# Botulinum Neurotoxin Type A–Cleaved SNAP25 Is Confined to Primary Motor Neurons and Localized on the Plasma Membrane Following Intramuscular Toxin Injection

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## INTRODUCTION

**Botulinum neurotoxin type A (BoNT/A) is used clinically for a growing number of indications. The primary reasons for the increased therapeutic application of BoNT/A is attributed to its marked clinical efficacy and proven safety record. The general mechanism of action for BoNT/A has been established and involves a 4-step sequential process: receptor-specific binding to the presynaptic nerve terminal, internalization into the terminal via endocytosis, translocation of the light-chain ($L_C$/A) portion into the cytosol and enzymatic cleavage of intracellular synaptosomal-associated protein of 25 kDa ($SNAP25_{206}$) to form the inactive $SNAP25_{197}$ cleaved product. Functionally, this mechanism leads to the inhibition of neurotransmitter/ neuropeptide release from nerve fiber endings and subsequent transient paralysis or quiescence of the associated end-organ being innervated.**

**Recent studies have proposed that BoNT/A catalytic activity is not strictly confined to presynaptic terminals at the peripheral site of injection. These investigations have indicated that at higher doses, BoNT/A activity can spread to central neurons via a mechanism of retrograde transport within primary neurons followed by transfer (transcytosis) of the active toxin to second-order neurons, where it can potentially cleave SNAP25 and inhibit transmitter release. Consequently, it was hypothesized that peripheral applications of BoNT/A can directly transcytose to neurons within the central nervous system. Nonetheless, questions have been raised regarding the tools, models, and BoNT/A dosing levels used in these studies.**

**We recently developed site-specific, recombinant monoclonal antibodies (rMAb) against the BoNT/A-cleaved SNAP25 epitope and demonstrated their superior specificity over polyclonal antibodies and other commercial monoclonal antibodies at recognizing $SNAP25_{197}$, especially with regard to immunohistochemistry[1] (IHC). Since many of the conclusions regarding BoNT/A retrograde transport and transcytosis were based on IHC analysis of the cleaved product, we resolved to explore this possibility utilizing one of our rMAbs (Ab632) in combination with high-resolution three-dimensional (3D) imaging and quantitative analysis to characterize $SNAP25_{197}$ expression in a rat motor neuron (MN) pathway, following intramuscular (i.m.) BoNT/A injections at various doses.**

## OBJECTIVE

**We performed a systematic evaluation to determine whether $SNAP25_{197}$ is confined to primary MNs or is also found in neighboring cells and/or nerve fibers within the spinal cord (SC). We selected the motor pathway leading from the rat hindlimb tibialis anterior (TA) muscle through the sciatic nerve and into the L4-L5 segment of the ventrolateral SC (VLSC). This anatomical pathway represents a relevant, in vivo system to study the effects of BoNT/A, as motor nerve terminals (MNT) are the target of numerous therapeutic indications.**

## MATERIALS AND METHODS

**Materials**: Primary antibodies used were recombinant human anti-$SNAP25_{197}$ (Ab632) rMAb (Allergan plc, Dublin, Ireland); rabbit anti-SNAP25 monoclonal antibody (Epitomics-Abcam, Cambridge, MA); rabbit anti-vesicular acetylcholine transporter polyclonal antibody (Sigma-Aldrich, St. Louis, MO); mouse anti-syntaxin 1 (sc-12736; Santa Cruz Biotechnology, Dallas, TX); mouse anti-choline acetyltransferase monoclonal antibody, rabbit anti-calbindin polyclonal antibody, mouse anti-vGlut1 monoclonal antibody, mouse anti-vGlut2 monoclonal antibody, mouse anti-GFAP monoclonal antibody, rabbit anti-GAD65+GAD67 polyclonal antibody (Abcam, Cambridge, MA); rabbit anti-tyrosine hydroxylase polyclonal antibody (EMD Millipore, Billerica, MA). Cholera toxin B subunit Alexa Fluor 488 conjugate (CTB AF-488) and α-bugarotoxin Alexa Fluor-488 conjugate (α-Bgt AF-488) were obtained from Invitrogen Life Technologies (Carlsbad, CA).

**Animals:** Male Sprague-Dawley rats (225–250 g; Charles River, Wilmington, MA) were group housed on a 12-hour light-dark cycle with food and water available ad libitum. Animal protocols and procedures were approved by the Allergan Institutional Animal Care and Use Committee and followed National Institutes of Health guidelines.

**BoNT/A Preparation and Injection Procedures:** Working solutions of BoNT/A (onabotulinumtoxinA, 900 kDa complex, Allergan plc, Dublin, Ireland) were prepared in 0.9% saline. For single-injection studies, 20 µL of BoNT/A (3, 10, 30 U/kg) in 0.9% saline or AF-488 conjugated CTB (10 µg/kg) was injected intramuscularly into the mid-belly of the TA muscle of the right hind limb. Doses of BoNT/A used were equivalent to ~22, 75, and 225 pg/kg, respectively (based on 150 kDa BoNT/A neurotoxin protein). Rats were sacrificed 1, 3, and 6 days after BoNT/A injection. For multi-injection studies, CTB AF-488 was first injected into 5 equidistant spots spanning the right TA rat muscle (10 µg/kg, 10 µL each). The following day, BoNT/A (10 U/kg) was injected into the mid-belly of the muscle. Animals were sacrificed 6 days after BoNT/A injection.

**Digit Abduction Score Assay:** The Digit Abduction Score (DAS) assay was performed as previously outlined.[1] Rats were scored for DAS response starting 24 hours after onabotulinumtoxinA injections by eliciting hind limb digit abduction. DAS response was induced by grasping the rat around the torso, lifting into the air, and simulating a drop or a return to a flat surface. The rat reflexively braces for impact by spreading the digits in its hind paws, and the DAS response was immediately scored with the animal facing up in a reclining position. The varying degrees of digit abduction were then scored on a 5-point scale (0 = normal to 4 = maximum reduction in digit abduction). The peak DAS responses at each dose, which occurred on days 3–4, were observed and recorded.

**Tissue Preparation and Immunohistochemistry:** We used both perfused, postfixed as well as fresh, postfixed neuronal tissue. The perfused, postfixed tissue was best for anatomic preservation of nerve fiber morphology within the SC, whereas fresh, postfixed tissue was necessary for double-labeling experiments to preserve the CTB AF-488 signal and other sensitive antigens. At scheduled time points, rats were either anesthetized and transcardially perfused with 4% paraformaldehyde (for fixed tissue) or euthanized with CO2 (for fresh-frozen tissue). Ipsilateral and contralateral TA muscles, sciatic nerves, lumbar and thoracic SC segments (T9–L5; ~1.2 cm) were then harvested and either OCT-embedded and frozen in liquid nitrogen, or postfixed in 4% paraformaldehyde for 2 hours, immersed in 30% sucrose, and OCT-embedded at –20°C. All tissues were stored at –80°C until use. Tissues were cryostat sectioned into 12, 16, or 40 µm-thick sections, mounted onto microscope slides, and postfixed with 4% paraformaldehyde for 10 minutes (for fresh-frozen tissue) followed by a wash in phosphate-buffered saline (PBS). Slide-mounted sections were first blocked for nonspecific signal in blocking buffer (1× PBS + 5% normal donkey serum) for 1 hour and then incubated with primary antibodies at desired concentrations in blocking buffer overnight at 4°C. Following several washes, sections were incubated with Alexa Fluor–labeled secondary antibodies (Jackson ImmunoResearch, West Grove, PA) diluted in blocking buffer for 2 hours at room temperature and washed again. Slide-mounted sections were coverslipped using ProLong Gold Antifade Mountant with 4′,6-diamidino-2-phenylindole (DAPI; Life Technologies, Grand Island, NY). Adjacent sections processed without primary antibodies served as negative controls to show background signal.

**Image Acquisition:** Muscle, sciatic nerve and SC images were acquired and analyzed using either an Olympus FV1200 confocal microscope (Olympus, Waltham, MA, USA) with Olympus Fluoview FV-10 ASW software (Version 4.1, Olympus, Center Valley, PA, USA, 2003-2013), a Zeiss LSM-710 confocal microscope (Carl Zeiss, Thornwood, NY, USA) with Zeiss ZEN software (Version 5.5, Carl Zeiss, Thornwood, NY, USA, 2009), or a Keyence BZ-9000E fluorescence microscope (Keyence Corp., Osaka, Japan). The resulting images were 3D reconstructed and analyzed using Imaris software (Bitplane, Concord, MA).

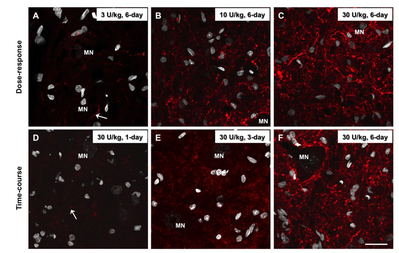
**Image Analysis:** Analysis of $SNAP25_{197}$ immunoreactive (IR) staining within rat VLSC was performed on 16 µm-thick sections collected from thoracic and lumbar regions of the SC (~1.2 cm total length). For T9-T10 and L1-L2 SC regions, 10 random sections from each region were qualitatively examined for $SNAP25_{197}$ staining per animal (n=3). For L4-L5 SC region, 100 consecutive sections per animal were collected for qualitative and quantitative analysis of $SNAP25_{197}$ staining. Quantitative analysis of the signal intensity in L4-L5 was performed on a total of ten Z-stack confocal images (12 bits, 1024×1024 px resolution, 8 planes) per animal acquired using a 30× silicon oil objective. Images were collected from every tenth section within the same general region of the ipsilateral VLSC. Signal intensity was determined using the Imaris 3D measurement algorithm. Image stacks were thresholded to remove background levels of fluorescence and retain the brighter pixels. The resulting $SNAP25_{197}$ mean signal intensity was calculated and recorded.

For colocalization analysis of $SNAP25_{197}$ staining in the VLSC with staining for other biomarkers, 300 consecutive sections were collected per animal (n=3) from the L4-L5 SC region. Depending on the biomarker, a total of 10–30 Z-stack confocal images (12 bits, 1024×1024 px resolution, 15 planes) per animal were acquired using a 40× silicon oil objective; 3–5 images were collected from every 15th section within the same general region of ipsilateral VLSC. Qualitative 3D analysis of $SNAP25_{197}$ signal was performed in multiple planes (XY, XZ, YZ) or oblique angles to examine precise colocalization with other biomarkers. Quantitative analysis of the colocalization patterns of $SNAP25_{197}$ with biomarkers was performed using the 'ImarisColoc' module. Image stacks from both channels were thresholded to remove background levels of fluorescence and retain the brighter pixels. The percentage of total $SNAP25_{197}$-positive voxels (3D pixels) colocalized with the other biomarkers was calculated and recorded.

**Statistical analysis:** All data were statistically analyzed using SigmaPlot v12.5 (Systat Software Inc., San Jose, CA). Statistical significance was determined by either a student-Newman-Keuls test comparing the mean intensity values between groups, or by one-way ANOVA followed by Dunn's post-hoc analysis.

## RESULTS

### 1 Dose- and time-dependent increase in $SNAP25_{197}$ immunostaining in the spinal cord following BoNT/A IM injections.

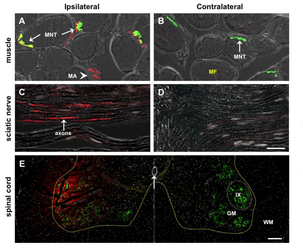



Confocal images in the ipsilateral VLSC showing an increase in $SNAP25_{197}$-IR signal (**A**–**C**) with increasing BoNT/A dose at 6 days post-injection and (**D**–**F**) with time post-intramuscular injection of 30 U/kg BoNT/A. Note that at 3 U/kg BoNT/A, 6 days (**A**) and 30 U/kg BoNT/A, 1 day postinjection (**D**), there is very light and sparse $SNAP25_{197}$-IR signal within the VLSC (arrows), whereas at 6 days post-BoNT/A (30 U/kg) injection (**C**, **F**), the signal is more concentrated and intense. DAPI-stained cell nuclei are in gray. BoNT/A=botulinum neurotoxin type A; DAPI=4′,6-diamidino-2-phenylindole; IM=intramuscular; IR=immunoreactive; MN=motor neuron; VLSC=ventrolateral spinal cord. Scale bar = 30 µm.

| Dose-Response | | | Time-Course | |
|---|---|---|---|---|
| BoNT/A Dose (U/kg) | DAS Score | Mean Intensity in VLSC (Mean ± SEM) | Days post-BoNT/A (30 U/kg) | Mean Intensity in VLSC (Mean ± SEM) |
| 3 | 2.0 | 3.2 ± 1.6† | 1-day | 11 ± 4 |
| 10 | 3.5 | 37 ± 10 | 3-day | 139 ± 4 |
| 30 | 4.0 | 225 ± 78* | 6-day | 225 ± 78+ |

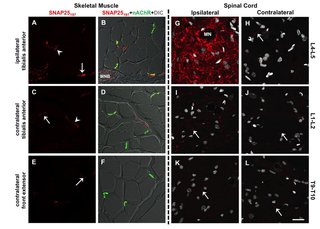
Quantitative analysis of $SNAP25_{197}$-IR staining intensity in the rat ipsilateral ventral SC following BoNT/A IM injections into the right TA under different conditions.The analysis was performed either at 6 days postinjection with increasing doses (3, 10, and 30 U/kg) of BoNT/A ("dose-response"), or at 3 different time points (1, 3, and 6 days) post-BoNT/A injection at a fixed dose (30 U/kg; "time-course"). The table shows corresponding DAS scores for the animals receiving increasing doses of BoNT/A. The same animals were used for analysis of BoNT/A dosed at 30 U/kg for 6-day time course parameters. Data represent mean ± SEM of n=3 rats; †n=2 animals, data presented as mean ± SD; *P<0.05 statistically different from 3 U/kg based on one-way analysis of variance with Dunn's post hoc analysis; +P<0.05 statistically different from 1 day based on student Newman-Keuls test; BoNT/A=botulinum neurotoxin type A; DAPI=4′,6-diamidino-2-phenylindole; DAS=Digit Abduction Score; IM=intramuscular; IR=immunoreactive; MN=motor neuron; SC=spinal cord; TA=tibialis anterior; VLSC=ventrolateral spinal cord.statistically different from 3 U/kg based on one-way analysis of variance with Dunn's post hoc analysis; +P<0.05 statistically different than 1 day based on student Newman-Keuls test.

### 2 $SNAP25_{197}$ immunostaining in ipsilateral TA muscle, sciatic nerve, and lumbar VLSC 6-days following BoNT/A IM injection (30 U/kg).



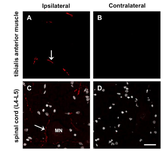

(**A**, **B**) Confocal images of cross-sections through the mid-belly of the ipsilateral (**A**) and contralateral (**B**) rat TA muscles 6 days after BoNT/A injection. The images show $SNAP25_{197}$-IR staining (red) in MNT, adjacent to α-Bgt-labeled nicotinic acetylcholine receptor clusters (green, arrows) and in motor axons (MA, arrowhead) in the ipsilateral, but not the contralateral muscle. Individual MF are delineated with DIC imaging. (**C**, **D**) Sagittal sections through the ipsilateral (**C**) and contralateral (**D**) rat sciatic nerve showing $SNAP25_{197}$-IR staining (red) aligned with the length of a small number of axons within the ipsilateral, but not the contralateral sciatic nerve. DAPI stained cell nuclei are in gray. (**E**) Low magnification (20×) image of the ventral SC at the L4-L5 region showing a diffuse circular pattern of $SNAP25_{197}$-IR staining (red) centered in laminae IX of the ipsilateral side. MN somas are delineated by ChAT and VAChT staining (green). Cell nuclei are labeled by DAPI staining (gray). Arrow points to the central canal of the spinal cord. The boundary between GM and WM is delineated. BoNT/A=botulinum neurotoxin type A; ChAT=choline acetyltransferase; DAPI=4′,6-diamidino-2-phenylindole; DIC=differential interference contrast; GM=gray matter; IM=intramuscular; IR=immunoreactive; MF=muscle fibers; MNT=motor nerve terminals; TA=tibialis anterior; VAChT=vesicular acetylcholine transporter; VLSC=ventrolateral spinal cord; WM=white matter. Scale bar = 20 µm in A–D, 200 µm in E.

### 3 Distal spread of BoNT/A activity is detected in muscle and SC at a high dose (30 U/kg) 6 days following toxin IM injection.



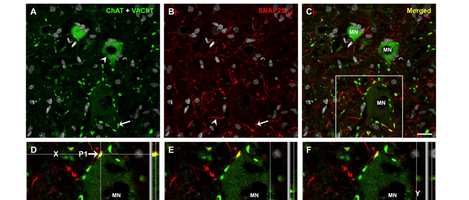

(**A**–**F**) Confocal images of cross-sections through the ipsilateral (**A**, **B**) and contralateral (**C**, **D**) TA and the contralateral front extensor (**E**, **F**) muscles showing $SNAP25_{197}$-IR staining (red) in motor nerve terminals (arrows), adjacent to α-Bgt-labeled nicotinic acetylcholine receptor clusters (green) and in motor axons (arrowhead). (**G**-**L**) Confocal images of cross-sections through the SC from the ipsilateral and contralateral side of the BoNT/A injection at L4-L5 (**G**, **H**), L1-L2 (**I**, **J**), and T9-T10 (**K**, **L**) showing $SNAP25_{197}$-IR staining (red) in neuronal processes (arrows). Note that the farther away from the muscle of injection (right hindlimb TA), the lighter and more sporadic the staining becomes. DAPI stained cell nuclei are in gray. BoNT/A=botulinum neurotoxin type A; DAPI=4′,6-diamidino-2-phenylindole; IM=intramuscular; IR=immunoreactive; MN=motor neuron; MNB=motor nerve bundle; SC=spinal cord; TA=tibialis anterior. Scale bar = 30 µm.

### 4 Distal spread of BoNT/A activity (based on $SNAP25_{197}$-IR) is not detected in contralateral muscle or SC at a low, physiologically relevant dose (3 U/kg) 6 days following toxin IM injection.



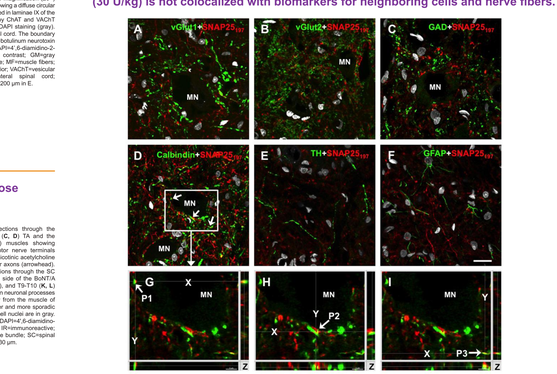

(**A**, **B**) Confocal images of cross-sections through the ipsilateral (**A**) and contralateral (**B**) TA muscle showing $SNAP25_{197}$-IR staining (red) in motor nerve terminals on the ipsilateral (arrow), but not the contralateral side. (**C**, **D**) Confocal images of cross-sections through the SC from the ipsilateral and contralateral side of the BoNT/A injection at the level of L4-L5 showing $SNAP25_{197}$-IR staining (red) in neuronal processes on the ipsilateral (arrow), but not the contralateral side. DAPI stained cell nuclei are in gray. BoNT/A=botulinum neurotoxin type A; DAPI=4′,6-diamidino-2-phenylindole; IM=intramuscular; IR=immunoreactive; MN=motor neuron; SC=spinal cord; TA=tibialis anterior. Scale bar = 30 µm.

### 5 $SNAP25_{197}$-IR signal in the lumbar SC, 6-days following BoNT/A IM injection (30 U/kg) is colocalized with biomarkers for cholinergic MNs.



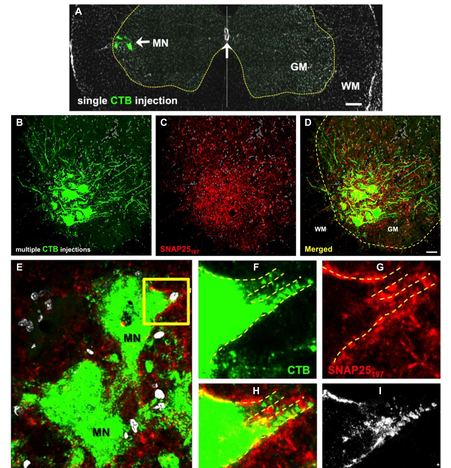

(**A**-**C**) Confocal images from ipsilateral ventral SC at L4-L5 showing MNs co-immunostained for (**A**) choline acetyltransferase (ChAT) and vesicular acetylcholine transporter (VAChT), (**B**) $SNAP25_{197}$ and (**C**) the two signals merged together. Note that ChAT-IR staining is found primarily in the MN cytoplasm (arrowhead in A), while VAChT-IR staining is found primarily at points of synaptic contact (arrow in **A**). In contrast, $SNAP25_{197}$-IR staining is found primarily in neurite processes (arrowhead in **B**) and at points of synaptic contact (arrow in **B**). (**D**-**F**) A 3D image analysis of the boxed region in (**C**) reveals three focal points of co-localization for VAChT-IR and $SNAP25_{197}$-IR staining (P1, D; P2, E; P3, F). The X, Y image stack reconstructions, depicted in the XZ and YZ planes demonstrate that the signals are in the same compartment for each one of the points analyzed. DAPI stained cell nuclei are in grey. MN, motor neuron. Scale bar = 20 µm in A-C; 10 µm in D-F. **Quantitative analysis demonstrated that 9 ± 0.6% (SEM) of the total $SNAP25_{197}$-IR signal was colocalized with VAChT-IR. Moreover, 3D image analysis of the stacked images in the XZ and YZ planes confirmed that these signals were indeed within the same neuronal compartments.**

### 6 $SNAP25_{197}$-IR signal in the lumbar SC, 6-days following BoNT/A IM injection (30 U/kg) is not colocalized with biomarkers for neighboring cells and nerve fibers.



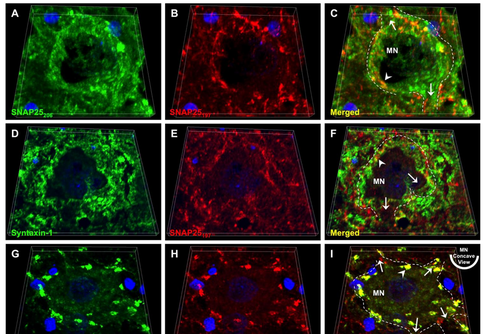

Confocal images from ipsilateral ventral SC at L4-L5 showing co-immunostaining of $SNAP25_{197}$ (red) with biomarkers (green) for (**A**, **B**) glutamatergic synaptic contacts (vesicular glutamate transporter, vGlut1/vGlut2), (**C**) inhibitory interneurons (glutamic acid decarboxylase, GAD65/67), (**D**) Renshaw cells (Calbindin D28k), (**E**) descending catecholamine pathways (tyrosine hydroxylase, TH) and (**F**) astrocytes (glial fibrillary acidic protein, GFAP). Note the lack of co-localization of red and green signals throughout the images. (**G**-**I**) A 3D image analysis of the boxed region in panel **D** reveals three focal points of apparent co-localization for calbindin D28K and $SNAP25_{197}$-IR staining (P1, G; P2, H; P3, I). However, the X, Y image stack reconstructions, depicted in the XZ and YZ planes demonstrate that the signals are, in fact closely adjacent to each other. DAPI stained cell nuclei are in grey. MN, motor neuron. Scale bar = 20 µm in A-F and 10 µm in G-I. **Quantitative analysis of the $SNAP25_{197}$ and calbindin D28k signals demonstrated that 7 ± 1.0% of the total $SNAP25_{197}$-IR signal was colocalized with calbindin D28k. However, 3D image analysis of these focal points in the XZ and YZ planes demonstrated that the signals were, in fact adjacent to each other (much like a synaptic contact) and not within the same neuronal compartments.**

### 7 Somatodendritic fields of SC-MNs innervating the TA muscle are delineated by retrograde tracing with labeled-CTB. Following BoNT/A (10 U/kg) TA injection, $SNAP25_{197}$-IR signal is found on the plasma membrane, adjacent to the CTB signal.



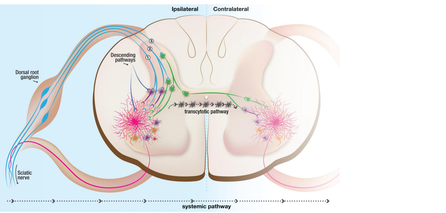

(**A**) Low magnification (20x) image of the ventral spinal cord at the L4-L5 region showing the delineation of MNs in VLSC 7-days following a single injection of labeled-CTB (green). Arrow points to the central canal of the SC. The boundary between grey matter (GM) and white matter (WM) is delineated. (**B**-**D**) Low magnification, confocal tiled images of the rat VLSC at L4-L5. (**B**) Delineation of MNs in the VLSC by labeled-CTB 7-days following multiple injections to the right TA muscle. (**C**) The same region of the VLSC showing $SNAP25_{197}$-IR staining 6-days following injection of BoNT/A (10 U/kg) into the right TA muscle (7-days after CTB injection). (**D**) The ipsilateral VLSC showing labelled-CTB and $SNAP25_{197}$-IR signals merged together. (**E**) Confocal image of several MNs illustrating the differential expression of labeled-CTB and $SNAP25_{197}$-IR signals. (**F**-**I**) High magnification analysis of the boxed region in (**E**) reveals that labeled-CTB (**F**) is localized primarily within the cellular cytoplasm, while $SNAP25_{197}$-IR (**G**) is expressed on the cellular membrane. Analysis of the merged signals (**H**) reveals that the majority of co-localization (I) is found on the plasma membrane. DAPI stained cell nuclei are in grey. MN, motor neuron. Scale bar = 30 µm in A-C; 200 µm in D; 20 µm in E and 5 µm in F-I.

### 8 $SNAP25_{197}$ is expressed in synaptic compartments on the MN plasma membrane.




Confocal images of individual 3D reconstructed MNs from the ipsilateral VLSC showing co-immunostaining of $SNAP25_{197}$ (red) with three different synaptic biomarkers (green): (**A**-**C**) Undesired SNAP25 ($SNAP25_{206}$), (**D**-**F**) syntaxin and (**G**-**I**) the cholinergic synaptic biomarker, VAChT, along with the merged signals. DAPI stained cell nuclei are in blue. Cells were 3D reconstructed from individual 2D images and these partial cells are all presented in a concave orientation (I). Cell borders are delineated by dashed lines. Arrowheads indicate potential regions of synaptic contact. Arrows point to dendritic processes coming off from the cell soma. MN, motor neuron. Scale bar = 10 µm.

### 9 Simplified diagram illustrating motor circuitry components within the ventral spinal cord and the potential pathways for spread of BoNT/A activity.




Motor neurons (pink) in the ventral SC have extensive dendritic arbors extending radially from the soma in all axes. The dendritic branches of MNs form a multitude of synaptic contacts with neighboring neurons and nerve fibers, including Renshaw cells (orange), excitatory (green) and inhibitory (purple) interneurons, sensory afferents from the dorsal root ganglia (light blue) and descending catecholamine fibers (dark blue), as well as resident glial cells (red). Collectively, this local circuitry within the SC helps mediate a number of sensorimotor reflex actions, including (1) the sensory (stretch) reflex arising from Ia afferent fibers originating in muscle spindles, (2) the Golgi tendon reflex arising from Ib afferent fibers originating at the junction of a muscle and tendon, and (3) the flexion reflex arising from cutaneous afferent nociceptors and resulting in withdrawal from painful stimuli. This latter reflex also stimulates an opposite reaction in the contralateral limb mediated by the crossed extension reflex. Potential pathways for BoNT/A spread from the ipsilateral to the contralateral side, either by transcytotic or systemic routes are defined (gray).

**Color-coded list of neuronal cell- and fiber-types in the ventral SC (above) and the different biomarkers used for identifying them.**

| Drawing | Cell Type | Neurotransmitter | Biomaker |
|---|---|---|---|
| | Motor neuron | Acetylcholine | ChAT / VAChT |
| | Renshaw cell | Glycine | Calbindin 28kD |
| | Excitatory neuron | Glutamate | vGlut1 / vGlut2 |
| | Inhibitory interneuron | GABA | GAD65 / GAD67 |
| | Sensory neuron | Glutamate | vGlut1 / vGlut2 |
| | DA efferents | Dopamine | TH |
| | Glial cell | --- | GFAP |

Table. For each cell type in the SC, the primary neurotransmitter and the identifying biomarker are listed. Also listed are the associated neurotransmitters (except for glial cells).

## CONCLUSIONS

- **$SNAP25_{197}$-IR staining in ipsilateral VLSC increased in a dose and time-dependent manner following BoNT/A peripheral injection.**
- **A high, saturating dose of BoNT/A (30 U/kg), but not a lower dose (3 U/kg), resulted in distal spread of toxin activity, giving rise to sporadic $SNAP25_{197}$ signal in the muscles and associated spinal cord regions with no evidence for transcytosis. Despite the spread in activity, functional effects were not detected in the distal muscles.**
- **Even at the high dose (30 U/kg), we found that $SNAP25_{197}$-IR staining was colocalized with biomarkers for motor neurons but was not colocalized with markers for neighboring neurons, nerve fibers, or glial cells.**
- **Under these experimental conditions, our data suggest that BoNT/A is confined to primary motor neurons and that any evidence of distal activity is the result of limited systemic spread of toxin at high doses and not through transcytosis within the spinal cord.**
- **At higher doses of BoNT/A, $SNAP25_{197}$ was expressed throughout the MN and was colocalized with synaptic markers on the plasma membrane even at 6 days posttreatment. These data support previous studies suggesting that $SNAP25_{197}$ may be incorporated into SNARE-protein complexes within the affected motor neurons.**

## REFERENCES

1. **Rheaume C, et al.** *Toxins.* **2015; 7:2354-2370.**
2. **Broide RS, et al.** *Toxicon.* **2013; 71:18-24.**

## DISCLOSURES

**Drs Broide, Cai, Francis, and Brin are full-time employees of Allergan plc.**

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