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Behavioral and immunohistochemical evidence for central antinociceptive activity of botulinum toxin A

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Abstract

Botulinum toxin A (BTX-A) is approved for treatment of different cholinergic hyperactivity disorders, and, recently, migraine headache. Although suggested to act only locally, novel observations demonstrated bilateral reduction of pain after unilateral toxin injection, and proposed retrograde axonal transport, presumably in sensory neurons. However, up to now, axonal transport of BTX-A from periphery to CNS was identified only in motoneurons, but with unknown significance.

We assessed the effects of low doses of BTX-A injected into the rat whisker pad (3.5 U/kg) or into the sensory trigeminal ganglion (1 U/kg) on formalin-induced facial pain. Axonal transport was prevented by colchicine injection into the trigeminal ganglion (5 mM, 2 μ l). To find the possible site of action of axonally transported BTX-A, we employed immunohistochemical labeling of BTX-A–truncated synaptosomal-associated protein 25 (SNAP-25) in medullary dorsal horn of trigeminal nucleus caudalis after toxin injection into the whisker pad.

Both peripheral and intraganglionic BTX-A reduce phase II of formalin-induced pain.

Antinociceptive effect of BTX-A was prevented completely by colchicine. BTX-A–truncated SNAP-25 in medullary dorsal horn (spinal trigeminal nucleus) was evident 3 days following the peripheral treatment, even with low dose applied (3.5 U/kg).

Presented data provide the first evidence that axonal transport of BTX-A, obligatory for its antinociceptive effects, occurs via sensory neurons and is directed to sensory nociceptive nuclei in the CNS.

Keywords: axonal transport; botulinum toxin A; synaptosomal associated protein 25; antinociceptive activity; sensory neurons.

Abbreviations: BTX-A, botulinum toxin A; SNAP-25, synaptosomal-associated protein 25; i.g., intraganglionic (in trigeminal ganglion); SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; CGRP, calcitonin gene-related peptide.

Apart from its well known therapeutic use in muscular hyperactivity and certain autonomic disorders (Ward et al., 2006, Truong et al., 2009), botulinum toxin A (BTX-A) was recently registered for migraine treatment (Dodick et al., 2010). Besides migraine, its beneficial effects not associated with cholinergic neurotransmission were reported in cluster headache, neuropathic pain, joint pain, back pain, etc (Querama et al., 2010). It was suggested that BTX-A, similarly to its activity in cholinergic neurons, inhibits the local neurotransmitter release from sensory nerve endings by peripheral SNAP-25 (Synaptosomal Associated Protein of 25 kDa) cleavage (Cui et al., 2004; Aoki, 2005).

Recent studies of rat “mirror pain” (muscular hyperalgesia) and polyneuropathy models (paclitaxel-induced polyneuropathy, diabetic neuropathy) demonstrated bilateral effects following unilateral BTX-A injection (Bach-Rojecky and Lacković, 2009; Favre-Guilmond et al., 2009; Bach-Rojecky et al., 2010). Obviously, such effects cannot be explained only by local action on the sensory nerve endings adjacent to the site of injection. Importantly, BTX-A effects were prevented by colchicine-induced blockage of axonal transport in the sciatic nerve, suggesting that retrograde axonal transport of BTX-A is necessary for its antinociceptive action (Bach-Rojecky and Lacković, 2009). However, neurons involved in axonal transport are unknown as well as the destination of transported BTX-A. So far, axonal transport of BTX-A from periphery to the CNS was demonstrated only in motoneurons (Antonucci et al., 2008), but with unknown functional significance.

Aims of this study were to investigate a.) in which neurons does BTX-A axonal transport,

essential for its antinociceptive effects, take place, and b.) to locate the destination of axonally transported BTX-A. Therefore, we examined the effects of low doses of BTX-A injected into the whisker pad or trigeminal ganglion on formalin-induced orofacial pain, and used intraganglionic colchicine to prevent the axonal transport in trigeminal sensory neurons. To investigate the possible site of BTX-A antinociceptive action we employed immunolabeling of BTX-A-truncated SNAP-25 in trigeminal nucleus caudalis.

1. EXPERIMENTAL PROCEDURES

1.1 Animals

Male Wistar rats (University of Zagreb School of Medicine, Croatia), weighing 300-400 g, kept on 12 h/12h light and dark cycle, were used in all experiments. The 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. Animal procedures were approved by the Ethical Committee of University of Zagreb School of Medicine (permit No. 07-76/2005-43).

1.2 BTX-A injections

For peripheral administration, conscious, restrained rats were injected unilaterally with 30 μ l of saline-diluted BTX-A 3.5 U/kg (Botox®, Allergan Inc., Irvine, CA, USA) into the whisker pad tissue using a 27¹/₂-gauge needle. 3.5 U/kg dose was chosen based on previous experiments by Cui et al., (2004) and from our laboratory (Bach-Rojecky and Lacković 2005, Bach-Rojecky et al., 2005). For intraganglionic (i.g.) injections, animals were anesthetized with chloral hydrate (Sigma, St. Louis, MO, USA; 300 mg/kg i.p.). Trigeminal ganglion was

injected as described by Neubert et al. (2005). In brief, 0-10 µl Hamilton syringe needle (Hamilton Microliter #701, Hamilton, Switzerland) was inserted through the skin into the infraorbital foramen, which lies in the medial part of zygomatic process, and through the infraorbital canal and foramen rotundum directly into the trigeminal ganglion. Saline-diluted BTX-A (1 U/kg, 2 µl) was slowly injected into the ganglion. Dose of 1 U/kg was chosen based on preliminary experiments and on the dose needed for antinociceptive effect after intrathecal application (Bach-Rojecky et al., 2010). Site of injection was verified by injecting 2 µl of methylene blue (Sigma, St. Louis, MO, USA) to 5 animals. The dye resided only in trigeminal ganglion.

1.3 Behavioral testing

Antinociceptive activity of BTX-A was assessed in a model of formalin-induced facial pain. Conscious rats were injected with 50 µl of saline-diluted 2.5% formalin (Kemika, Zagreb, Croatia) (0.92 % formaldehyde) into the whisker pad ipsilateral to BTX-A pretreatment and placed in transparent cages for observation (45 min). Observer was blind to the animal treatment (however, experienced observer could see slightly atonic rat whisker movement in BTX-A peripherally treated animals). The number of seconds of formalin-induced ipsilateral facial rubbing/grooming was measured in 3 min periods during phases I and II of formalin-induced pain (Raboisson and Dallel, 2004). Phase I (0-12 min) represents the acute nociceptive pain characterized by direct stimulation of nerve endings with formalin, while phase II (12-45 min) is characterized by the release of inflammatory mediators and sensitization (Cui et al., 2004, Raboisson and Dallel, 2004).

-For testing of peripherally applied BTX-A (3.5 U/kg), rats were divided in 3 groups (5-6 animals per group): 1. saline (i.g.) + saline peripherally, 2. saline (i.g.) + BTX-A peripherally, 3. colchicine (i.g.) + BTX-A peripherally.

-For intraganglionic BTX-A testing (1U/kg), rats were divided into 5 groups (4-7 animals per group): 1. saline (i.g.), 2. BTX-A (i.g.) 1 day, 3. BTX-A (i.g.) 2 days, 4. colchicine (i.g.) + BTX-A (i.g.) 2 days, 5. colchicine (i.g.) + saline (i.g.) 2 days.

Colchicine (Sigma, St. Louis, MO, USA) was injected i.g. (2 µl), as described above, 24 h prior to second injection (BTX-A or saline), ipsilaterally. To reduce the number of animals used, effects of intraganglionic colchicine (colchicine + saline group) on formalin-induced pain were tested only in the experiment with intraganglionic BTX-A application. Rats were tested 3 days after peripheral BTX-A injection based on data of Antonucci et al. (2008) and our preliminary experiments, and 1 and 2 days after intraganglionic BTX-A injection.

1.4 Characterization of the antibody specificity to BTX-A-cleaved SNAP-25 by Western blot

The antibody used for immunohistochemical detection of BTX-A-cleaved SNAP-25 (a kind gift from Assist. Prof. Ornella Rossetto, University of Padua, Italy) was used previously in study from Antonucci et al. (2008). One of the questions to the authors, regarding the specificity of the antibody to cleaved SNAP-25, is the lack of controls to differentiate between cleaved and non-cleaved SNAP-25. Thus, using the similar protocol as Antonucci et al. (2008), we injected BTX-A (4 U/rat) in rat dorsal hippocampus. One day following the treatment rats were sacrificed and hippocampus excised. Protein isolation, SDS-PAGE and Western blots were performed as described previously (Antonucci et al., 2008, Constantin et al., 2005). To visualize cleaved SNAP-25, and then the total SNAP-25, two sequential Western blots were performed on the same membrane. Membranes were blocked and incubated firstly with rabbit anti-cleaved SNAP-25 (1:500) in blocking solution overnight at 4°C, and then with goat-anti-rabbit HRP-conjugated secondary antibody (Biosource, Invitrogen, Carlsbad, CA, USA). After development in chemoluminescent (Super Signal West Femto, Pierce, Rockford, IL, USA) and visualization by ECL camera (Biorad, Hercules, CA, USA), membrane was washed and incubated with the mouse monoclonal antibody to total SNAP-25 (1:5000, overnight at 4°C). Antibody to total SNAP-25 (SMI-81, Sternberger Monoclonals, Baltimore, MD, USA) is well characterized and recognizes both intact and BTX-A-cleaved SNAP-25 (Jurasinski et al., 2001). Then, the membrane was incubated with goat anti-mouse secondary antibody (BD Pharmingen, San Diego, CA, USA) and visualized.

As shown in a Figure 1., cleaved SNAP-25 (approximately 24 kDa band) was visible only in BTX-A-treated hippocampus and positioned under non-cleaved SNAP-25 (25 kDa band).

Figure 1

1.5 Immunohistochemistry

For the time-course experiment rats were injected into the whisker pad with 15 U/kg BTX-A, and sacrificed 1, 3, or 5 days after BTX-A injection. To access the BTX-A effects at different peripheral doses, rats were injected with 3.5 U/kg, 15 U/kg, and 30 U/kg into the whisker pad and sacrificed after 5 days. Doses were chosen based on study from Cui et al. (2004). Rats were deeply anesthetized using chloral hydrate (300 mg/kg, i.p.) and transcardially perfused with 250 ml saline, followed by 250 ml of fixative (4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA) in 0.01 M PBS, pH 7.4). Brainstems with upper cervical spinal cords were dissected and cryoprotected at 4°C overnight in 15% sucrose in fixative, followed by 30 % sucrose in PBS the next day, until the tissue sank. Immunohistochemical protocol was similar to that previously described (Antonucci et al., 2008). 40 µm coronal sections (medullas caudal from obex and upper cervical spinal cords) were cut on a cryostat and collected for free floating in PBS. Sections were washed 3 x 5 minutes in 0.25% Triton X-100 in PBS (PBST), blocked in 10% normal goat serum (Monosan, Uden, Holland) (NGS) (1h) and incubated overnight at room temperature with 1:400 anti-BTX-A-cleaved SNAP-25 rabbit polyclonal antibody in 1% NGS. The next day, sections were washed, blocked with 1% NGS for 30 minutes and further incubated with 1:400 goat anti-rabbit Alexa Fluor-555 ((Invitrogen, Carlsbad, CA, USA)), in the dark for 2h at room temperature. Sections were then washed with PBST, mounted on glass slides with anti-fading agent (FluoroGel, Electron Microscopy Sciences, Hatfield, PA, USA) and visualized with fluorescent microscope (Olympus BX51, Olympus, Tokyo, Japan) connected to digital camera (Olympus DP-70, Olympus, Tokyo,

Japan).

To obtain high-resolution image of the whole section, parts of brainstem sections were photographed using 10x magnification, and subsequently connected using Microsoft Paint software. Images were processed for brightness and contrast using Adobe Photoshop software.

1.6 Statistical analysis

The results of orofacial formalin test were presented as mean \pm S.E.M. Between-group differences were analyzed by the Newman-Keuls post hoc test. $P < 0.05$ was considered significant.

2 RESULTS

2.1 BTX-A reduces formalin-induced orofacial pain: necessity of axonal transport in sensory neurons

BTX-A had no significant antinociceptive effects during phase I of formalin-induced pain. However, peripheral BTX-A pretreatment (3.5 U/kg) significantly reduced the time of facial grooming during phase II of formalin induced pain (measured 3 days post BTX-A injection). Injection of colchicine (5 mM) into trigeminal ganglion abolished the effect of subsequently applied BTX-A (Figure 2).

Intraganglionic BTX-A (1 U/kg) reduced the formalin-induced face grooming 2 days after the injection (Figure 3). Pain was not significantly reduced when BTX-A was applied i.g. 1 day before the formalin test. Intraganglionic pretreatment with 5 mM colchicine prevented the antinociceptive effect of intraganglionic BTX-A. Intraganglionic injection of colchicine (5 mM) alone did not alter formalin-induced pain (Figure 3).

Figure 2

Figure 3

2.2 Central SNAP-25 cleavage in TNC after BTX-A peripheral application

Following application of BTX-A into the rat whisker pad (15 U/kg), fiber-like cleaved SNAP-25 immunoreactivity, apparently with varicosities, appeared in dorsal horn of ipsilateral TNC (Figures 4 and 5). Occurrence of truncated SNAP-25 in dorsal horn was evident starting on day 3 after the injection but not on day 1 (Figure 5 A). Truncated SNAP-25 in TNC also appeared at lower and higher peripheral doses (3.5 and 30 U/kg) (Figure 5B). We did not quantify the immunoreactivity, however, our impression was that 3.5 U/kg dose produces less intensive cleaved SNAP-25 immunofluorescence than higher doses (15 U/kg and 30 U/kg). Cleaved SNAP-25 was present throughout the rostro-caudal length of the TNC and confined to the middle part of the medullar dorsal horn coronal section (TNC according to rat brain atlas (Paxinos and Watson, 2005)). This is in accordance with the dorso-ventral somatotopic organization of spinal trigeminal nuclei, whose middle portion belongs to maxillary branch of trigeminal nerve, which innervates the whisker pad (Florence and Lakshman, 1995; Capra and Dessem, 1992). In some sections only few immunoreactive fiber-like structures were visible in contralateral dorsal horn. Some immunoreactivity was also observed inconsistently in C1 upper cervical dorsal horn.

Figure 4

Figure 5

3 Discussion

Cleavage of SNAP-25 at neuromuscular junctions or autonomic synapses, induced by enzymatic activity of BTX-A light chain, results in blockade of acetylcholine (ACh) release. Analogous mechanism was proposed as an explanation of antinociceptive activity in peripheral sensory neurons (Cui et al., 2004; Aoki, 2005). In formalin-induced inflammatory

pain, peripheral BTX-A pretreatment had no effect on the acute nociceptive pain in phase I but it reduced pain during inflammatory phase II, accompanied by lowered peripheral glutamate release and reduction of edema in inflamed paw tissue. The most logical explanation was that both antinociceptive and anti-inflammatory actions of BTX-A are mediated by blockage of neurotransmitter and inflammatory mediator release from sensory nerve endings, as a consequence of peripheral SNAP-25 cleavage (Aoki, 2005). However, further studies with other inflammatory pain models showed dissociation between anti-inflammatory and antinociceptive activity of BTX-A. When applied in doses that effectively reduced pain, BTX-A had no effect on capsaicin-induced neurogenic inflammation and carrageenan-induced edema (Bach-Rojecky and Lacković, 2005; Bach-Rojecky et al., 2007; Favre-Guilmond et al., 2009). Since inflammation is a peripheral phenomenon, the lack of the BTX-A effect on inflammation while concomitantly reducing pain brought into question the inhibition of peripheral exocytosis as a main mechanism of the antinociceptive action.

3.1 Involvement of CNS and importance of axonal transport for BTX-A antinociceptive activity

Evidence of antinociceptive activity distant from the site of peripheral unilateral BTX-A injection was found in paclitaxel- induced polyneuropathy (Favre-Guilmond et al., 2009), acidic saline-induced muscular hyperalgesia (Bach-Rojecky and Lacković, 2009) , and diabetic neuropathy (Bach-Rojecky et al., 2010). In those reports, apart from the injected side, BTX-A reduced the pain on contralateral side, too. Also, BTX-A injection into the distally cut sciatic nerve was still able to reduce contralateral pain in a model of bilateral muscular hyperalgesia, thus excluding the involvement of peripheral nerve endings (Bach-Rojecky and Lacković, 2009). In this model, effect of peripheral BTX-A was prevented by colchicine

injection into the sciatic nerve (Bach-Rojecky and Lacković, 2009). These observations demonstrated the necessity of retrograde axonal transport for BTX-A antinociceptive activity, and probably a central site of toxin's action. In line with that, antinociceptive activity of BTX-A is obtained with lower doses and with faster onset after intrathecal than after peripheral injection (Bach-Rojecky et al., 2010). Recently, it was found that increased vesicular release from trigeminal ganglionic cells acutely isolated from rats with experimental trigeminal neuropathy was prevented if animals were pretreated with peripherally applied BTX-A (Kitamura et al., 2009). The authors suggested BTX-A retrograde transport from periphery and transcytosis within ganglionic somata as a possible explanation. Most recent studies of BTX-A effects on regenerative processes in sciatic nerve and neuroimmunological changes in dorsal root ganglia and lumbal dorsal horn of rats with experimental neuropathy also suggest the possible direct BTX-A action distant from the site of injection due to retrograde axonal transport (Marinelli et al., 2010, Mika et al., 2011, Pavone and Luvisetto, 2010). Observations described up to now suggest that antinociceptive action of BTX-A is centrally mediated and axonal transport-dependent. To verify the hypothesis that BTX-A is axonally transported through sensory neurons, in present experiments we investigated BTX-A effects in trigeminal sensory system. In a model of pain induced by formalin injection into the whisker pad, peripherally applied BTX-A (3.5 U/kg) reduced inflammatory phase II of formalin pain. Effects of peripheral BTX-A were completely abolished by colchicine injection into the trigeminal sensory ganglion (Figure 2). Intraganglionic BTX-A in a dose of 1 U/kg in our experiment had similar effect like peripheral dose of 3.5 U/kg BTX-A. These observations confirm the assumption that in present experiments, axonal transport of BTX-A occurs via trigeminal sensory neurons and rules out the importance of axonal transport via motor or sympathetic neurons for antinociceptive effects of BTX-A.

Since Kitamura et al. (2009) suggested blockage of vesicular release and BTX-A transcytosis

within ganglionic somata, we investigated whether BTX-A antinociceptive activity is caused by its actions inside the ganglion. We speculated that direct i.g. delivery of BTX-A would cause the spread of toxin within the ganglion and should enable fast antinociceptive effect. However, in the present experiment this action was delayed, occurring after 2 days, opening the possibility that the ganglion itself is not the main site of BTX-A antinociceptive activity. Moreover, by blocking axonal transport with colchicine pretreatment, effect of intraganglionic BTX-A was prevented (Figure 3), suggesting that BTX-A, even when delivered directly to the ganglion, still requires axonal transport to exert its antinociceptive effects. Thus, we hypothesized that BTX-A could be axonally transported, via sensory root, to trigeminal nociceptive projections in central nervous system.

3.2 Occurrence of truncated SNAP -25 in the CNS after BTX-A injection in trigeminal innervation area

To verify our assumption about the axonal transport of peripherally applied BTX-A to central trigeminal projections, we employed immunohistochemical labeling of truncated SNAP-25 in trigeminal nucleus caudalis (TNC), the region that predominantly receives facial nociceptive input. By demonstrating SNAP-25 cleavage in TNC we found that peripherally applied BTX-A, or its catalytically active fragments, most probably reach central projections of primary sensory neurons by axonal transport (Figures 4 and 5). Time of occurrence of truncated SNAP-25 in our experiment (Figure 5A) is similar to the time course of BTX-A traffic in central neurons and motoneurons (Antonucci et al., 2008).

Theoretically it might be possible that, instead of BTX-A, the truncated SNAP 25– product of its proteolytic activity, was axonally transported to central trigeminal nuclei. However, previously it was shown that BTX-A injection into the sciatic nerve, which was cut distally to

the place of toxin's injection, still reduces pain on contralateral side (Bach-Rojecky and Lacković, 2009). This experiment rules out the possibility that cleaved SNAP-25, transported from peripheral nerve endings, would be involved in antinociceptive effects of BTX-A. Possibility of contribution of SNAP-25 cleavage in trigeminal ganglion cannot be ruled out completely.

Confinement of cleaved SNAP-25 immunoreactivity to medullary dorsal horn excludes possible systemic spreading of BTX-A. Moreover, animals injected into whisker pad with 3.5 and 15 U/kg did not exhibit impaired rotarod performance (results not shown). However, in some sections, few immunoreactive fiber-like structures were also visible in contralateral dorsal horns (data not shown), which could be associated with contralateral crossing of central afferent terminals (Jacquin et al., 1990).

Occurrence of truncated SNAP-25 in TNC suggest that peripherally applied, axonally transported BTX-A can affect second order central sensory neurons, either presynaptically by SNAP-25 cleavage in central terminals of primary afferent neurons, or following transcytosis. Transcytosis of BTX-A to second-order synapses has been suggested in retinal ganglionic cells after axonal transport within visual system (Antonucci et al., 2008). Recently Kitamura et al. (2009) on the basis of in vitro experiments proposed existence of BTX-A transcytosis in trigeminal ganglia, too.

SNAP-25 cleavage in medullary dorsal horn (Figures 4 and 5) suggests that BTX-A can alter central nociceptive transmission of presently unknown neurotransmitters, which requires further investigation. There is evidence that, when applied directly to brainstem slices or intrathecally at the level of spinal cord, BTX-A alters release and expression of CGRP (Calcitonin gene related peptide), a neuropeptide involved in pain transmission (Meng et al., 2009; Lee et al., 2010).

Importantly, we found that BTX-A-truncated SNAP-25 appeared in TNC even at 3.5 U/kg,

the lowest peripheral antinociceptive dose (Bach-Rojecky et al., 2005, Bach-Rojecky and Lacković, 2005). Therefore, the axonal traffic of BTX-A from periphery to the CNS is not a phenomenon occurring only at high doses, as proposed by Alexiades –Armenakas (2008). Although comparison of doses in rats and humans can always be questioned, dose used in our experiment (3.5 U/kg) corresponds to 245 U dose in 70 kg human. BTX-A doses typically used in migraine treatment range from 100 to 260 U (Aurora et al., 2007), and in recent study which resulted in FDA approval for migraine treatment, from 155 to 195 U (Dodick et al., 2010).

4 Conclusion

Antinociceptive effect of BTX-A requires axonal transport through sensory neurons and it is associated with occurrence of truncated SNAP-25 in central sensory nociceptive nuclei.

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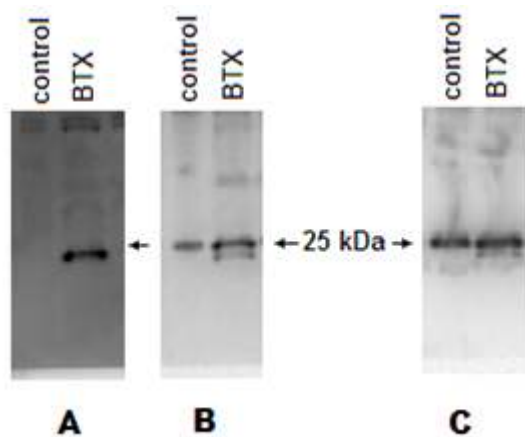


Figure 1. Characterization of specificity of polyclonal antibody to cleaved SNAP-25 fragment.

A) Western blot membrane processed with primary antibody to cleaved SNAP-25 (1:500 dilution) and appropriate secondary antibody; B) the same membrane subsequently, before secondary antibody, incubated with antibody to total SNAP-25 (SMI-81, 1:5000 dilution) which recognizes both cleaved and intact SNAP-25; C) Separate experiment with the membranes incubated only with primary antibody to total SNAP-25 (the same as in experiment B) and appropriate secondary antibody. Control– hippocampus from saline-injected animal; BTX– hippocampus injected with BTX-A

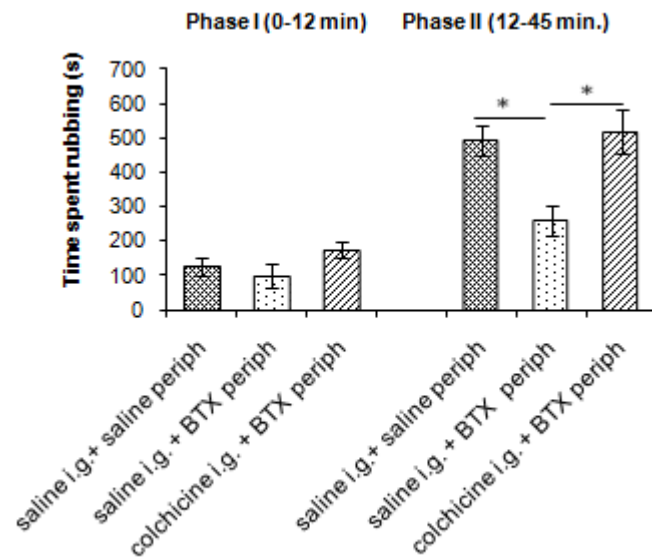


Figure 2. Effects of peripherally applied BTX-A on pain (facial rubbing) in first and second phase of orofacial formalin test and essential role of axonal transport.

Facial pain was produced by formalin injection into the whisker pad (2.5% formalin, 50 μ l).

BTX-A (3.5 U/kg) was also applied into the whisker pad. Colchicine was injected into the

trigeminal ganglion (5 mM, 2 μ l) 24 h prior to BTX-A or saline injection into the whisker pad.

Measurements were performed 3 days after BTX-A injection. i.g. – intraganglionic

application into the trigeminal ganglion; periph – peripheral application into the whisker pad.

Data are represented as mean \pm SEM, n=5-6, * - $P < 0.05$ (Newman-Keuls post hoc).

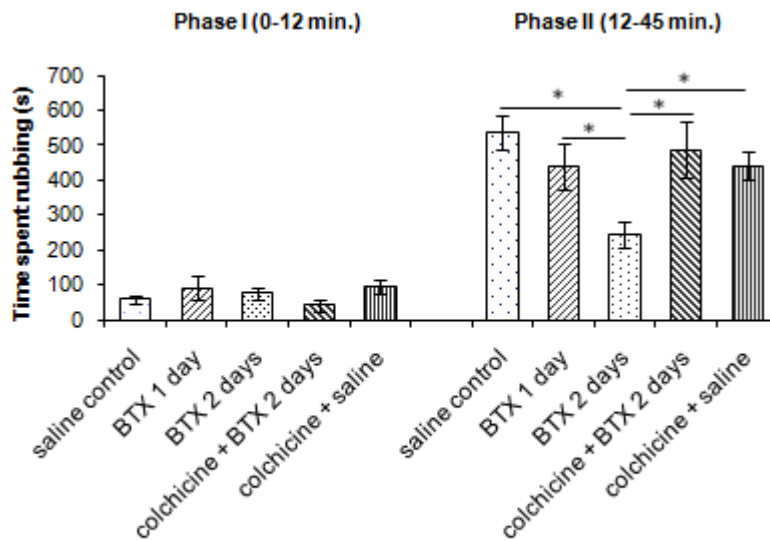


Figure 3. Effects of intraganglionic BTX-A on pain (facial rubbing) in first and second phase of orofacial formalin test and essential role of axonal transport.

Facial pain was produced by formalin injection into the whisker pad (2.5% formalin, 50 μ l). BTX-A (1U/kg) was injected into the trigeminal ganglion. Colchicine was also injected into the trigeminal ganglion (5 mM, 2 μ l) 24 h before BTX-A or saline. Effect of BTX-A were measured on first and second day after the application.. BTX 1 day – BTX-A applied 1 day before the formalin test; BTX 2 days – BTX-A applied 2 days before the formalin test. Data are represented as mean \pm SEM, n=4-7, * = P< 0.05 (Newman-Keuls post hoc).

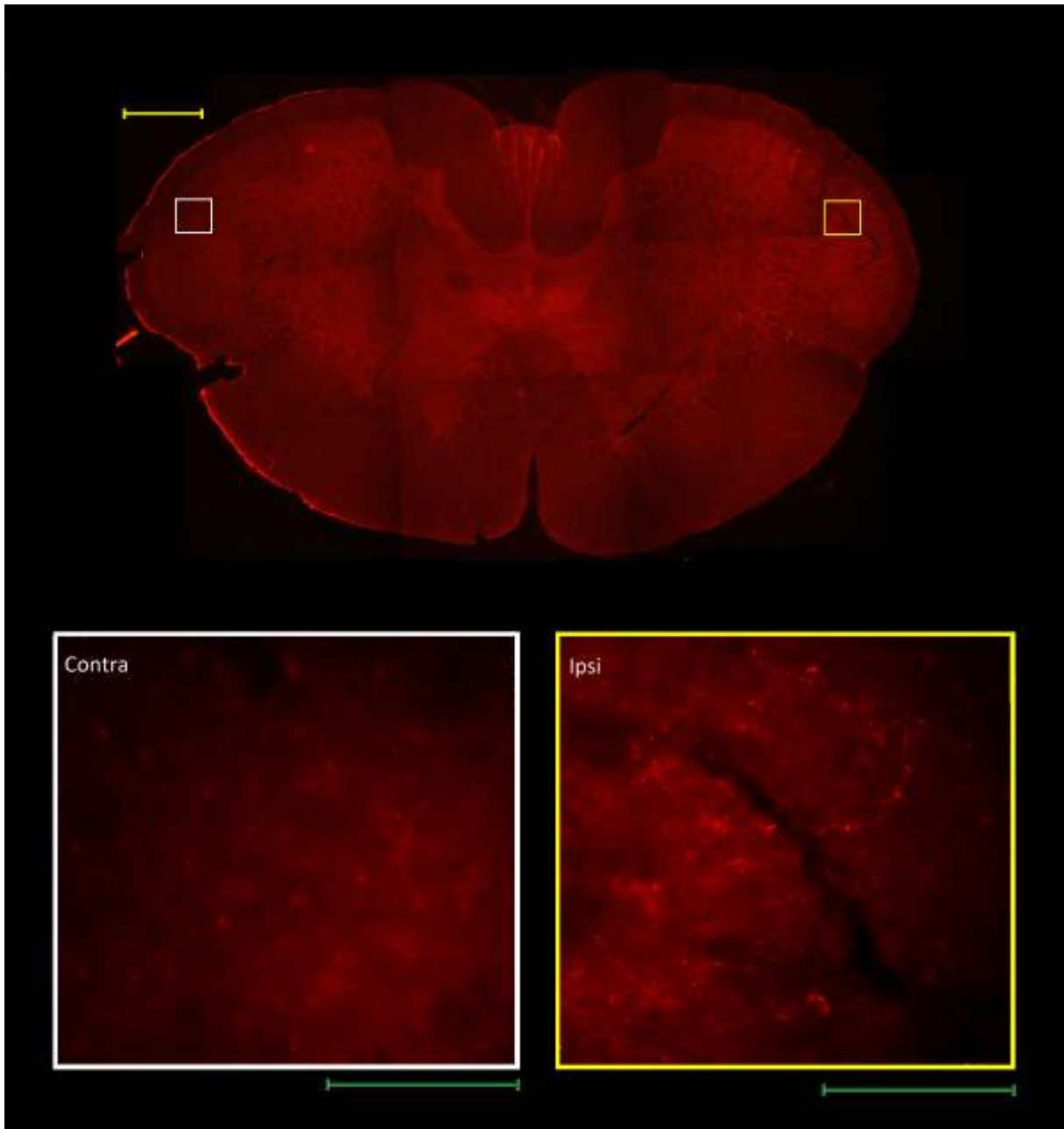


Figure 4. Immunofluorescently labeled truncated SNAP-25 (light red) in coronal sections of rat caudal medulla 5 days after BTX-A (15 U/kg) injection into the whisker pad.

Contra=TNC contralateral to the site of injection. Ipsi= TNC ipsilateral to the site of injection; Section approx. 1.4 mm caudal from obex. Yellow scale bar, 500 μm (10x magnification); green scale bar, 100 μm (40x magnification).

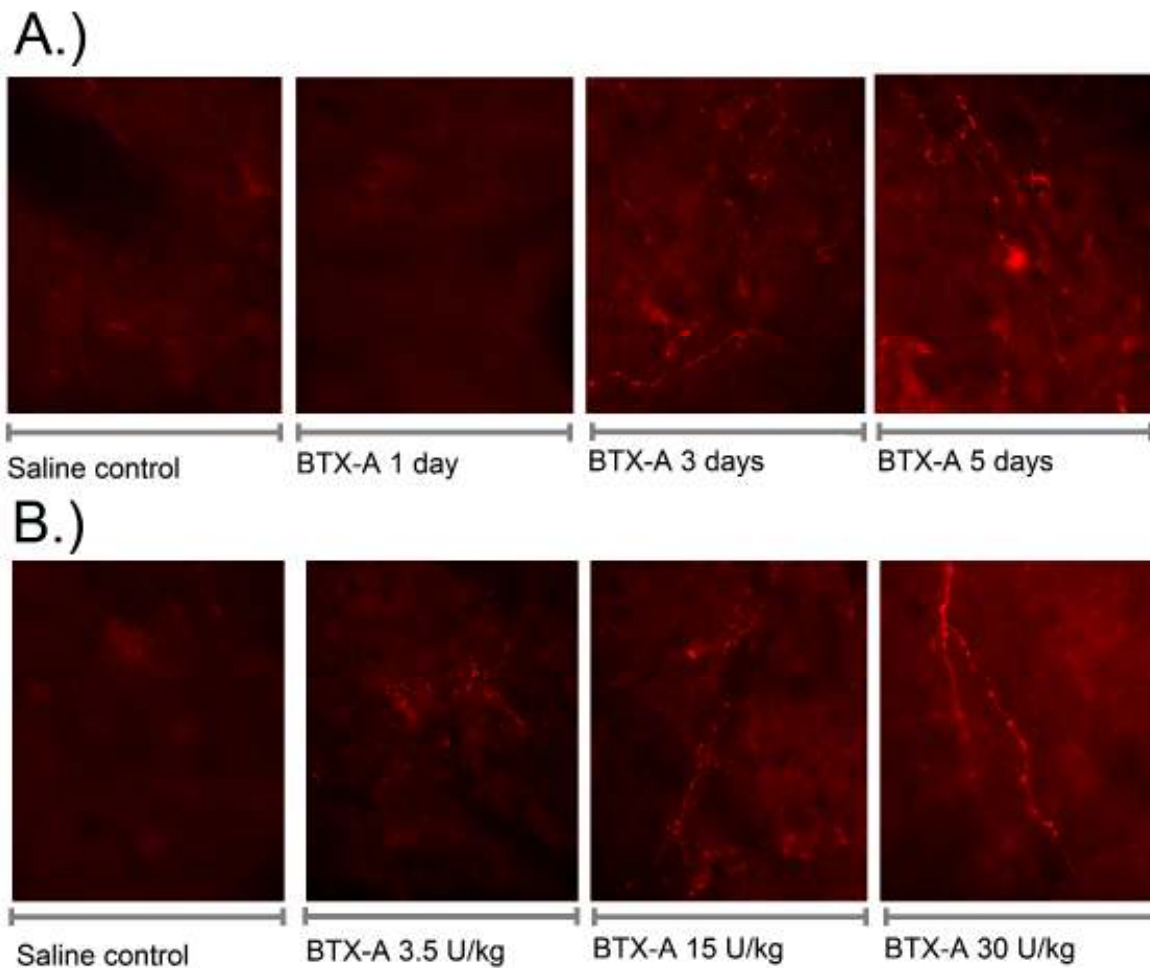


Figure 5. Evidence of central enzymatic activity of BTX-A in ipsilateral TNC after peripheral application.

A.) Time course: Occurrence of truncated SNAP-25 1, 3 and 5 days after BTX-A injection into the whisker pad (15 U/kg). B.) Effect of different doses: truncated SNAP-25 in ipsilateral TNC 5 days after peripheral BTX-A application at different doses (3.5, 15 and 30 U/kg).

Sections approx. 1-1.5 mm caudal from obex. 40 x magnification; scale bar (gray line), 100 μm .