Evidence for central antispastic effect of botulinum toxin type

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EVIDENCE FOR CENTRAL ANTISPASTIC EFFECT OF BOTULINUM TOXIN TYPE A

Running title: Central effects of botulinum toxin on spastic paralysis

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Author contributions

IM: Conception and design the study. Acquisition and analysis of the data. Draft of the manuscript and figures. Approval of the final manuscript.

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Bullet point summary

What is already known

- Supposedly, botulinum toxin A (BoNT/A) relieves spasticity and hyperkinesias due to its peripheral muscular actions
- Animal studies reported detection of BoNT/A-truncated enzymatic products in central motor regions after peripheral injections

What this study adds

- Preclinical data showed reduction of local spastic paralysis by BoNT/A's direct central activity in rats
- The BoNT/A effects were dependent on toxin's retrograde axonal transport and transcytosis within ventral horn

Clinical significance

- BoNT/A efficacy in spasticity and movement disorders might involve central reduction of abnormal muscle tone

ABSTRACT

Background and purpose: Botulinum toxin type A (BoNT/A) injections into hyperactive muscles enable efficient treatment of spasticity and dystonias, presumably due to its local effects on extrafusal and intrafusal motor fibers. A recent discovery of toxin's retrograde axonal transport to CNS might suggest additional action sites. However, in comparison to cholinergic peripheral terminals, functional consequences of BoNT/A direct central action on abnormally increased muscle tone are presently unknown. To address this question, the central effects of BoNT/A were assessed in experimental local spastic paralysis.

Experimental approach: Local spastic paralysis was induced by tetanus toxin (TeNT) injection into rat gastrocnemius (1.5 ng). Subsequently, BoNT/A (5 U kg^{-1}) was applied i.m. into the spastic muscle, or intraneurally (i.n.) into the sciatic nerve to mimic the action of axonally transported toxin. Possible role of BoNT/A transcytosis in spinal cord was evaluated by lumbar intrathecal (i.t.) application of BoNT/A-neutralising antitoxin. BoNT/A effects were studied by behavioural motor assessment and cleaved synaptosomal-associated protein 25 (SNAP-25) immunohistochemistry.

Key results: TeNT-evoked muscular spasm evoked a sustained rigid hind-paw extension and resistance to passive ankle flexion. Subsequent BoNT/A i.m. or i.n. injections reduced the TeNT-evoked spastic paralysis. Beneficial effect of i.n. BoNT/A and occurrence of cleaved SNAP-25 in ventral horn were prevented by i.t. antitoxin.

Conclusions and implications: Axonally transported BoNT/A relieves muscle hypertonia induced by TeNT, dependently on its trans-synaptic movement in CNS. Present results suggest that such direct, centrally mediated reduction of abnormal muscle tone might contribute to BoNT/A efficacy in spasticity and hyperkinetic movement disorders.

List of unapproved abbreviations:

BoNT/A, botulinum toxin type A; TeNT, tetanus toxin; i.n., intraneural; SNAP-25, synaptosomal-associated protein 25; DAS; digit abduction score

INTRODUCTION

Botulinum toxin (BoNT) serotypes A-G, potent neurotoxins from *Clostridium botulinum*, inhibit synaptic neurotransmitter release by proteolytic cleavage of soluble N-ethylmaleimide-sensitive factor attachment protein receptor proteins involved in Ca^{2+}-dependent neuroexocytosis (Pirazzini *et al*., 2017). Serotype A (BoNT/A) is applied intramuscularly (i.m.) in low doses for lasting reduction of abnormal skeletal muscle tone and/or hyperkinesia in hyperkinetic movement disorders and spasticity. It is approved for blepharospasm, hemifacial spasm, oromandibular and cervical dystonias, upper and lower limb spasticity (Jankovic *et al*., 2017; Safarpour and Jabbari, 2018). Currently, it is believed that its beneficial effects in motor disorders are mediated entirely by local muscular neuroparalysis of both extrafusal and intrafusal cholinergic nerve endings, with indirect normalization of spinal reflexes and central processing of movement (Kaňovský and Rosales, 2011). However, based on immunodetection of BoNT/A-truncated synaptosomal-associated protein of 25 kDa (SNAP-25), rodent studies demonstrated that enzymatically active BoNT/A is axonally transported from injected muscle to the motor nuclei of brainstem and spinal cord (Antonucci *et al*., 2008; Matak *et al*., 2012; Restani *et al*., 2012; Koizumi *et al*., 2014; Caleo *et al*., 2018). While the central action of BoNT/A is causally involved in its antinociceptive activity (Bach-Rojecky and Lacković, 2009; Matak *et al*., 2011), functional role of the toxin central activity on normal motor function or abnormal muscle tone has not been investigated up to now.

Muscle hypertonia and hyperkinesia in dystonia and spasticity involves impaired balance of excitatory vs inhibitory input to α-motor neurons, and the pharmacological treatment aimed at controlling the excitatory drive or increasing the inhibitory transmission at the spinal cord level reduces their symptoms (Kita and Goodkin, 2000; Halett *et al*., 2011). Tetanus toxin (TeNT), a homologue of BoNTs, exerts spastic paralysis by blocking the synaptic inhibitory input to α-motor neurons (Restani *et al*., 2012; Matthew *et al*., 2014). Quantification of local spastic paralysis induced by low dose i.m. TeNT might be used to study the effects of antispastic drugs (Kutschenko *et al*., 2012), however, this possibility has not been examined so far. Here, we employed TeNT-induced local spastic paralysis of the gastrocnemius muscle in conscious rats to examine the possible central effects of BoNT/A on abnormal muscle tone.

MATERIALS AND METHODS

Animals

Male Wistar Han rats (University of Zagreb School of Medicine, Croatia), 3-4 months old, 300-400 g weight, kept on 12 h/12h light and dark cycle, 3 animals per cage with free access to food and water, were used in all experiments. Experiments were conducted in accordance with the European Union Directive 2010/63/EU, and approved by institutional review board (University of Zagreb School of Medicine) and Croatian Ministry of Agriculture ethical committees (permit no. EP 24-2/2015). Animal studies performed have been designed and reported in compliance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al*., 2010), and the BJP guidelines (Curtis *et al*., 2018).

Pharmacological treatment

For i.m. injections, rats were anesthetized with ketamine/xylazine (70/7 mg kg^{-1} i.p.). By employing 0-50 µL luer tip Hamilton syringe coupled to 0.45 mm x 16 mm disposable needles, rats were percutaneously injected with 20 µL of neurotoxin divided into 2 injection sites (10 µL each site) into lateral and medial bellies of right gastrocnemius. The doses of TeNT (1.5 ng) or BoNT/A (5 U kg^{-1}) were chosen based on previously used non-systemic doses (Cui *et al*., 2004; Matak *et al*., 2012; Matthew *et al*., 2014), and preliminary experiments.

For intraneural (i.n.) injection of BoNT/A/saline, a lateral skin incision was made on the right thigh, and sciatic nerve vas blunt-dissected through the muscles of anesthetized animals. BoNT/A (5 U kg^{-1}, 2 µL) was slowly injected into the nerve trunk with a 0-10 µL Hamilton syringe needle (Cat. No. #701, Hamilton, Bonadouz, Switzerland), as previously described (Bach-Rojecky and Lacković, 2009; Matak *et al*., 2012).

In experiment examining the effect of GABA-B agonist R(+) baclofen, 3 mg kg^{-1} dose was injected i.p. to conscious, briefly restrained animals. The rats were then returned to their home cages prior to behavioural measurment. The dose was chosen based on previously employed non-sedative doses (Lorrai *et al*., 2014).

In experiments examining the BoNT/A transcytosis, the animals were anesthetized with ketamine/kylazine, and administered i.t. with 20 i.u. of BoNT/A antitoxin (National Institute for Biological Standards and Control, Potters Bar, UK) or equal volume (20 µL) of normal horse serum (Gibco, ThermoFisher Scientific, Waltham, MA, USA). The injection was performed by employing luer tip Hamilton syringe coupled to 26 G sterile disposable needle, similar to as described previously (Bach-Rojecky and Lacković, 2009). Correct site of

injection between the lumbar vertebrae was confirmed by the brief movement of rat tail and/or leg after the needle entered into the vertebral canal at the level of cauda equina. The dose of BoNT/A antiserum was chosen based on our previous study (Caleo *et al*., 2018).

Behavioural motor tests

Resistance to passive ankle flexion

To assess the magnitude of TeNT-evoked local rigidity, the rats were lifted by the experimenter's hand wrapped around the rat waist, and the ankle joint was flexed by pressing the hind-paw interdigital pad area against a rectangular plastic platform (1.5 x 4 x 4 cm) mounted on the flat metal surface of zeroed digital scale. When a dorsiflexion with tibiotarsal angle of 90º was reached, the pressure exerted by experimenter was slowly relieved, and the resistance value in grams (g) was noted just before further elevation of the animal elevated the tibiotarsal angle above 90º. After the rats were accustomed to handling for three separate training sessions, the resistance measured at 90º dorsiflexion was consistently low (around 10 – 20 g). Based on the preliminary testing of spastic animals, the cut-off value was set to 150 g to prevent pain and discomfort. Two trials were made per measurement session and the mean value calculated.

Digit abduction score

To assess local spastic paralysis of the hind-limb, the toe spreading reflex was measured upon lifting the animal from the ground by holding it around the waist. The reflex was quantified according to a semi-quantitative scale, termed digit abduction score (DAS) (Broide *et al*., 2013). The DAS scale is defined as 0= separation of all toes; 1= separation of 4 toes; 2=separation of 3 toes; 3 separation of 2 toes; 4=no toe separation.

Narrow beam walking

The animals were trained to walk across an elevated horizontal narrow beam (2.5 cm x 2.5 x 100 cm, elevated 50 cm above the ground), connecting a rectangular platform (10x10 cm) exposed to lamp light and a box-like dark platform (25x25 cm) with narrow entrance (10x10 cm), as described by Carter *et al*. (2001). The beam was marked at 10 cm distance from both platforms, and the transit time between the markings was measured (80 cm distance). Two trials per measurement were made and the mean value calculated. Measurements were repeated if animal stumbled or stopped during the transit.

Rota-rod

The animals were trained to maintain balance on a rota-rod device with 8 cm diameter rod rotating at 10 rpm. The cut-off time (latency to fall) was set to 120 seconds. Two trials were made per measurement session and the mean value calculated.

Experimental protocol

Experimental design was made in adherence with BJP guidelines (Curtis *et al*. (2018). In all experiments, animals were assigned randomly into different experimental groups by using block randomization. The experimenter who performed the motor tests was blinded to the animal treatment. However, a particular visual appearance of hind limbs attributable to a certain treatment (e.g. hind paw extension in TeNT-treated animals, limp appearance of the hind-paw in BoNT/A i.m.-treated animals) could not be masked during the animal manipulation.

Upon the end of all experiments, the animals were killed by deep ketamine/xylazine anaesthesia followed by transcardial perfusion with saline and buffered 4% paraformaldehyde fixative, or deep anaesthesia followed by decapitation.

Experiment 1: Effect of i.m. BoNT/A on TeNT-induced calf muscle spasticity

The animals were injected into the gastrocnemius muscle with vehicle or 1.5 ng TeNT. Saline/BoNT/A 5 U kg^{-1} was injected ipsilaterally into the gastrocnemius muscle on day 7 post vehicle/TeNT. Motor behaviour was measured on days 0, 7 (prior to saline/BoNT/A), 8, 10 and 13 post TeNT. Number of animals per group = 6 in control groups (vehicle + saline, vehicle + BoNT/A) and 7 in experimental spastic groups (TeNT + saline, TeNT + BoNT/A).

Experiment 2: Effect of i.n. BoNT/A injection into the sciatic nerve on TeNT-induced spasticity

Vehicle or TeNT i.m.-injected animals (1.5 ng TeNT dose) were injected i.n. with BoNT/A (5 U kg^{-1} in 2 µl) 7 days post TeNT. Motor parameters were measured on days 0 (prior to vehicle/TeNT), 1, 3, 7, (prior to saline/BoNT/A), 10, 13 post TeNT. Number of animals per group= 7 in experimental control groups (vehicle + saline, vehicle + BoNT/A) and 9 in experimental spastic groups (TeNT + saline, TeNT + BoNT/A).

Experiment 3: Effect of baclofen on TeNT-induced spasticity

Vehicle or TeNT i.m.-injected animals (1.5 ng TeNT dose) were injected with i.p. (R+) baclofen (3 mg kg^{-1}) on day 7 post TeNT. Motor behaviour was measured prior to saline/baclofen, and 1 hour post baclofen. Number of animals per group = 7- in experimental

control groups (vehicle + saline, vehicle + baclofen) and 9 in experimental spastic groups (TeNT + saline, TeNT + baclofen).

Experiment 4:Role of transcytosis in the effect of intrasciatic BoNT/A on TeNT-induced spasticity

I.m. TeNT-injected animals (1.5 ng TeNT dose) were injected i.n. with BoNT/A (5 U kg-1//2 µl) 7 days post TeNT. The next day, 20-24 h after BoNT/A, animals were injected i.t. with BoNT/A-antiserum. Motor parameters were measured on day days 0 (prior to vehicle/TeNT), 1, 3, 7, (prior to saline/BoNT/A), 8 (prior to horse serum/antitoxin), 10, 12, 14 post TeNT. Number of animals per group= 7 in experimental control groups (vehicle + saline, vehicle + baclofen) and 9 in experimental spastic groups (TeNT + BoNT/A + horse serum, TeNT + BoNT/A + antitoxin)

Experiment 5: *Examination of BoNT/A transcytosis in spinal cord after BoNT/A i.m. injection*

In 5 animals, BoNT/A (5 U kg-1) was injected into the left gastrocnemius, and 4 days after they were injected with another 5 U kg-1 injection into right gastrocnemius. One day after second BoNT/A injection, animals were administered i.t. with BoNT/A antiserum. The animals were further kept for additional 14 days, until they were sacrificed by saline/fixative perfusion.

Cleaved SNAP-25 immunohistochemistry

Animals from experiments no. 4 and 5. were sacrificed by tissue-fixation perfusion. Lumbar spinal cords were removed, and cryoprotected in sucrose (15% in fixative for 1 day and 30% in PBS for 2 days). Spinal cord coronal sections (35 µm) were cut on a freezing microtome and transferred to PBS-filled wells for free floating.

Following inactivation of endogenous peroxidase and blocking in 10% normal goat serum, the sections were incubated with 1: 2000 rabbit polyclonal IgG antibody raised against the C terminal peptide of BoNT/A-cleaved SNAP-25 (provided by prof. Ornella Rossetto, University of Padua, Italy), overnight at room temperature. The antibody binds specifically to BoNT/A-cleaved SNAP-25 and not the intact SNAP-25 (Matak *et al*., 2011). The next day, slices were processed with Alexa Fluor 555 Tyramide Superboost Kit (cat no. B40923; Invitrogen, Carlsbad, CA, USA), which contains the HRP-conjugated goat anti-rabbit secondary antibody and Tyramide-Alexa 555 substrate, according to manufacturer's

instructions. Controls with omitted primary antibody showed no specific staining, suggesting the lack of non-specific binding of secondary antibody and/or Tyramide reagent. Sections were washed with PBS, mounted on glass slides with anti-fading agent (Fluorogel, Electron Microscopy Sciences, Hatfield, PA, USA), and visualized with fluorescence microscope (Olympus BX51, Olympus, Tokyo, Japan) coupled to digital camera (Olympus DP70, and equipped with cellSens Dimension visualizing and quantification software (Olympus, Tokyo, Japan, RRID:SCR_014551). Images shown in the figures were composed and processed for brightness and contrast using Adobe Photoshop Elements 9 (Adobe Systems, San Jose, CA, USA, RRID:SCR_014199). The average cleaved SNAP-25 immunoreactivity (IR) area for a single animal was quantified based on 3 randomly chosen L4 slices. High magnification images (20x, obtained by employing 40x objective and 0.5x camera adapter) were used under constant low exposure time (18 milliseconds) for lower background. The area covered by cleaved SNAP-25 IR was quantified by employing constant manual threshold range (117-256) of red channel pixel intensity in all images. In a single slice, cleaved SNAP-25 IR was quantified and summated in 3 non-overlapping visual fields located in the lateral parts of ventral horn gray matter (3x 0.14 mm^2). Upon analysis, the images were coded for blinding to the animal treatment. The experimental details conform to the BJP guidelines for reporting of Western blot or immunohistochemical studies (Alexander *et al.*, 2018).

Statistical analysis of data

Results were presented as mean ± S.E.M and analysed by two-way ANOVA for repeated measurements, followed by Bonferroni's *post hoc* test ($p<0.05$ considered significant). The *post hoc* tests were performed only if F was significant ($p<0.05$) and there was no variance inhomogeneity. Non-parametric data (DAS) were presented as median and analysed by Kruskal Wallis non-parametric one-way ANOVA, followed by Dunn's multiple comparison *post hoc* test ($p<0.05$ was considered significant). The cleaved SNAP-25 IR data were analysed by non-parametric Mann Whitney t-test (two group comparison) or Kruskal Wallis non-parametric one-way ANOVA, followed by Dunn's multiple comparison *post hoc* test ($p<0.05$ was considered significant). GraphPad Prism 5 (GraphPad Software, Inc, La Jolla, CA, USA, RRID:SCR_002798) was used for statistical analyses and graph drawing. The sample size and minimal number of animals per treatment group was pre-determined based on preliminary experiments to predict the effect size for different measurements, and according to *a priori* power analysis using free statistical software (G*power version 3.1., University of Düsseldorf, Germany, RRID:SCR_013726) (Charan and Kanthara, 2013). The number of

animals per group was calculated to be 5 or 6 based on estimated effect size of F=0.4, α error probability = 0.05, power (1-β) = 0.9, statistical test: analysis of variance (ANOVA): repeated measures, within-between interaction. A possible attrition due to various reasons was also taken into account, and higher number of animals per group than required by power analysis was used in all experiments (however, there was no loss of animals or data exclusion from analysis in experiments). Due to expected large differences in measured parameters between control and spastic treatments, a smaller number of rats was used in control non-spastic groups to reduce the total number of animals used. The study complies with the recommendations on experimental design and analysis in pharmacology (Curtis et al, 2018).

Materials

Lyophilized tetanus toxin (Sigma Aldrich, St Louis, MO, USA) was reconstituted in 0.9% saline vehicle containing 2% bovine serum albumin (Sigma Aldrich), stored in concentrated aliquots on -80ºC, and further diluted with vehicle to obtain the necessary concentration for i.m. injections. Lyophilized BoNT/A (Botox®, Allergan, Irvine, CA, USA) was reconstituted in physiological saline. Baclofen (R(+) Baclofen) (Sigma Aldrich) was dissolved in saline to obtain the required dose. Lyophilized polyclonal equine IgG-based BoNT/A antitoxin (Botulinum type A antitoxin from National Institute for Biological Standards and Control, NIBSC code 14/174, Potters Bar, UK, validated by Li *et al.*, 2012) was reconstituted in 0.9% saline to 1000 i.u. mL^{-1} concentration.

Nomenclature of Targets and Ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding *et al*., 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander *et al.*, 2017).

RESULTS

Effect of intramuscular and intrasciatic BoNT/A on TeNT-evoked spastic paralysis

In conscious animals, TeNT injection into the gastrocnemius muscle induces unilateral rigid extension of the hind paw. This resulted in unilateral increased resistance to ankle dorsiflexion and impaired narrow beam and rota-rod latencies. The deficits started to develop at day 3 post TeNT injection.

Injection of BoNT/A 5 U kg^{-1} i.m. into the gastrocnemius of spastic animals gradually reduced the ankle flexion resistance from day 1 to day 3 post BoNT/A. Thereafter, the ankle flexion resistance remained low (similar to control-non spastic group), until the end of experiment. (Figure 1 A). Toxin injection into the sciatic nerve reduced the resistance to passive ankle flexion, as well. The effect was evident at day 3, and remained similarly low, thereafter, until the end of observation period (Figure 1 B). The antispastic drug R(+) baclofen reduced the ankle flexion resistance 60 minutes after systemic i.p. treatment, as well (Figure 1 C).

In i.m. BoNT/A-treated animals, unilateral flaccid paralysis was evident as impaired toe spreading reflex, assessed by DAS (Figure 1 D). In contrast to that, BoNT/A i.n. did not significantly impair the DAS (Figure 1 E).

Figure 1

BoNT/A or R(+) baclofen exerted no observable benefits in beam walking or rotarod latencies (Figure 2 A C). Similarly, BoNT/A i.n. did not improve beam walking, however it slightly improved the rota-rod performance at days 3 and 5 post BoNT/A treatment (Figure 2 B). In control, non-spastic animals, neither i.m. nor i.n. BoNT/A, or R(+) baclofen impaired rota-rod or beam walking performances, excluding the systemic effects of BoNT/A and sedative effects of R(+) baclofen.

The antispastic effect of i.n BoNT/A is prevented by i.t. antitoxin treatment

BoNT/A-mediated benefits were evident as gradual reduction of ankle flexion resistance starting 2 days following its i.n. injection (Figure 3 B), resulting in a markedly improved voluntary use of the hind-limb during standing and walking (Supplementary video S1). Intrathecal lumbar injection of BoNT/A-specific antitoxin prevented beneficial effects of i.n. BoNT/A on rigid hind-paw extension, dorsiflexion resistance, and hind-limb use during standing and walking (Figure 3 A, Figure 3 B, Supplementary video S2).

Figure 3

Cleaved SNAP-25 occurrence in ventral horn after low dose i.n. and i.m. BoNT/A is prevented by spinal intrathecal antitoxin

After BoNT/A injection into the sciatic nerve and subsequent horse serum i.t. treatment, cleaved SNAP-25 immunofluorescence was observed in the ipsilateral ventral horn. In animals treated with i.t. antitoxin 24 h after BoNT/A, very few individual neurites were

observed in the ventral horn, suggesting the prevention of transcytosis of enzymatically active BoNT/A by the antitoxin (Figure 4).

Figure 4

After i.m. injections of BoNT/A at different times relative to the i.t. antitoxin treatment (5 vs. 1 d before the antitoxin), cleaved SNAP-25 was visible only in the left ventral horn ipsilateral to earlier BoNT/A treatment. Very little or no cleaved SNAP-25 was observed in the right ventral horn ipsilateral to the BoNT/A injected i.m. 1 day prior to antitoxin (Figure 5). The antitoxin injection into the intrathecal space did not alter the pre-established flaccid paralysis on either side treated with BoNT/A (median DAS in both legs = 4, data not shown).

Figure 5

DISCUSSION

In present study, local muscle hyperactivity was evoked by low dose-TeNT i.m. injection into the rat gastrocnemius. TeNT, unlike BoNT/A and other BoNT serotypes, induces muscular spasm following its axonal transport and transcytosis into ventral horn inhibitory synapses which control the motor neuron activity (Brooks *et al*., 1957; Restani *et al*., 2012; Matthew *et al*., 2014). Although the muscular hyperactivity in hyperkinetic movement disorders and spasticity is etiologically different, it is similarly associated with impaired inhibitory control of lower motor neurons (Halett, 2011; Kita and Goodkin, 2000). Converging mechanisms of muscle hypertonia in dystonia, spasticity, and tetanus are in line with therapeutic efficacy of common drugs (Kita and Goodkin, 2000; Hassel, 2013), and supported by the efficacy of GABA-B agonist baclofen in TeNT-evoked spastic paralysis (Figure 1 C). Thus, TeNT-evoked local spastic paralysis could be employed to induce the motor neuron disinhibition and exaggerated excitatory drive, however, TeNT-induced local muscle spasm does not reflect the full complexity of clinical disorders. The most common form of naturally occurring spasticity caused by upper motor neuron lesion is resulting from complex compensatory plastic changes at different levels such as altered supraspinal inputs and/or consequent dysfunction of segmental spinal modulation (Segal, 2018). Thus, the use of TeNT-evoked local spasm as a model of naturally occurring spasticity may be limited only to its certain aspects, such as the disinhibition of local spinal interneurons.

In rats with established TeNT-evoked focal spastic paralysis, BoNT/A was injected i.m. into the gastrocnemius, and, to mimic the effect of axonally transported toxin, i.n. into the sciatic

nerve. Intrasciatic injection was chosen based on previous studies, which demonstrated the occurrence of cleaved SNAP-25 in spinal cord without observable ipsilateral flaccidity of the hind limb (Matak *et al*., 2012), and centrally mediated antinociceptive effect (Bach-Rojecky and Lacković, 2009), suggesting the retrograde axonal transport of BoNT/A from the nerve trunk. Quantification of toe spread reflex by DAS scale was used to evaluate the BoNT/A-mediated local muscular action, as previously described (Broide *et al*., 2013). Both modes of BoNT/A application relieved the TeNT-evoked spastic paralysis. In line with local neuromuscular effect on the neuromuscular junction, i.m. BoNT/A produced a quick-onset spasm relief (within 24 h), which was concurrent with prominent DAS impairment (Figure 1 A; Figure 1 D). After i.n. BoNT/A, the relief of local spastic paralysis was observed after 48-72 h, without significant DAS impairment (Figure 1 B; Figure 1 E, Figure 3 B), suggesting a spasm-relieving effect not related to the peripheral flaccid paralysis.

Previously, we showed that the occurrence of cleaved SNAP-25 within facial motor nucleus after toxin application into the whisker pad muscles is prevented by BoNT/A-specific antitoxin applied into lateral ventricles or cisterna magna. These experiments demonstrated BoNT/A trans-synaptic migration into secondary synapses via the extracellular fluid (Caleo *et al*., 2018). In present study, BoNT/A-antitoxin injected i.t. into the lumbar spinal canal prevented the i.n. BoNT/A-mediated relief of TeNT-induced spastic paralysis (Figure 3 A, Figure 3 B; Supplementary video S1, Supplementary video S2). The antitoxin also reduced the i.n. BoNT/A-induced SNAP-25 cleavage in the ventral horn (Figure 4). Mentioned observations indicate a modulation of motor function by retrogradely transported and transcytosed enzymatically active BoNT/A. Similarly, i.t. antitoxin prevented the occurrence of cleaved SNAP-25 in the ventral horn after BoNT/A injection into the gastrocnemius (Figure 5), while the DAS impairment resulting from BoNT/A muscular action was unaffected by the antitoxin. Present observations suggest that, after retrograde axonal transport and transcytosis of i.m.-injected BoNT/A, peripheral and central effects co-occur. However, central BoNT/A effects might not be easily distinguished since they are preceded by prominent flaccid paralysis.

In line with reduction of overt muscle tone observed here, and selectivity of BoNT/A action in excitatory synapses (Grumelli *et al*., 2010), it is likely that transcytosed BoNT/A primarily reduces the excitatory inputs onto motor neurons. Previous colocalization studies show that, after toxin i.m. injection, BoNT/A-cleaved SNAP-25 in motor nuclei of facial and hind-limb muscles was dominantly present in ChAT- positive neurites surrounding the motor neurons

and vesicular acetylcholine transporter (VAChT)- expressing synaptic contacts with their somas (Matak *et al*., 2012; Cai *et al*., 2017; Caleo *et al*., 2018). Cholinergic synapses with direct excitatory input on motor neurons, known as C-boutons or C-terminals, reduce the after-hyperpolarisation of motor neurons via muscarinic M_2 receptors (Miles *et al*., 2007). The BoNT/A effect on C-boutons or other synapses in the ventral horn awaits further functional characterization.

The clinical significance of central BoNT/A effects is presently unknown. Peripheral effects could be more dominant or mask the central toxin effects. However, clinical benefits exerted by i.m. BoNT/A often do not follow the degree of peripheral weakness. Several clinical observations are difficult to explain only by local peripheral action of BoNT/A:

- Distant effects in non-injected muscles and limbs. A reduction of motor neuronal excitability was reported in hand muscle of patients treated into neck muscles for spasmodic torticollis Wohlfarth *et al*., 2001), and in lower facial muscles of patients treated into orbicularis oris for hemifacial spasm (Ishikawa *et al*., 2010). In spastic patients, observed reduction of recurrent inhibition of distant non-injected muscle cannot be explained by peripheral action on BoNT/A on either local or distant neuromuscular junction or muscle spindle (Marchand-Pauvert *et al*., 2013; Aymard *et al*., 2013). Distant, even contralateral motor benefits have been reported in patients treated for focal dystonias and stiff man syndrome, which were interpreted either as toxin systemic diffusion or a direct central effect (Giladi, 1997; Girlanda *et al*., 1996; Liguori *et al*., 1997).

- Reduction of spasm frequency in focal dystonias (such as blepharospasm and torticollis) and stiff person syndrome, which cannot be interpreted by toxin action on muscle relaxation alone (Giladi, 1997; Liguori *et al*., 1997; Valls Sole *et al*., 1991).

- Clinical benefits not necessarily concurrent with the extent and duration of muscular paralysis. In patients treated with BoNT/A for dystonia and spasticity, the apparent muscle weakness was negligible or lasted shorter than the clinically observed beneficial effects (Priori *et al*., 1996; Bjornson *et al*., 2007; Eek and Himmelman, 2016). In writer's cramp patients, a greater BoNT/A-induced weakness was observed after writing-induced muscle activity in comparison to no muscle activity immediately after toxin injection (Chen *et al*., 1999). This observation suggested that the increased muscle activity leads to increased BoNT/A entry into the neuromuscular junctions. However, the time-point of maximal muscle weakness did not correlate with the time point of greatest therapeutic benefit, and there was

no significant difference in the observed therapeutic benefit in both patient groups (Chen *et al*., 1999).

Beyond the neuromuscular relaxation, BoNT/A may modify motor neuron activity by neuroparalytic action on 1. intrafusal nerve endings of muscle spindles and consequent modification of stretch reflexes (Giladi, 1997; Rosales and Dressler, 2010) and/or 2. cholinergic synapse between recurrent axonal collaterals and Renshaw interneurons involved in recurrent inhibition, provided by axonal transport (Marchand-Pauvert *et al*., 2013; Mazocchio and Caleo, 2014). However, these mechanisms cannot account for aforementioned effects in blepharospasm and hemifacial spasm, since facial muscles and facial motor neurons lack their respective muscle spindles and recurrent axonal collaterals (Whitehead *et al*., 2005; Kitai *et al*., 1972).

Present results suggest that modification of motor function following BoNT/A transcytosis within CNS might contribute to the antispastic action of i.m. BoNT/A. Central effects might participate in the fine-tuning of motor activity in movement disorders and augment the toxin's peripheral effects (Matak *et al*., 2016). In particular, central BoNT/A action might mediate prolonged duration of beneficial action after the resolution of peripheral weakness (Mazocchio and Caleo, 2014), or reduced excitability of motor neurons innervating the distant, non-injected muscles. In conclusion, present results point to the existence of BoNT/A central action on pathologically increased muscle tone, involving a mechanism that is distinct from its well-known action in cholinergic peripheral terminals.

Acknowledgements

Supported by European Social Fund and Croatian Ministry of Science, Education and Sport (project no. HR.3.2.01-0178).

Antibody to cleaved SNAP-25 and the BoNT/A-specific antitoxin were kindly provided, respectively, by prof. Ornella Rossetto (University of Padua, Padua, Italy), and dr. Thea Sesardic (National Institute for Biological Standards and Control, Potters Bar, United Kingdom). Zdravko Lacković and Maja Relja (University of Zagreb School of Medicine, Zagreb, Croatia) and Matteo Caleo (CNR Institute of Neuroscience, Pisa, Italy) provided advices regarding the data representation and preparation of the manuscript. Božica Hržan (University of Zagreb School of Medicine, Zagreb, Croatia) gave excellent technical support regarding animal treatment and randomization.

Author contributions

IM: Conception and design the study. Acquisition and analysis of the data. Draft of the manuscript and figures. Approval of the final manuscript

Conflict of interest

The author declares no conflict of financial interests related to the study.

Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for Design & Analysis, Immunoblotting and Immunochemistry, and Animal Experimentation, and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

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FIGURE LEGENDS AND FIGURES

Figure 1

Botulinum toxin type A (BoNT/A) and baclofen reduce the hind limb rigidity evoked by TeNT i.m. injection. TeNT (1.5 ng, i.m.)-evoked resistance to ankle dorsiflexion (achieving 90º tibiotarsal angle) is reduced by BoNT/A intramuscular (i.m.) injection into gastrocnemius (A.) and intraneural injection (i.n.) into sciatic nerve (B.). TeNT-evoked increase in force required for ankle flexion is also reduced by intraperitoneal (i.p.) injection of spasmolytic drug R(+) baclofen (3 mg kg^{-1}) (C.). BoNT/A (5 U kg^{-1}) significantly impairs the digit abduction score after i.m. injection (D.), but not after i.n. injection (E.). The DAS was measured 7 days after i.m. or i.n. BoNT/A, and 14 d after TeNT i.m. injection. Veh; vehicle; sal, saline intramuscular treatment; dotted horizontal line indicates time points of TeNT i.m., BoNT/A (i.m./ i.n.), and baclofen (i.p.) treatments. A.)-C.) mean ± SEM, *-$p<0.05$ vs. veh + sal, +-$p<0.05$ vs. TeNT + sal (two-way RM ANOVA followed by Bonferroni's post hoc test, $p<0.05$ considered significant); D.),E.) Data are represented as individual values and median (horizontal line) +-$p<0.05$ vs. veh + sal (one-way non-parametric ANOVA (Kruskal Wallis) followed by Dunn's post hoc, $p<0.05$ considered significant).

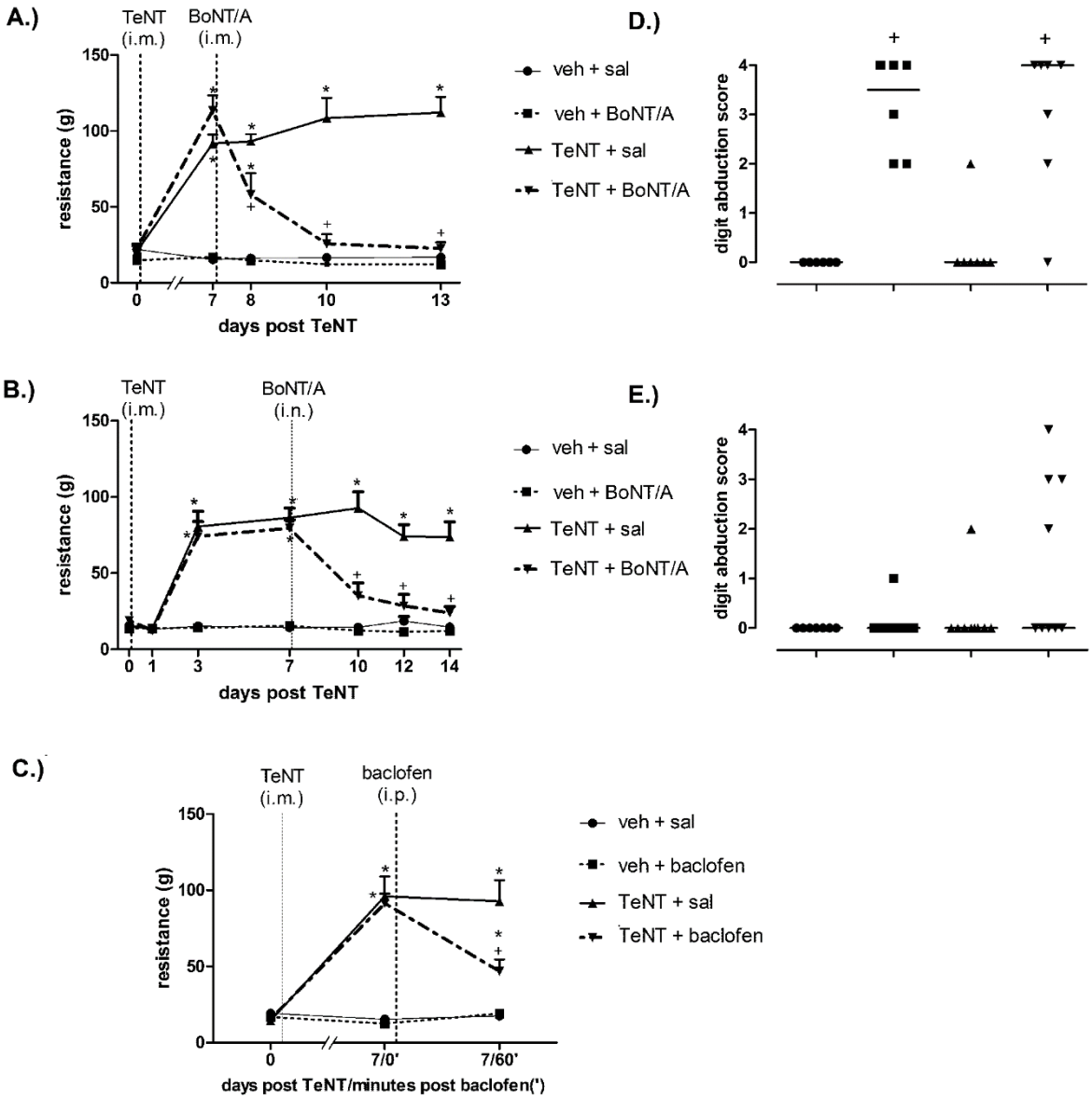

A.)
TeNT (i.m.)
BoNT/A (i.m.)
resistance (g)
days post TeNT
D.)
veh + sal
veh + BoNT/A
TeNT + sal
TeNT + BoNT/A
digit abduction score
B.)
TeNT (i.m.)
BoNT/A (i.n.)
resistance (g)
days post TeNT
E.)
veh + sal
veh + BoNT/A
TeNT + sal
TeNT + BoNT/A
digit abduction score
C.)
TeNT (i.m.)
baclofen (i.p.)
resistance (g)
days post TeNT/minutes post baclofen(')
veh + sal
veh + baclofen
TeNT + sal
TeNT + baclofen

Figure 2

Effects of BoNT/A and baclofen on TeNT (1.5 ng i.m.)-induced functional motor deficits. Intramuscular BoNT/A (5 U kg-1, i.m.) and intraperitoneal baclofen do not affect TeNT-evoked impairment in beam walking and rota-rod latencies ((A.) and C.)). Intraneural BoNT/A (5 U kg-1, i.n). does not improve TeNT-evoked impairment in beam walking latency, but it slightly improves rota-rod latency (B.) Veh, vehicle intramuscular treatment; sal, saline treatment; vertical lines indicate time point of TeNT, BoNT/A or baclofen treatments; Mean ± SEM, *-$p<0.05$ vs. veh + sal, +-$p<0.05$ vs. TeNT + sal (two-way RM ANOVA followed by Bonferroni's post hoc test, $p<0.05$ considered significant).

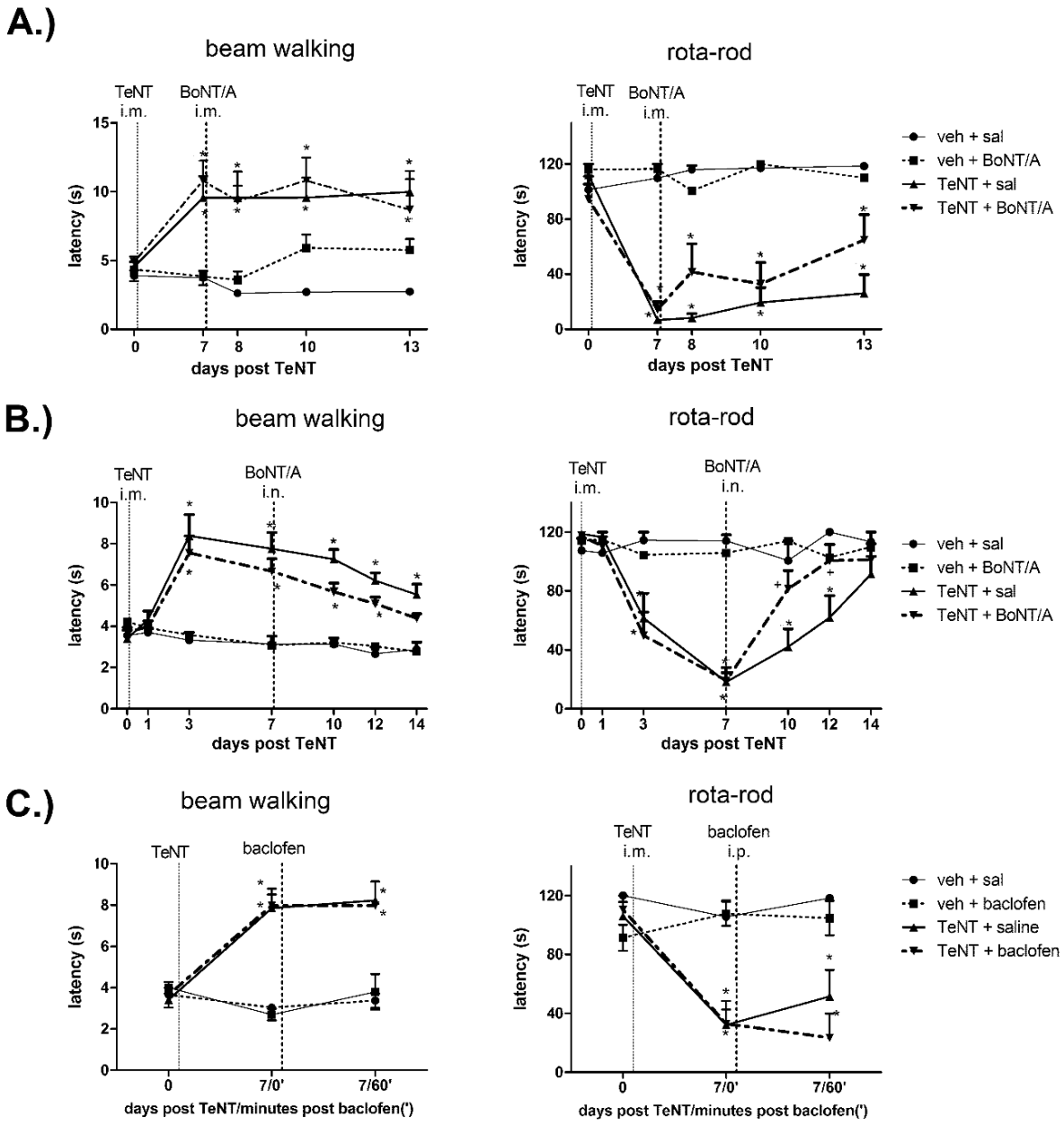


Figure 3

Beneficial effect of axonally transported BoNT/A on TeNT-induced muscle hypertonia is dependent on BoNT/A transcytosis. The BoNT/A-specific antitoxin (20 i.u.) injected i.t. at the level of lumbar spinal canal prevents intraneural (i.n.) BoNT/A (5 U kg^{-1})-mediated reduction of the TeNT-evoked right hind-paw extension (A.) and resistance to 90º ankle dorsiflexion (B.). Upper panel above the photographs shows the time-course of TeNT, BoNT/A and antitoxin treatments. A.) The middle and lower panel show the photo of same animal taken from different angle (representative of 7-9 animals per group). Photographs of calm, hand-held animals were taken on day 14 post TeNT/7 post BoNT/A. B.) On the graph, vertical lines indicate time point of TeNT, BoNT/A and antitoxin treatments. N=7-9 animals/ group; mean ± SEM, +-p<0.05 vs. TeNT + saline + horse serum. #-p<0.05 vs TeNT + BoNT/A + horse serum (two-way RM ANOVA followed by Bonferroni's post hoc test).

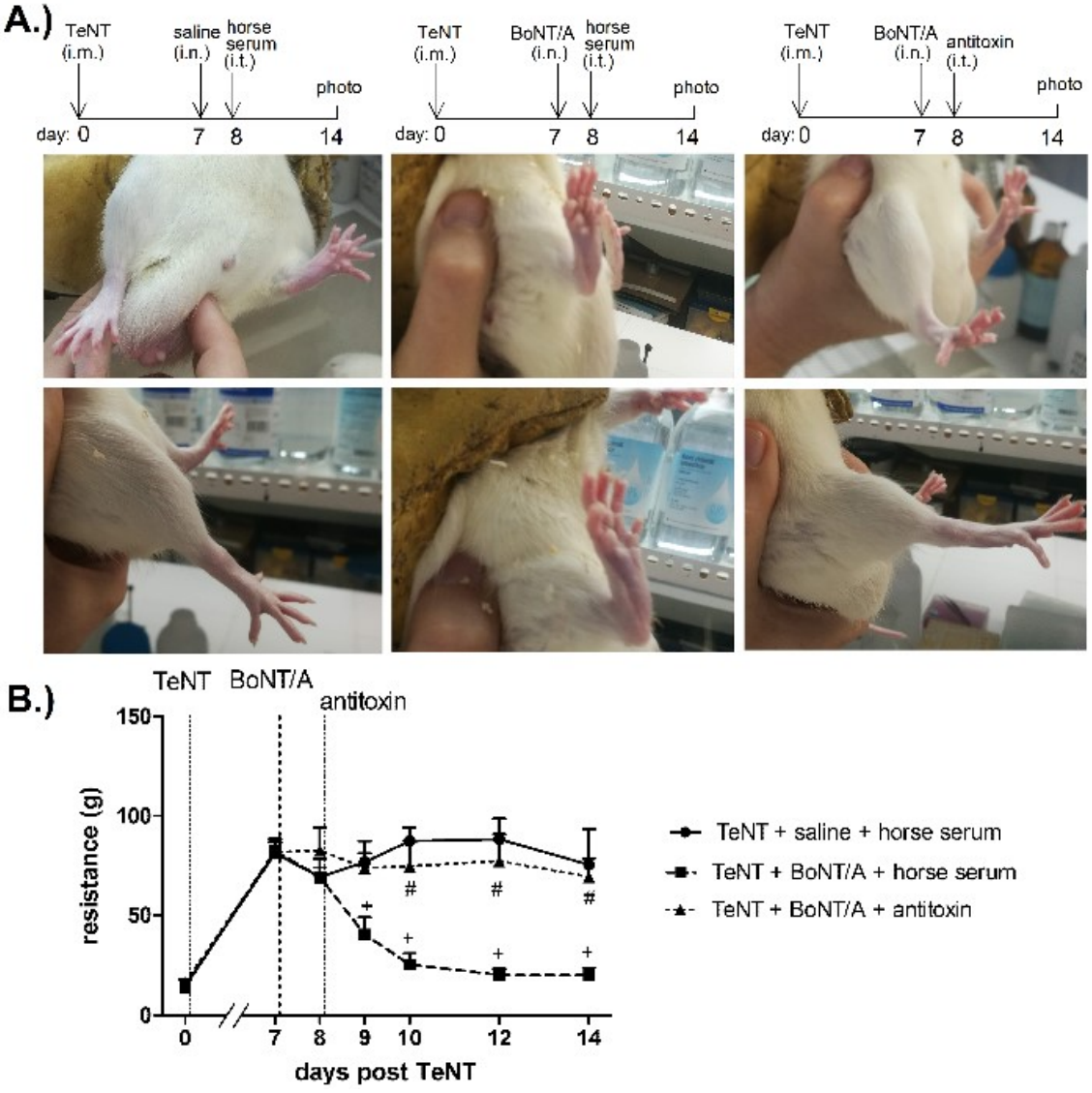


Figure 4

Occurence of BoNT/A enzymatic activity in the spinal cord after toxin intraneural (i.n.) injection is dependent on BoNT/A transcytosis. (A.) Intrathecal lumbar administration of BoNT/A-antitoxin (20 i.u.) reduces the occurrence of BoNT/A cleaved SNAP-25 (indicated by arrows, immunofluorescent Alexa 555-tyramide signal amplification) in the ventral horn when injected 1 day after BoNT/A application into the sciatic nerve (5 U kg^{-1}, right lower panel). Upper panel above the microphotographs shows the time-course of TeNT, BoNT/A and antitoxin treatment, and the tissue preparation by perfusion. Scale bar = 250 µm.B. Quantitative analysis of pixel intensity-thresholded area of cleaved SNAP-25 immunoreactivity in 3 non-overlapping high magnification visual fields (433 µm x 323 µm = 0.14 mm^2) located in lateral L4 ventral horn, average of 3 slices per animal (N= 6 animals per group). Mean ± SEM; + $p<0.05$ vs TeNT + saline + horse serum (one-way non-parametric ANOVA followed by Dunn's post hoc, $p<0.05$ considered significant).

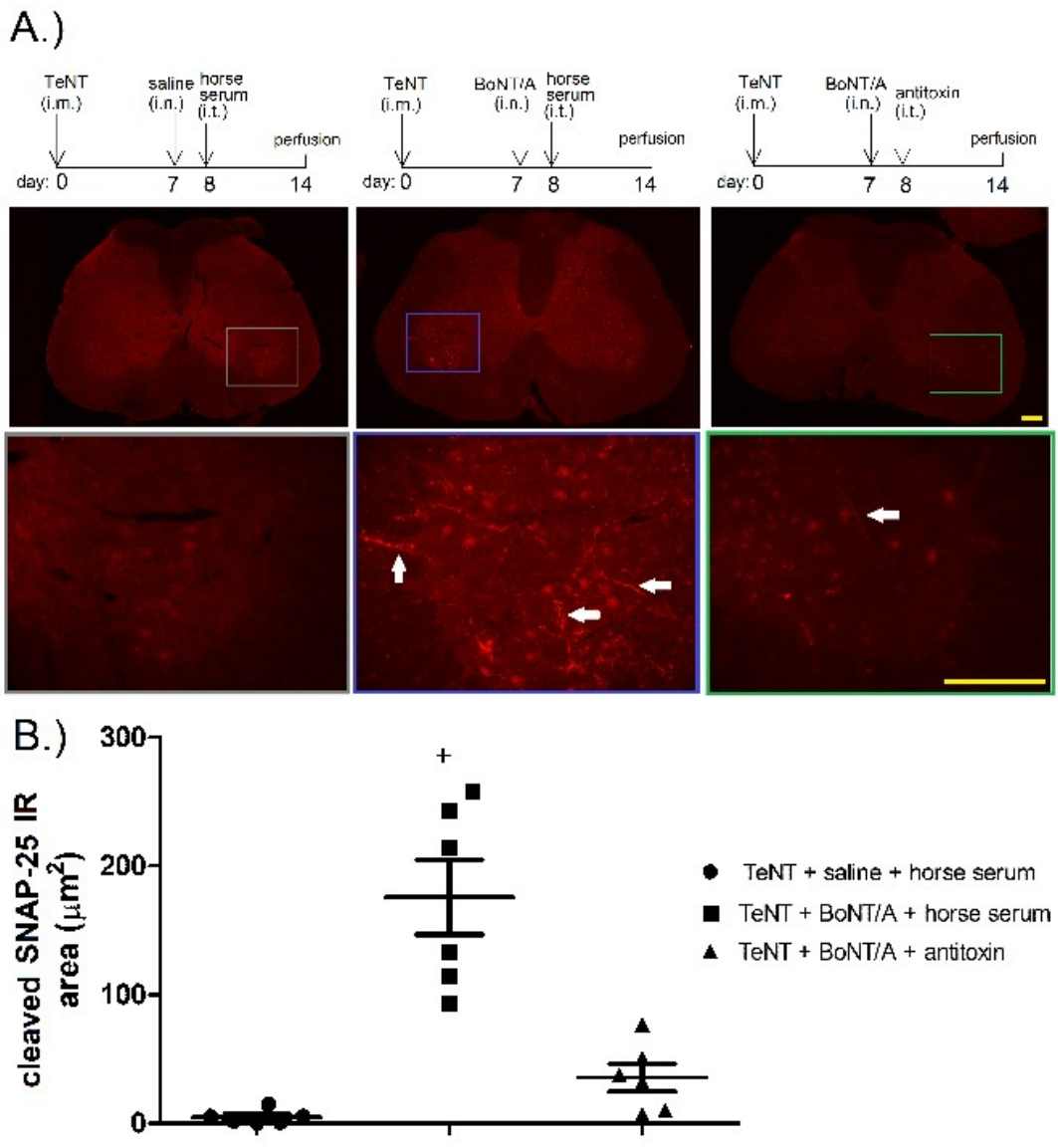
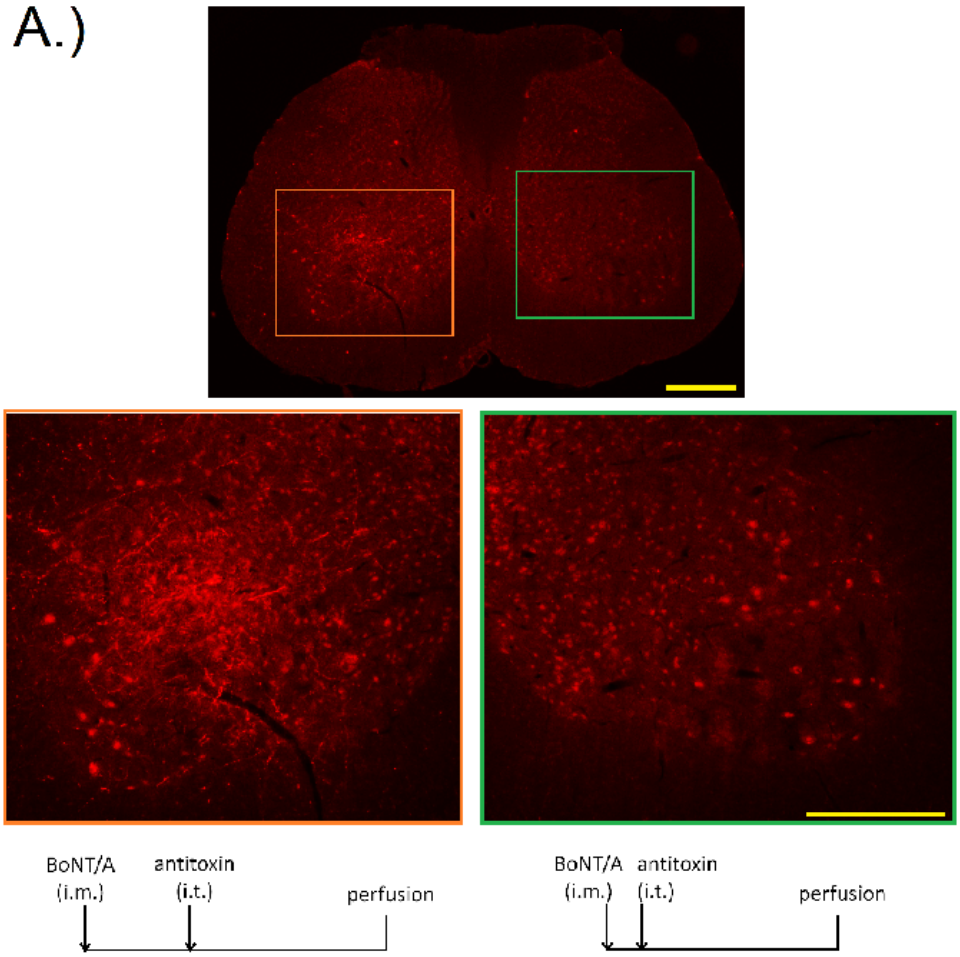

A.)
TeNT (i.m.)
saline (i.n.)
horse serum (i.t.)
perfusion
day: 0
7
8
14
TeNT (i.m.)
BoNT/A (i.n.)
horse serum (i.t.)
perfusion
day: 0
7
8
14
TeNT (i.m.)
BoNT/A (i.n.)
antitoxin (i.t.)
perfusion
day: 0
7
8
14
B.)
cleaved SNAP-25 IR area (μm²)
300
200
100
0
TeNT + saline + horse serum
TeNT + BoNT/A + horse serum
TeNT + BoNT/A + antitoxin

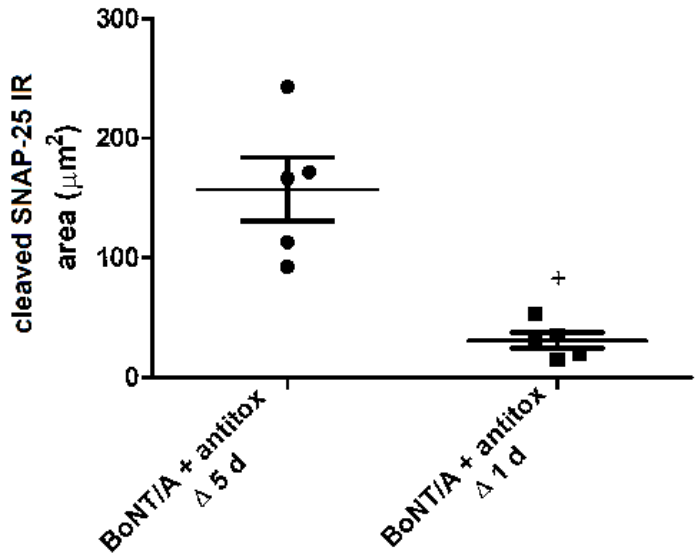
Figure 5

Occurrence of cleaved SNAP-25 in spinal cord following BoNT/A intramuscular injection depends on toxin transcytosis. A. Lumbar intrathecal (i.t.) administration of BoNT/A-specific antitoxin (20 i.u.) prevents the occurrence of cleaved SNAP-25 (bright immunofluorescence, Alexa 555-tyramide signal amplification) in the right ventral horn ipsilateral to BoNT/A injected 1 day before antitoxin (5 U kg-1, right gastrocnemius, ventral horn on the right side of the coronal section). In comparison to that, left ventral horn ipsilateral to BoNT/A (5 U kg^{-1}, left gastrocnemius) injected 5 days before antitoxin, shows abundant cleaved SNAP-25. The panel below the magnified ventral horn images indicate the time-course of BoNT/A and antitoxin treatments on the respective side. Scale bar = 500 μm. B.) Quantitative analysis of pixel intensity-thresholded area of cleaved SNAP-25 immunoreactivity in 3 non-overlapping high magnification (20 x) visual fields (433 μm x 323 μm = 0.14 mm^2) per single L4 slice, average of 3 slices per animal (N= 5 animals per group). BoNT/A + antitox Δ 5d, BoNT/A injected 5 days before antitoxin; BoNT/A +antitox Δ 1d, BoNT/A injected 1 days before antitoxin. + $p<0.05$ vs BoNT/A Δ 5d (two-tailed Mann Whitney U test, $p<0.05$ considered significant).

A.)


BoNT/A
(i.m.)
antitoxin
(i.t.)
perfusion
5 days
14 days
BoNT/A
(i.m.)
antitoxin
(i.t.)
perfusion
1 day
14 days

B.)


cleaved SNAP-25 IR
area (μm²)
300
200
100
0
BoNT/A + antitox
Δ 5 d
BoNT/A + antitox
Δ 1 d

SUPPLEMENTARY MATERIAL LEGENDS

Supplementary video S1

Intraneuronal (i.n.) sciatic injection of BoNT/A improves the hind-limb use in TeNT-evoked spasticity. The video shows representative external appearance and short walking sequence of a single rat with TeNT-evoked spastic paralysis of the right gastrocnemius muscle prior to, and 7 days post-treatment with i.n. BoNT/A (5 U kg^{-1})/6 days post i.t. control treatment with normal horse serum . Note the extended position and the inability to flex the hind paw, resulting in disuse of TeNT-injected hind-limb (day 0, seconds 0-11 of the video). Marked improvement of the local spastic paralysis restoring the ability to flex the hind limb, ground placement of the hind paw, and use during walking is visible 7 days after i.n. BoNT/A (seconds 12-21 of the video).

Supplementary video S2

BoNT/A specific antitoxin prevents beneficial effects of i.n. BoNT/A on hind limb use in TeNT-induced spastic paralysis. The video shows representative external appearance and short walking sequence of a single rat with TeNT-evoked spastic paralysis prior to, and 7 days post-treatment with i.n. BoNT/A (5 U kg^{-1})/6 days post intrathecal lumbar treatment with BoNT/A-specific antitoxin (20 i.u. in 20 µL). Local spastic paralysis and disuse of the hind-limb was similar at day 0 (seconds 0-4 of the video), and day 7 post BoNT/A/day 6 post antitoxin (seconds 5-17 of the video).