to this puzzle since the contralateral spread of hyperalgesia in this model of “mirror pain” is not sufficiently understood.

4.2. Is antinociceptive effect of BTX-A independent of peripheral nerve endings?

There is theoretical possibility that BTX-A produces antinociceptive effect acting primarily on SNAP-25 in the peripheral nerve endings, while indirectly triggering some long lasting changes in the CNS. Several reports have indeed described changes at the level of the CNS in man and animals treated intramuscularly with BTX-A (Garner et al., 1993; Giladi, 1997; A bbruzzese and Berardelli, 2006). These changes were usually ascribed to plastic rearrangements subsequent to denervation or alterations in the sensory input after the toxin local application. To elaborate participation of periphery, BTX-A was injected directly into the sciatic nerve which was cut distally to the site of injection. In this experiment attention was paid that no BTX-A leaks outside the nerve. Even after such an injection, BTX-A produced a significant antinociceptive effect on the contralateral side (Fig. 3). In line with the common knowledge, transection of the sciatic nerve produced flaccid paralysis on the ipsilateral side. Because nerve transection prevents BTX-A to reach the peripheral nerve endings on that side, this experiment demonstrates that the antinociceptive effect of BTX-A could not be associated with the ipsilateral SNAP-25 cleavage in the peripheral cholinergic or any other peripheral nerve endings. It appears that the only explanation for the observed phenomenon might be that the antinociceptive effect on the contralateral side results from the central action of BTX-A after its retrograde axonal transport from the nerve trunk.

4.3. Evidence of an axonal transport of BTX-A

Antonucci et al. (2008) have recently detected a time-dependent bilateral SNAP-25 cleavage and blockade of neuronal activity after a unilateral toxin injection (0.2 μl of 10 nM toxin solution which corresponds to ~0.3 ng, i.e. 6 U of the toxin per animal) into the rat hippocampus. Additionally, retrograde appearance of the BTX-A truncated SNAP-25 in the retina after the toxin injection into the optic tectum was prevented by the microtubule depolymerizing agent colchicine. Furthermore, a cleaved SNAP-25 appeared in the facial

nucleus after the injection of the toxin (135 pg ~ 2.8 U) into the rat whisker muscles. Although using an indirect approach, Antonucci et al. (2008) were the first to offer novel pathways of BTX-A trafficking in neurons. From a clinical point of view, these findings raise the question whether BTX-A injected into muscles or cutis might induce unexpected central actions, and whether these actions might have clinical relevance (Currà and Berardelli, 2009). Nowadays there is evidence that after i.m. injection BTX-A might exert CNS effects, partially ascribed to plastic rearrangements subsequent to the peripheral blockade and partially due to retrograde axonal transport and direct BTX-A central effects (Caleo et al, 2009). At present, the functional consenquence of BTX-A axonal transport through motor and central neurons is not understood. Up to now, to our knowledge, there has been neither molecular nor behavioural evidence for the axonal transport of BTX-A within the sensory nerves.

Results of our experiments cannot be explained without the assumption that BTX-A is transported from the site of injection to the CNS. In our experiments, when colchicine, an axonal transport blocker, was injected into the sciatic nerve before the BTX-A application into the hindpaw pad, it completely prevented the effect of BTX-A on both sides (Tab. 1). To exclude the theoretical possibility of anterograde axonal transport of BTX-A from the CNS to the contralateral peripheral nerve endings, in one group of animals colchicine was injected into the sciatic nerve on the side contralateral to the s.c. BTX-A injection. In that experiment colchicine did not affect the toxin's antinociceptive activity on either side (Tab.1), thus eliminating possible contribution of the contralateral peripheral nerve endings to the bilateral BTX-A effect.

4.4. Antinociceptive effect of BTX-A after intrathecal application

A BTX-A intrathecal injection in a dose 1 U/kg abolished the acidic saline induced mechanical hypersensitivity on both sides (Fig. 4). Luvisetto et al. (2006) were the first to demonstrate antinociceptive effect after central (intracerebroventricular) injection of small

doses of BTX-A (1.8-3.5 pg/mice). They suggested that BTX-A might act not only at peripheral, but also at the central level. Although this is only a circumstantial argument, our results after intrathecal injection in the model of "mirror pain" are in line with suggestion of Luvisetto et al. (2006) and additionally support the central site of the BTX-A antinociceptive action.

4.5. Conclusion

The only explanation for the experiments presented here is that after the peripheral application of BTX-A, the toxin through axonal transport comes to the CNS, where it exhibits its antinociceptive effect. The precise site and mechanism of BTX-A action in the CNS remains to be elucidated.

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