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# **BOTULINUM TOXIN'S AXONAL TRANSPORT FROM PERIPHERY TO THE SPINAL CORD**

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Abbreviations used in the text <sup>1</sup>

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<sup>1</sup> BTX-A (botulinum toxin A), SNAP-25 (synaptosomal-associated protein 25), s.c. (subcutaneous), i.m. (intramuscular), i.n. (intraneural), PBS (phosphate-buffered saline), NGS (normal goat serum), SDS (sodium dodecyl sulphate), BTX-E (botulinum toxin E)

**Abstract:**

Axonal transport of enzymatically active botulinum toxin A (BTX-A) from periphery to the CNS has been described in facial and trigeminal nerve, leading to cleavage of synaptosomal-associated protein 25 (SNAP-25) in central nuclei. Aim of present study was to examine the existence of axonal transport of peripherally applied BTX-A to spinal cord via sciatic nerve. We employed BTX-A-cleaved SNAP-25 immunohistochemistry of lumbar spinal cord after intramuscular and subcutaneous hind limb injections, and intraneural BTX-A sciatic nerve injections. Truncated SNAP-25 in ipsilateral spinal cord ventral horns and dorsal horns appeared after single peripheral BTX-A administrations, even at low intramuscular dose applied (5 U/kg). Cleaved SNAP-25 appearance in the spinal cord after BTX-A injection into the sciatic nerve was prevented by proximal intrasciatic injection of colchicine (5 mM, 2  $\mu$ l). Cleaved SNAP-25 in ventral horn, using choline-acetyltransferase (ChAT) double labeling, was localized within cholinergic neurons.

These results extend the recent findings on BTX-A retrograde axonal transport in facial and trigeminal nerve. Appearance of truncated SNAP-25 in spinal cord following low-dose peripheral BTX-A suggest that the axonal transport of BTX-A occurs commonly following peripheral application.

**Highlights:** > Peripheral BTX-A cleaves SNAP-25 in dorsal and ventral horn of the spinal cord.  
> Axonal transport of BTX-A occurs at low intramuscular dose.> BTX-A retrograde transport occurs via peripheral nerves and is microtubule-dependent. > In ventral horn truncated SNAP-25 was localized within cholinergic neurons

**Keywords:** botulinum toxin A, synaptosomal-associated protein 25, retrograde axonal transport, spinal cord, peripheral nerve, immunohistochemistry

## 1. INTRODUCTION

It is a textbook knowledge that botulinum toxin type A (BTX-A) in botulism, as well as in therapeutic applications, exerts the neuromuscular paralysis by enzymatic cleavage of peripheral synaptosomal-associated protein 25 (SNAP-25), involved in neuroexocytosis. Unlike related tetanus toxin, which is known to be retrogradely transported and transcytosed to second-order synapses (Schwab et al., 1979), it was generally accepted that BTX-A acts directly only on peripheral nerve endings. Nevertheless, already in the 70-ties some authors reported axonal transport of radioactively labeled botulinum toxin A (BTX-A) within peripheral nerves to spinal cord (Habermann, 1974; Wiegand et al., 1976). Those observations remained forgotten and questioned in later studies (Tang Liu et al., 2003). Main objections to these early studies were that it was not known if radioactively labeled BTX-A retained the enzymatic activity by the time it reached spinal cord. However, axonal transport of functional BTX-A molecules was recently found in hippocampus, visual system and in facial motoneurons (Antonucci et al., 2008). BTX-A axonal transport followed by enzymatic cleavage in CNS has been demonstrated in trigeminal sensory neurons (Matak et al., 2011). Recent *in vitro* study suggests spread of BTX-A within cell bodies and distal processes of cultured sympathetic neurons (Lawrence et al., 2011).

Retrograde axonal transport of low dose BTX-A in spinal sensory neurons has been suggested by behavioral experiments in models of bilateral muscular hyperalgesia (Bach-Rojecky & Lacković, 2009) and diabetic neuropathy (Bach-Rojecky et al., 2010).

In present study we found the enzymatic activity of BTX-A in rat motor and sensory regions of the spinal cord after intramuscular, subcutaneous, or intraneural toxin application.

## **2. MATERIALS & METHODS**

### **2.1 Animals**

18 male Wistar rats (University of Zagreb School of Medicine, Croatia), weighing 300-400 g, kept on 12 h/12h light and dark cycle with unlimited access to food and water, 3 months old, were used. Experiments were conducted according to the European Communities Council Directive (86/609/EEC). Animal procedures were approved by the Ethical Committee of University of Zagreb, School of Medicine (permit No. 07-76/2005-43). All efforts were made to reduce the suffering of animals and the number of animals used.

### **2.2 Experimental procedure**

Animals were injected unilaterally with BTX-A (Botox, Allergan, Irvine, CA , USA) diluted in 0.9% saline. One international unit (1 U) of BTX-A, equal to mouse LD<sub>50</sub>, contains approximately 48 pg of 900 kDa neurotoxin. Animals were divided into 6 treatment groups (3 animals per group): group 1: BTX-A injected subcutaneously (s.c.) into the plantar side of hind-paw pad (30 U/kg, in volume of 30 µl); groups 2 and 3: BTX-A injected intramuscularly (i.m.) into the gastrocnemius (5 U/kg and 30 U/kg, 30 µl). In groups 4 and 5 BTX-A was injected intraneurally (i.n.) into the sciatic nerve (10 U/kg, 2 µl). 2 µl of 5 mM axonal transport blocker colchicine (Sigma, St. Louis, MO, USA) (group 4) or saline (group 5) were injected into the sciatic nerve 24 h prior to the more distal i.n. injections of BTX-A. To reduce the number of

animals in the experiment, single control group (group 6) consisting of 0.9% saline (i.m.) - treated animals was used.

5 U/kg low dose and 30 U/kg BTX-A high dose was chosen based on previous experiments (Cui et al, 2004; Bach-Rojecky et al., 2005), and 10 U/kg dose was chosen based on preliminary data. 5 U/kg dose in humans (350 U for a 70 kg average human) is within the dose-range regularly used for treatment of spasticity (Intiso, 2012).

For i.m. and s.c. injections rats were restrained, while for the i.n. injections animals were deeply anesthetized (chloral hydrate, Sigma, St. Louis, MO, USA; 300 mg/kg intraperitoneally). Sciatic nerve was exposed after skin incision at mid-femoral level and blunt dissection through the thigh muscles. Special care was made to check for possible leakage by placing piece of parafilm under the nerve prior to i.n. injection. 0-10 µl Hamilton needle (Hamilton, Bonaduz, Switzerland) was used to inject saline/colchicine and BTX-A into the nerves. 3 minutes following the treatment, parafilm was removed, the nerve returned to previous position and the skin sutured. After the operation animals were left to recover from anesthesia under warm bulb light and returned to their cages.

### **2.3 Immunohistochemistry**

Animal preparation and immunohistochemistry was performed similarly as previously described (Matak et al., 2011, Antonucci et al., 2008). In brief, 5 days after BTX-A s.c and i.m. injections or 3 days following i.n. injections, rats were deeply anesthetized with chloral hydrate and transcardially perfused with 0.9 % saline followed by fixative (4% paraformaldehyde in 0.01 M phosphate-buffered saline (PBS)). Lumbal spinal cords were removed, cryoprotected in sucrose (15% in fixative for 1 day and 30% in PBS for 2 days), and kept on -80 °C until further use. 40

µm spinal cord coronal sections were cut on a freezing microtome and transferred to PBS-filled wells for free floating. Following blocking in PBS-diluted 10% normal goat serum (NGS), the sections were incubated with 1: 600 anti-BTX-A-cleaved SNAP-25 rabbit polyclonal antibody (a kind gift from prof. Ornella Rossetto, University of Padua, Italy) diluted in PBS with 1% NGS, overnight at room temperature, and the following day with fluorescently-labeled secondary antibody in dark (goat anti-rabbit Alexa-Fluor 555, Invitrogen, Carlsbad, CA, USA). Sections were then blocked again and counterstained with mouse monoclonal antibody for neurons (Anti-NeuN, Millipore, Temecula, CA, USA) (1:500 dilution overnight at 4°C), and the next day with secondary anti-mouse Alexa-Fluor 488 (Invitrogen, Carlsbad, CA, USA).

Sections were washed with PBS, mounted on glass slides with antifading agent (Fluorogel, Electron Microscopy Sciences, Hatfield, PA, USA), and visualized with fluorescence microscope equipped with appropriate filters (Olympus BX51, Olympus, Tokyo, Japan) and digital camera (Olympus DP-70). Double-label images were composed with Olympus DP Manager software, assembled with Microsoft Paint and then processed for brightness and contrast using Adobe Photoshop. We checked for the appearance of cleaved SNAP-25 immunoreactivity in 20-25 lumbar spinal cord sections from each animal. In the figures, to show the data of one experimental group, representative image from single animal was chosen.

#### **2.4 Colocalization study**

. L5 spinal cord sections from animals injected with 30U/kg s.c. were blocked with donkey serum and incubated overnight at room temperature with primary antibodies for cleaved SNAP-25, and for choline acetyltransferase (Millipore, Temecula, CA, USA), produced in goat, 1:100 dilution. The next day sections were incubated with secondaries: donkey anti-rabbit Alexa Fluor 488 and donkey anti-goat 546. In second experiment sections were blocked with goat serum and



incubated with anti-cleaved SNAP-25 and mouse anti-gial fibrillary acidic protein (Sigma, St. Louis, MO, USA, 1:1000) overnight at room temperature. The next day sections were incubated with secondary goat anti-mouse Alexa Fluor 546 and goat anti-rabbit Alexa Fluor 488 (Invitrogen, Carlsbad, CA, USA). Spinal cord ventral horns were visualized with confocal laser scanning microscope (Leica TCS SP2 AOBS), using 488 and 543 nm lasers.

### **3. RESULTS**

Slight ipsilateral flaccidity of rat distal hind limb was visible only following the i.m. treatment (5 and 30 U/kg of BTX-A), but not following the i.n. or s.c. injections.

BTX-A-cleaved SNAP-25 immunoreactivity appeared in ipsilateral lumbal ventral horns of s.c., i.m. and i.n.- BTX-A treated animals (Figures 1 and 2). Cleaved SNAP-25 immunoreactivity was visible around motoneuronal nuclei of lamina 9 in the form of dense, small fibers, and long neuronal processes, which also extended into the lamina 7 (Figures 1 and 2). Following i.m. and i.n. injections, truncated SNAP-25 occurred in L4, mainly in laminae 7 and 9, while following s.c. injection, strongest immunolabeling occurred in L5 segment ventral horn lamina 9 (Figure 1). Following intrasciatic saline + BTX-A injection at mid-thigh level, truncated SNAP-25 immunoreactivity was more widespread rostro-caudally (L3-L5).

Intramuscularly applied low-dose BTX-A (5 U/kg) also resulted in ventral horn SNAP-25 cleavage (Figure 2B). Intensity of cleaved SNAP-25 in ventral horn following 5 U/kg i.m. injection, although not quantified, was apparently lower than after higher doses (30 U/kg i.m. and s.c).

BTX-A enzymatic activity in the form of individual fibers appeared in ipsilateral dorsal horn even at small peripheral i.m. (5 U/kg) dose (Figure 2A), and following higher dose i.m., i.n. and s.c. injections (not shown), thus, indicating the axonal transport in spinal sensory neurons.

Intrasciatic colchicine pretreatment abolished the cleaved SNAP-25 immunoreactivity in spinal cord of BTX-A (i.n.) treated animals, thus, demonstrating the microtubule-dependent retrograde axonal transport of BTX-A through sciatic nerve (Figures 2C and D).

To examine the cellular localization of cleaved SNAP-25 in spinal cord motor region, we performed colocalization study with markers of cholinergic neurons and astrocytes. Cleaved SNAP-25 was found to colocalize with choline acetyl transferase (ChAT) - positive fibers, most likely belonging to proximal dendrites of motor neurons (Figure 3A). Cleaved SNAP-25 did not colocalize with glial fibrillary acidic protein (GFAP), marker of astrocytes (Figure 3B).

Figure 1

Figure 2

Figure 3

## **4. DISCUSSION**

### **4.1 Occurrence of cleaved SNAP -25 in the spinal cord after BTX-A peripheral injections**

Cleavage of central SNAP-25 in ipsilateral spinal cord segments after single peripheral application of BTX-A suggests the long-distance axonal traffic of enzymatically active BTX-A fragments from periphery to the spinal cord. These results extend recent findings on retrograde axonal transport of functionally active BTX-A in cranial nerves (Antonucci et al., 2008, Matak et al., 2011). Importantly, apart from cranial nerves in facial region with relatively short axons

which project to the rat brainstem, this study shows BTX-A axonal transport to CNS over longer distances. Present immunohistochemical evidence of axonal transport to spinal cord is in line with previous behavioral findings, where inhibition of axonal transport in the rat sciatic nerve abolished BTX-A antinociceptive effects in a model of bilateral pain (Bach-Rojecky and Lacković, 2009).

To examine the traffic within peripheral nerves we applied BTX-A directly into the sciatic nerve. Occurrence of truncated SNAP-25 in spinal cord after i.n. injection, preventable by proximally applied colchicine, shows that BTX-A is retrogradely transported through the peripheral nerve by means of microtubule-dependent axonal transport.

The effect of intrasciatic injection of BTX-A on pain behavior was reported before (Bach-Rojecky and Lacković, 2009). How the BTX-A enters the sciatic axons is unknown. BTX-A uptake into the peripheral nerve endings is mediated by SV2C-receptor mediated endocytosis (Mahrhold et al., 2006). If the similar mechanism exists in axons is not known. Another possibility is that BTX-A diffuses into the axoplasm through damaged axons, caused by i.n. injection itself.

In this study we found that cleaved SNAP-25 was localized in ChAT- positive cholinergic fibers surrounding the motoneuronal cell bodies. This finding indicates that, after axonal transport from periphery, botulinum toxin cleaves SNAP-25 most likely in primary motor neurons. However, the possibility that botulinum toxin, following transcytosis, enters cholinergic nerve terminals of neurons other than motoneurons cannot be ruled out completely. Novel study described the cleavage of SNAP-25 in the muscles of contralateral forelimb following peripheral BTX-A forelimb injection, thus, indicating the possibility of transcytosis within the motor neurons (Torii et al., 2011).

In this study an indirect method of BTX-A detection (by cleaved SNAP-25 immunolabeling) was used. Antibody specificity to BTX-A-truncated and not to the intact SNAP-25 was previously confirmed by BTX-A injections into the rat hippocampus and SDS polyacrylamide gel electrophoresis followed by Western blot (Matak et al., 2011). Single 24 kDa band corresponding to truncated SNAP-25 was visible only in BTX-A-treated animals, and position of that band was confirmed using antibody which recognizes both cleaved and intact SNAP-25. This experiment excluded possible immunostaining due to the non-specific binding to intact SNAP-25, or due to endogenously cleaved SNAP-25 (Figure 1 from Matak et al., 2011).

It can be argued that, instead of BTX-A, truncated SNAP-25 was transported from periphery to the spinal cord. Axonal transport of BTX-A was demonstrated previously in the visual system. Antonucci et al. (2008) injected BTX-A into the superior colliculus, and examined the occurrence of truncated SNAP-25 in the rat retina. Then, they cut the optic nerve and depleted the retina from truncated SNAP-25 by transiently active botulinum toxin E (BTX-E), which cleaves both intact and BTX-A-cleaved SNAP-25. After completion of BTX-E effects, BTX-A-cleaved SNAP-25 re-appeared in the retina, demonstrating the presence of BTX-A protease.

Low dose BTX-A (0.5 U/kg) injection into the distally cut rat sciatic nerve reduced contralateral pain in a bilateral pain model (Bach-Rojecky and Lacković, 2009), which, obviously, cannot be associated with peripheral SNAP-25 cleavage.

In sensory system, occurrence of truncated SNAP-25 in CNS seems to be associated with toxin's antinociceptive activity (Matak et al., 2011). On the other hand, significance of SNAP-25 cleavage in central motor regions remains to be investigated.

## **5. Conclusion**

Appearance of truncated SNAP-25 in spinal cord following low dose i.m. BTX-A (5 U/kg) administration suggests that the axonal transport of toxin to CNS commonly occurs following peripheral administration.

## **6. Acknowledgements**

Experiments were performed at the Department of Pharmacology, University of Zagreb School of Medicine, and Clinics and Polyclinics of Psychiatry, University of Würzburg. This work was supported by Croatian Ministry of Science, Education and Sport, (Project No. 108-1080003-0001 to Z. Lackovic) and Deutscher Akademischer Austausch Dienst (DAAD, to J. Deckert and Z. Lackovic). Antibody to BTX-A–cleaved SNAP-25 was a kind gift from Prof. Ornella Rossetto (University of Padua, Italy). The authors declare no conflict of interest.

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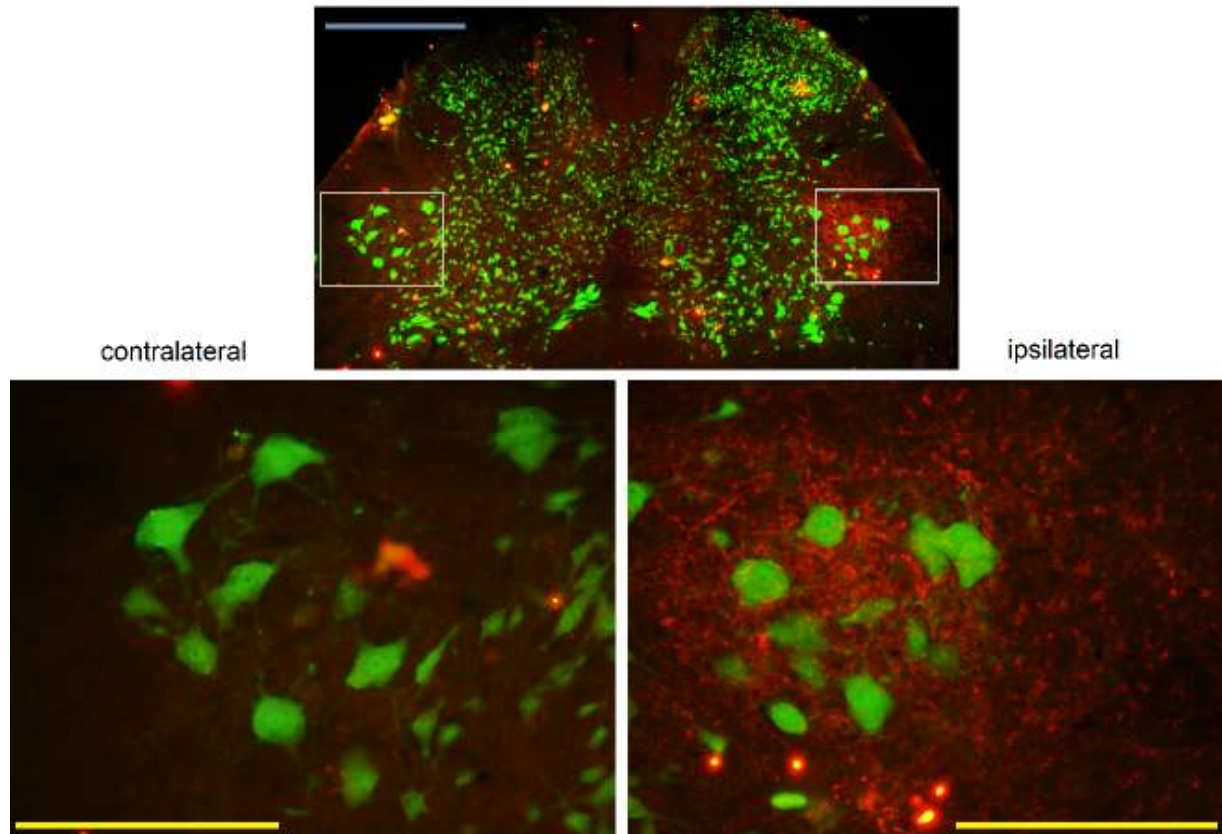


Figure 1. Immunofluorescently labeled truncated SNAP-25 (red) in ipsilateral ventral horn of rat spinal cord (L5 segment) 5 days after subcutaneous BTX-A (30 U/kg) injection into the hind-paw pad. Green represents NeuN neuronal staining. Contralateral= ventral horn contralateral to the site of injection; ipsilateral= ipsilateral to the site of injection. Blue scale bar, 500  $\mu\text{m}$ ; yellow scale bar, 200  $\mu\text{m}$ .



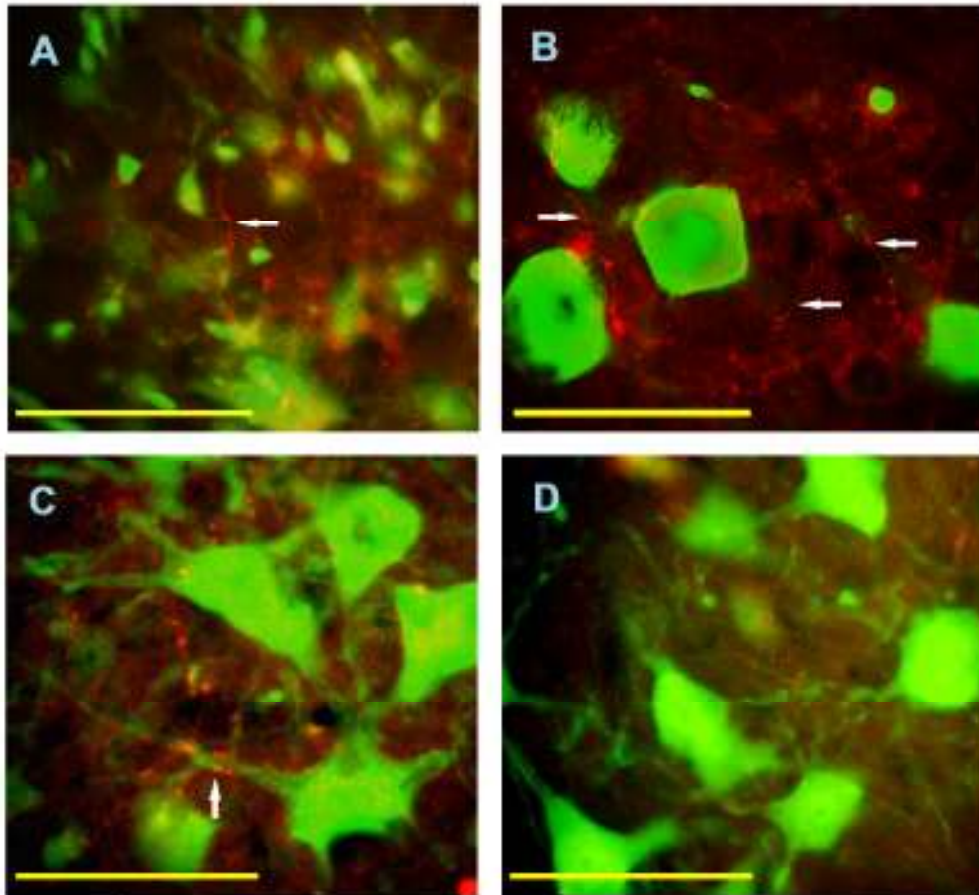


Figure 2 Truncated SNAP-25 in ipsilateral L4 spinal cord segment. A.) Dorsal horn after intramuscular BTX-A (5 U/kg) injection into the gastrocnemius; B.) Ventral horn after intramuscular BTX-A (5 U/kg) injection into the gastrocnemius; C.) Ventral horn after saline and BTX-A (10U/kg) injection into the sciatic nerve and D.) Ventral horn after colchicine (5mM) and BTX-A (10U/kg) injection into the sciatic nerve. White arrows point to cleaved SNAP-25 fibers (red immunoreactivity). Green represents NeuN neuronal staining. Scale bar, 100  $\mu$ m.

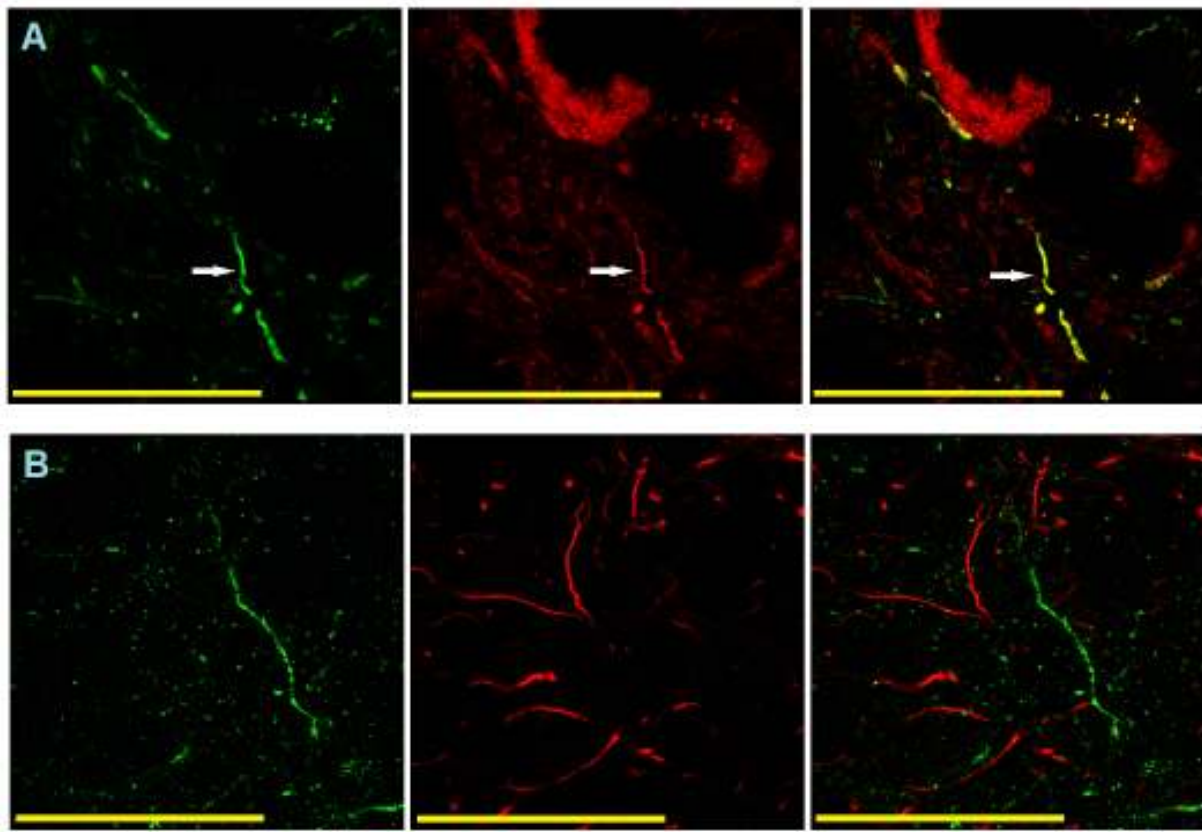
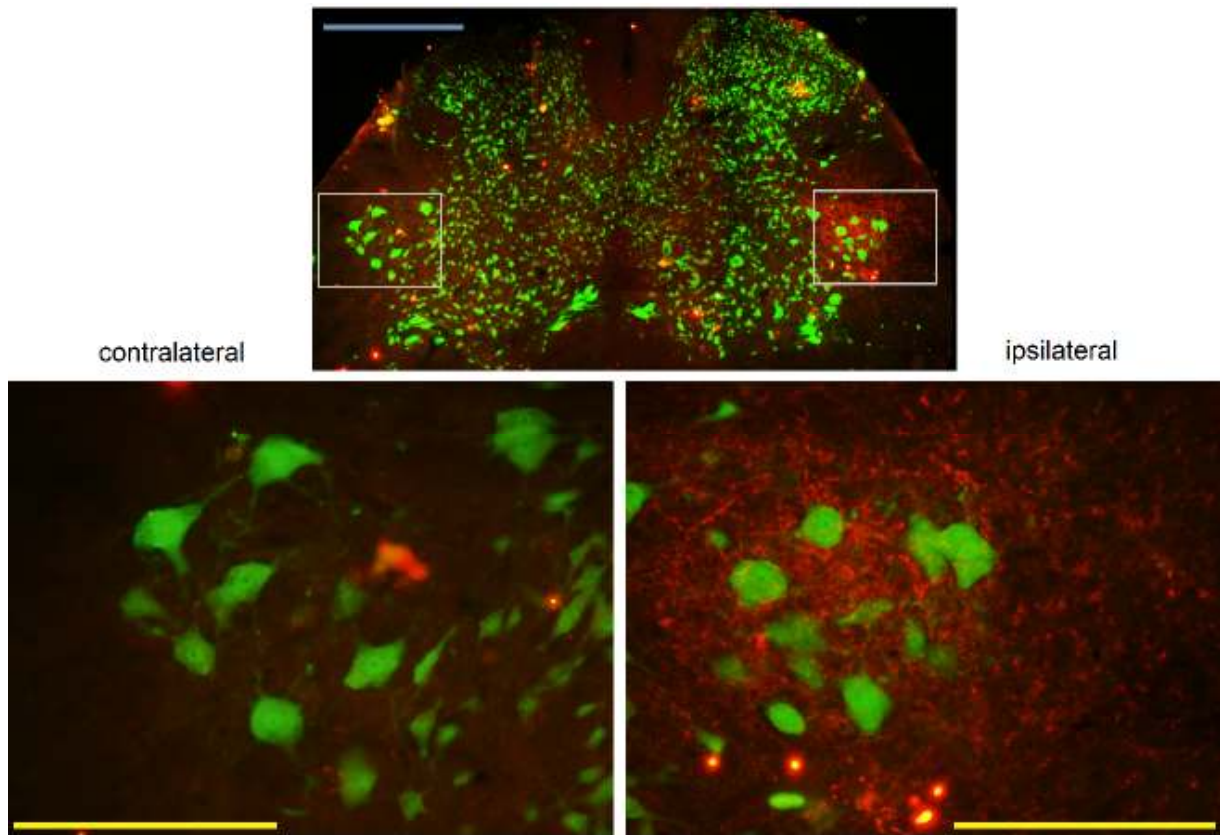


Figure 3 Truncated SNAP-25 colocalizes with cholinergic neurons, but not astrocytes.

A. Confocal image of 2 µm optical section of ventral horn ipsilateral to BTX-A (30U/kg, sc.) treatment, showing colocalization of cleaved SNAP-25 (green immunofluorescence) and ChAT (red).

B. Cleaved SNAP-25 (green) did not colocalize with GFAP (red), marker of astrocytes. Scale bars, 50 µm.

## Graphical abstract



**Occurrence of cleaved SNAP-25 in ipsilateral lumbar ventral horn following botulinum toxin A (30 U/ kg) subcutaneous injection into the rat hind paw**