Cell Biology

Botulinum toxin intoxication requires retrograde transport and membrane translocation at the ER in RenVM neurons

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Abstract

Botulinum neurotoxin A (BoNT/A) is a highly potent proteolytic toxin specific for neurons with numerous clinical and cosmetic uses. After uptake at the synapse, the protein is proposed to translocate from synaptic vesicles to cytosol through a self-formed channel. Surprisingly, we found that after intoxication proteolysis of a fluorescent reporter occurs in the neuron soma first and then centrifugally in neurites. To investigate the molecular mechanisms at play, we use a genome-wide siRNA screen in genetically engineered neurons and identify over three hundred genes. An organelle-specific split-mNG complementation indicates BoNT/A traffic from the synapse to the soma-localised Golgi in a retromer dependent fashion. The toxin then moves to the ER and appears to require the Sec61 complex for retro-translocation to the cytosol. Our study identifies genes and trafficking processes hijacked by BoNT/A, revealing a complex route for efficient intoxication that contradicts the currently accepted model of BonT intoxication.

eLife assessment

In this **valuable** manuscript, Yeo et al. describe new methods for assessing the intracellular itinerary of Botulinum neurotoxin A (BoNT/A), a potent toxin used in clinical and cosmetic applications. The current manuscript challenges previously held views on how the catalytic portion of the toxin makes its way from the endocytic compartment to the cytosol, to meet its substrates. The approach taken is deemed innovative and the experiments are carefully performed, presenting **solid** evidence for some of the drawn conclusion; however, the conclusions one may draw from the experimental results are somewhat limited, as it is possible that the scope of their findings could be restricted to the specific neuron model and molecular tools that were used. This paper could be of interest to both cell biologists and physicians.

Introduction

Botulinum toxins (BoNTs) are the most potent toxins known, with a lethal dose of less than a microgram; paradoxically they are also one of the most widely used drugs today for neuromuscular conditions and various aesthetic procedures. BoNTs block overactive muscles involved in neuromuscular disorders or wrinkles by preventing nerve stimulation. The high potency of BoNTs allows for precise localized treatments through injection of small quantities of toxins. Their long-lasting effects make them attractive treatments despite the required injection. These remarkable properties of BoNTs are derived from their unique intoxication biology. Yet, key features of this biology remain poorly understood.

BoNTs target synapses at the neuro-muscular junction, where they cleave specific cytosolic proteins involved in the release of the acetylcholine neurotransmitter in neurons (Dong et al., 2019; Montecucco and Schiavo, 1994). As BoNTs are proteins of ~150kDa, they cannot diffuse through cellular membranes. The key biological features of BoNTs are: 1) high binding specificity for neurons, specifically neuronal synapses, 2) ability to reach the cytosol of neurons, 3) high specificity for a molecular target once in the cytosol. Of these three features, the second is probably the least understood.

BoNTs are naturally expressed by the gram-positive bacterium *Clostridium botulinum* (*C. botulinum*) as a single ~150kDa polypeptide chain, then post-translationally cleaved to produce a 50kDa N-terminal catalytic light (L) chain, which is a zinc metalloprotease and a 100kDa C-terminal heavy (H) chain. The L and H chains are linked via a disulfide bond and other noncovalent interactions to form the active toxin. *C. botulinum* produces seven serotypes of BoNTs, A to G, and while they all interfere with neurotransmission, they have different cell surface receptors and, interestingly, different kinetics of action across subtypes (Pellett et al., 2015b; Rasetti-Escargueil and Popoff, 2020).

BoNT/A is the most clinically-used serotype, it binds to the synaptic membrane of neurons with high specificity, with synergistic binding to the synaptic vesicle protein 2 (SV2) and the trisialogangloside GT1b (Dong et al., 2006; Yowler and Schengrund, 2004). After internalization and translocation to the cytosol, the light chain of BoNT/A binds and cleaves the SNARE (Soluble N-ethylmaleimide-sensitive factor Attachment protein REceptor) SNAP25 at the Q167R residue. As SNAP25 is essential for the release of synaptic vesicles, its inactivation results in the inability of neurons to release acetylcholine, the key neurotransmitter at neuromuscular junctions (Dong et al., 2019; Montecucco and Schiavo, 1994). As the toxin proteolytic activity is highly specific, the neuron remains viable and able to recover once the toxin has been cleared (Peng et al., 2013).

The current model for intoxication postulates cell surface binding and internalisation in endosomes, then, due to changes of pH there are conformational changes in the heavy chain that leads to the formation of a channel which ushers the L chain into the cytosol of the axonal bouton (Azarnia Tehran et al., 2017; Pirazzini et al., 2014). The heavy chain is proposed to contain an N-terminal translocation domain (HN) in addition to the C-terminal receptor-binding domain (HC). Once or while the L chain is translocated, it is released from the H chain by disulfide bond reduction, mediated by the thioredoxin reductase 1 (TXNRD1) (Dong et al., 2019; Pirazzini et al., 2013; Rossetto et al., 2021). After refolding of the L chain, it binds and cleaves SNAP25 at a specific residue.

Parts of this scenario have been challenged by studies showing that there are no distinct structural changes to the H and L domains upon acidification and by the difficulty in observing in vitro a self-translocation process (Araye et al., 2016; Galloux et al., 2008). Studies have also shown

that BoNT/A is trafficked beyond the axon boutons in non-acidic and non-recycling vesicles (Antonucci et al., 2008; Harper et al., 2016; Restani et al., 2012). In addition, the current model fails to explain the delayed onset of action, with hours to days between the time of injection and muscle paralysis (Ledda et al., 2022). Arguably, the main problem of the model is its failure to propose a thermodynamically consistent explanation for the directional translocation of a polypeptidic chain across a biologial membrane. Other known instances of polypeptide membrane translocation such as the co-translational translocation into the ER indicate that it is an unfavorable process, which requires cellular energy (Alder and Theg, 2003).

Other similar toxins, like the Cholera toxin, Ricin and Pseudomonas exotoxin A, follow a complex intracellular trafficking route, first from endosomes to the Golgi apparatus, then to the endoplasmic reticulum where they translocate across the membrane. The translocation event itself relies on the host translocon machinery or other ER endogenous complexes (Moreau et al., 2011; Nowakowska-Gołacka et al., 2019; Zhang et al., 2013).

In this study, we present a novel assay based on genetically modified human progenitors able to generate functional neurons (Donato et al., 2007; Song et al., 2019). The reporter assesses BoNT/A proteolytic activity in live cells and is amenable to a high throughput screening. Complete cleavage of the reporter requires nearly 72h of exposure. Surprisingly, we find that BoNT/A proteolytic activity is first detected in the soma of neurons, 24 to 48 hours before reaching the end of neurites. To investigate the underlying reasons, we performed a genome-wide RNAi survey of the host factors required for BoNT/A intoxication. Our results reveal a high number of genes linked to membrane trafficking, suggesting that BoNT/A follows a complex intracellular route. We use another set of reporters, based on GFP reconstitution to retro-axonal traffic is required for BoNT/A to reach the ER, where it translocates to the cytosol using the Sec61 translocon. These findings help explain the delayed effect of the toxin and could pave the way to improved therapeutics.

Results

ReD SNAPR: neuronal cells expressing a BoNT/A reporter derived from SNAP25

To establish a high-throughput assay for BoNT/A activity, we selected an abundant and consistent source of neuronal cells, the ReNcell VM, a v-myc-transformed human neuronal stem cell line. This cell line has the ability to differentiate into neurons in about two weeks after withdrawal of EGF and bFGF from the culture medium. Over this period, neurites are formed and the neuronal markers, beta3-tubulin and MAP2 increase significantly in differentiated cells (**Supplementary Figure 1A**). In parallel, the low levels of the oligodendrocyte marker CNPase expressed in the stem cells are further diminished.

To detect BoNT/A activity, we generated a chimeric reporter protein composed of SNAP25 flanked by the red fluorescent protein called tagRFPT (tRFPT) and the green fluorescent protein tagGFP (tGFP) at its N-terminus and C-terminus respectively (Shaner et al., 2008). The construct was named SNAPR (SNAP25 tagged with RFP and GFP). Using lentiviral transduction, we generated ReNcell VM cells stably expressing SNAPR (**Figure 1A**). We coined this cell line Red-SNAPR for ReNcell-derived, expressing SNAPR. After BoNT/A cleaves the SNAP25 moiety at Q197R, the C-terminal tGFP-containing moiety is rapidly degraded while the rest of the construct is preserved (**Figure 1A**).

After incubation with 100 nM BoNT/A for 48 hours, tGFP fluorescence was noticeably diminished compared to tRFP, whose signal remains stable. By western blot, a ~75 kDa SNAPR construct was detected by both tRFP and tGFP antibodies, and cleaved to a 50 kDa product only detected by tRFP

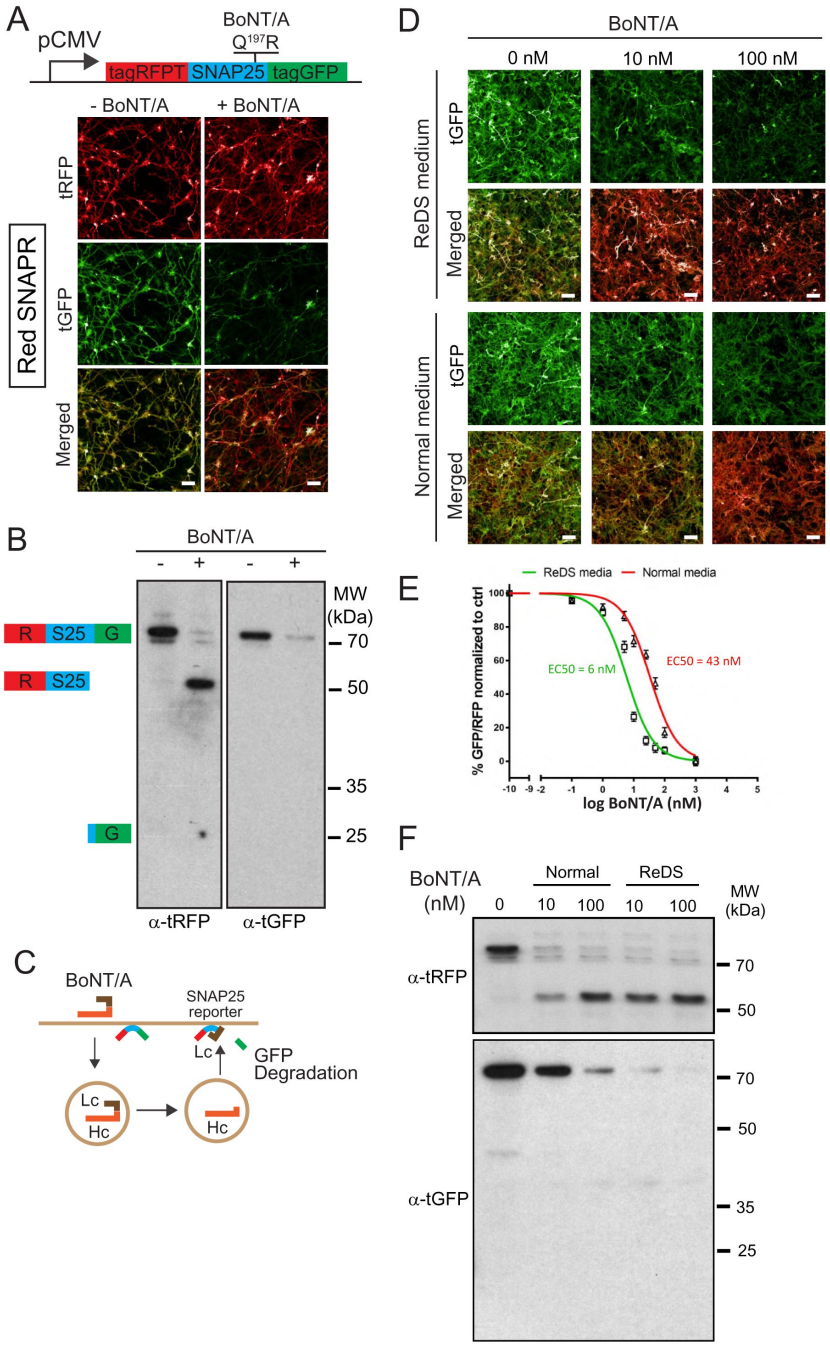
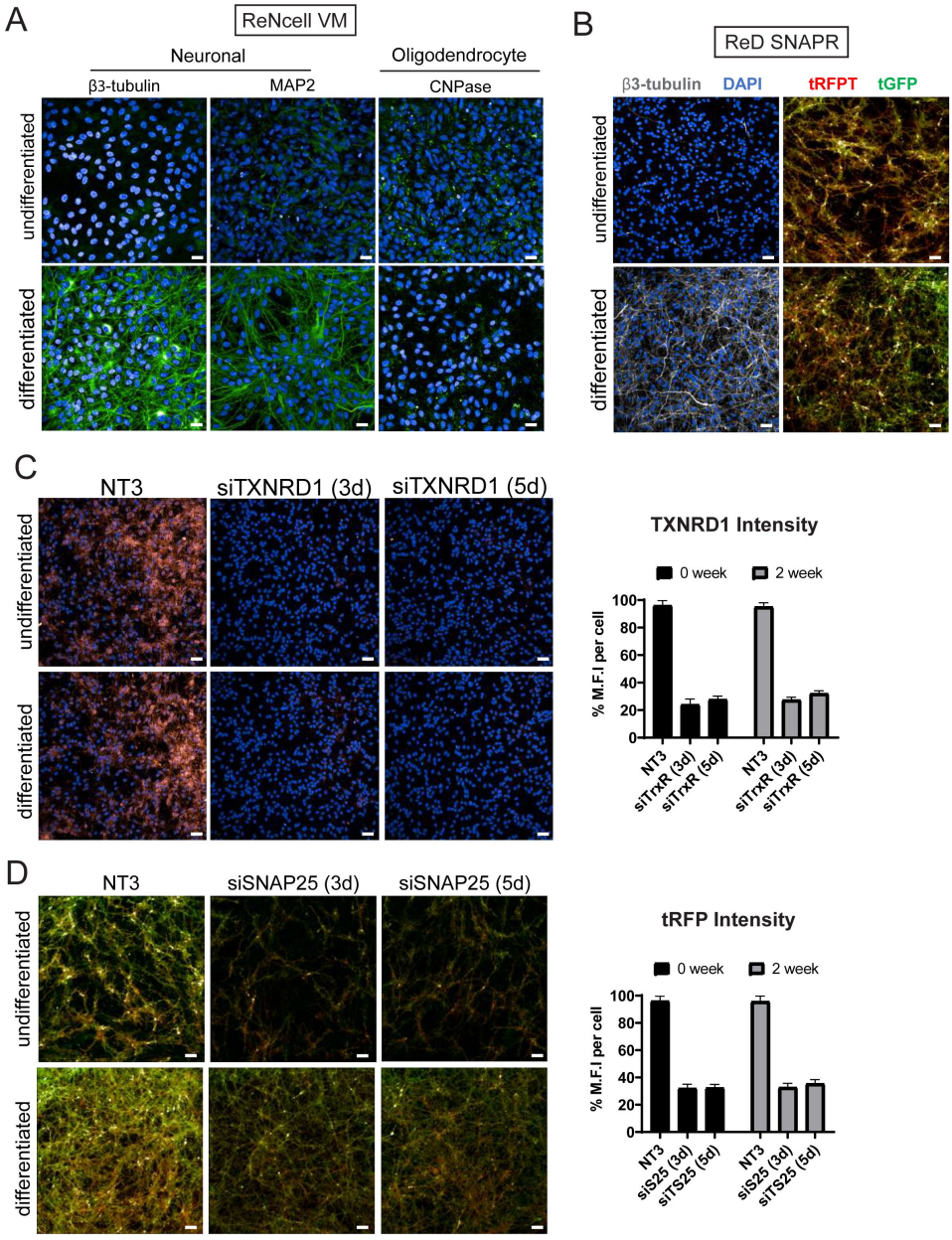


Figure 1.

Differentiated BoNT/A reporter cell line, Red SNAPR, is highly sensitive to BoNT/A intoxication.

(A) Schematic diagram of the BoNT/A reporter construct, SNAPR. Representative images of ReNcell VM cell line, Red SNAPR, stably expressing SNAPR and incubated with 100nM BoNT/A for 48 hours. **(B)** Western blot of cell lysates from **(A)** probed with tRFPT and tGFP antibodies. **(C)** Schematic diagram of the BoNT/A intoxication assay. **(D)** Red SNAPR differentiated in normal or ReDS medium, then incubated with 0, 10 and 100 nM BoNT/A for 48 hours. Quantification of EC_{50} dose response of BoNT/A in ReD SNAPR cells incubated with 0 - 100nM BoNT/A in normal and ReDS medium using GFP/RFP ratio readout. **(E)** Western blot of cell lysates from **(D)** probed with tRFPT and tGFP antibodies. Scale bars 50 μm.



Supplementary Figure 1.

Differentiation and siRNA depletion dynamics in ReNcell VM.

(A) ReNcell VM differentiated for 2 weeks with staining for nuclei (in blue) and neuronal markers b3-tubulin MAP2 (green) and for oligodendrocyte marker CNPase (2′,3′-Cyclic-Nucleotide3′-Phosphodiesterase). Scale bars 20 μm. **(B)** ReD SNAPR cell line differentiated for 2 weeks with staining of b3-tubulin (white). Expression of SNAPR construct is detected in the RFP and GFP channels. Scale bars 50 μm. **(C)** Undifferentiated and differentiated ReNcell VM transfected with siNT3 (Non Targeting 3) siRNA or siRNA against thioredoxin reductase 1 (TXNRD1) for 3 and 5 days showing persistent TXNRD1 protein depletion. Scale bars 50 μm. **(D)** Undifferentiated and differentiated ReD SNAPR cells transfected with siNT3 or SNAP25 siRNA for 3 and 5 days showing reduction of the SNAPR reporter. Scale bars 20 μm.

antibody, while the expected ~25 kDa tGFP fragment was undetectable (**Figure 1B**). This stable cell line has unaltered differentiation potential (**Supplementary Figure 1B**).

To test whether BoNT/A intoxication was dependent on neuronal activity, we used the neurotrophic factors GDNF and BDNF to enhance neuronal differentiation. We further supplemented the medium with high salt (KCl and CaCl) for neuronal stimulation (Harper et al., 2011; Pellett et al., 2015a). This differentiation and stimulation media (ReDS media) resulted in an improvement of sensitivity by an order of magnitude in the imaging assay (**Figure 1D, E**). This increased sensitivity was confirmed by western blot in which the EC_{50} of BoNT/A intoxication improved from 43 nM to 6 nM (**Figure 1F**). This strongly suggests that toxin binding and uptake occurs at the level of active synapses, as is the case in vivo.

BonT/A activity is first detected in the soma of neurons

During the initial experiments, we noticed that optimal degradation of the reporter required 48h after adding the toxin. This delay was surprising considering the model of rapid translocation after internalisation. As the reporter allows live detection of BonT/A proteolytic activity, we were curious to observe the distribution of BonT/A activity within the neurons over time. We thus imaged cells at 24, 48 and 72hr after intoxication. To facilitate imaging of individual neurites, a co-culture of Red-SNAPR to ReNcell VM cells (1:4 ratio) was implemented (**Figure 2A**). Surprisingly, at 24 hours, there was no loss of GFP signal in the terminal part of neurites but visible degradation at the neurite hillock. At 36 hours, tGFP degradation progressed towards the neurite terminals and most of the tGFP signal was eliminated by 48 hours. The pattern suggests a slow distribution of BoNT/A from the cell body to the terminus of neurites (i.e. ~16µm/h) (**Figure 2B**).

BoNT/A protein is also detected first in neuronal soma

SNAPR reports on BoNT activity and it is conceivable that BoNT/A might not be active immediately after translocation, thus potentially affecting the spatiotemporal pattern of proteolytic activity. To directly detect BoNT protein in the cytosol, we generated a ReNcell VM cell line expressing an HA-tagged split monomeric NeonGreen (mNG) protein targeted to the cytosol (Cyt-mNG_{1-10}) (Feng et al., 2017). We verified that Cyt-mNG_{1-10} was expressed using the HA tag, the expression was homogeneously distributed in differentiated neurons and we observed no GFP signal (**Figure 2C**). We also generated and produced the complementary mNG_{11} fused to BoNT/A (BoNT/A-mNG_{11}) with three beta-strands of mNG in tandem (**Figure 2C**).

We next imaged the neurons after different intoxication times (**Figure 2D**). After 12h, the first signal was observed in the cell body of neurons, in concordance with the pattern of BoNT activity. The fluorescence appeared in specks first, then converted to a more diffuse pattern. At 24h and 36h, the GFP pattern became more diffuse and started to spread in the neurites. The initial pattern might reflect reconstitution of GFP proteins at the site of extrusion from membranes. By 48h, the GFP signal had filled up the neurites, indicating that BoNT/A had diffused throughout the cell (**Figure 2D**). Quantification of the GFP signal confirmed an initial accumulation in the soma followed by an increase in neurites (**Figure 2D**). The late (>24h) and gradual accumulation in neurites was further confirmed by quantification of intensity correlation between BoNT/A LC-mNG and α-Tuj1, a neuronal marker enriched in neurites (**Figure 2D**).

Altogether, the data indicates that BoNT/A translocates into the cytosol at the level of the soma. Based on the dependency of the toxin on neuronal activity and current knowledge of BoNT/A receptors, it is likely that BoNT/A is internalised at the tip of neurites, where synapses form. Thus, the data would suggest that BoNT/A requires retrograde trafficking before it can translocate.

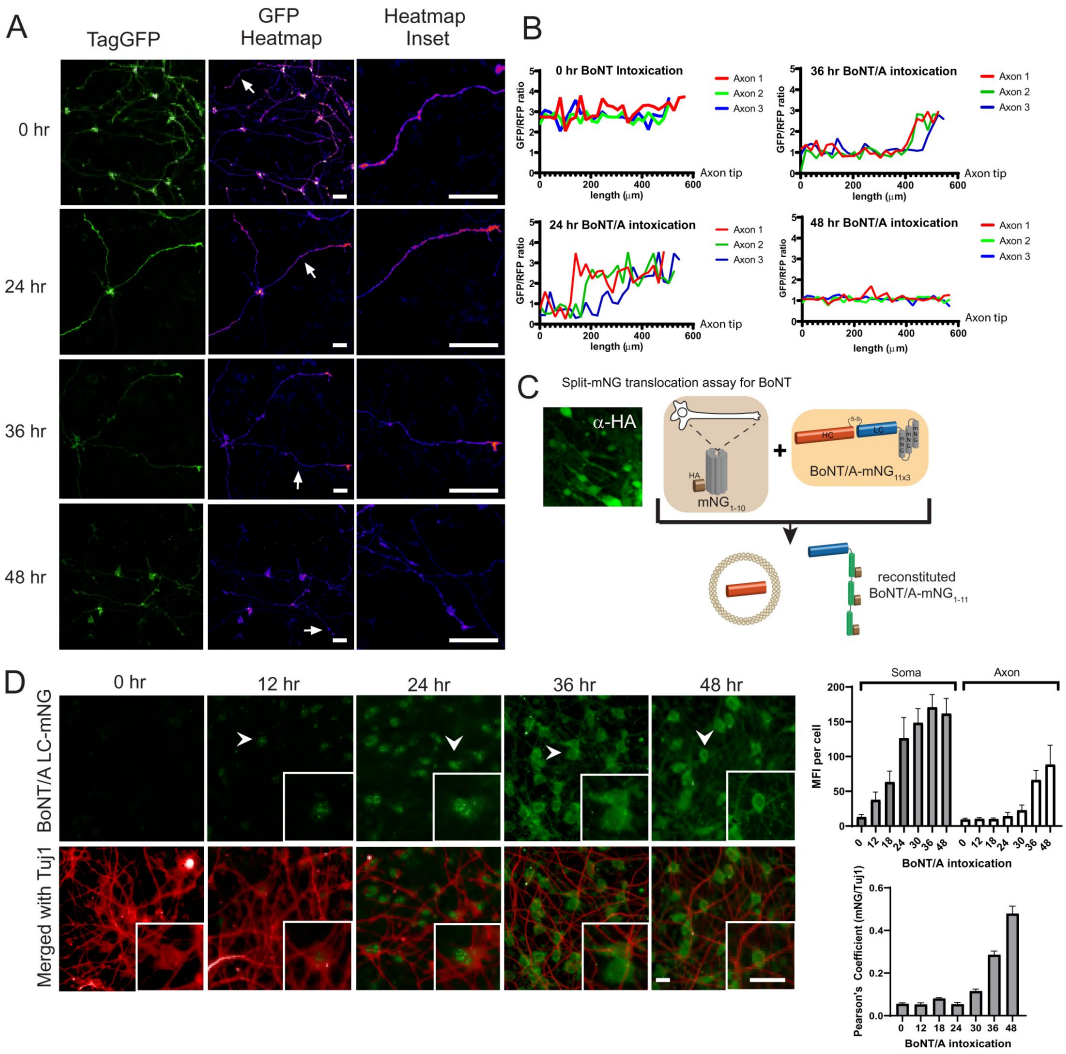


Figure 2

BoNT is first detected in the neuronal soma then emanate to the axons.

(A) Red SNAPR cells co-cultivated at ¼ with ¾ of unlabeled Ren-VM were imaged for 48h after addition of BoNT/A. The GFP color-coded intensity signal is displayed in the second column. **(B)** Quantification of GFP/RFP signal along the length of individual neurites (Axon 1,2,3) at various time points. **(C)** Schematic diagram of split-mNG (NeonGreen Fluorescent Protein) detection system, consisting of ReNcell VM expressing cytosolic mNG_{1-10} and mNG_{11}-tagged BoNT/A. Fluorescence occurs after binding of mNG_{11}-tagged BoNT/A to mNG_{1-10}. **(D)** Time-course of fluorescence reconstitution after exposure to mNG_{11}-tagged BoNT/A and quantification of Mean Fluorescence Intensity in the soma and neurites of cells. Scale bars 20 μm.

A genome-wide RNAi screen reveals numerous positive and negative regulators of BoNT intoxication

To elucidate the molecular mechanisms required for BoNT/A intoxication and understand the surprising pattern of appearance, we decided to systematically survey the genes required for BoNT/A translocation.

We first optimized the liposome-mediated delivery of siRNA in the Red-SNAPR cells (**Supplementary Figure 1C**). As a positive control, we targeted TXNRD1 as a factor known to be required. Cells differentiated for 2 weeks were transfected with TXNRD1 siRNA for 3 to 5 days and analysed by image analysis. Undifferentiated and differentiated ReNcell VM displayed ~70% knockdown compared to non-targeting control as measured by image analysis of TXNRD1 staining. We also targeted SNAPR using an siRNA targeting SNAP25. This approach achieved an 80% reduction in signal (**Supplementary Figure 1D**).

As neurons cannot be passaged after differentiation, a forward siRNA transfection pipeline was developed (**Figure 3A**). The genome-wide screen was carried out in duplicate using pools of 4 siRNAs per gene and targeting 21,121 human genes in total. Differentiated Red-SNAPR cells on laminin-coated 384-well imaging plates were incubated with siRNA complexes for 3 days and intoxicated with BoNT/A for 2 days before imaging. The positive control siTXNRD1 rescued the tGFP signal reproducibly (**Figure 3B**).

We analyzed the two replicates of the whole genome-wide screen using the ScreenSifter software (Kumar et al., 2013) (**Supplementary Figure 2A**). The data was converted to average plate Z-score, revealing low plate-to-plate variations (**Figure 3C**). The controls such as the BoNT/A-treated, siNT3 (NT3+), non BoNT/A-treated siNT3 (NT3-) and BoNT/A-treated, siTXNRD1 (TXNRD+ in figure) were tightly grouped (**Figure 3D**). The assay had a robust Z-factor of 0.81. The two replicates were correlated with an R-value of 0.74. Genome-wide plots for individual replicates are shown in **Supplementary Figure 2B**. The tight clustering of the controls demonstrates high reproducibility between experiments. The data was ranked to establish cut-offs for hits selection (**Figure 3E**).

Most hits (363) were genes required for BoNT/A intoxication (red dots); interestingly a significant fraction of hits (76) resulted in enhanced intoxication (blue dots) (**Figure 3F**). Many of the positive regulators resulted in higher rescue than the siTXNRD1 control, suggesting that previously unidentified molecular processes are critical for BoNT/A intoxication.

To exclude potential indirect effects, we first used nuclei counts and identified 289 genes that significantly affect neuronal survival (**Supplementary Figure 2C**). 80 positive and 8 negative genes were sifted out of the hit list (**Supplementary Figure 2D**). Next, we carried out a duplicate deconvoluted siRNA screen on the hit list to exclude potential off-target siRNAs (Jackson and Linsley, 2010) (**Supplementary Figure 2E**). Using this approach, 35 genes could not be confirmed by two independent siRNAs and thus were sifted out. To ensure the hit list only contains genes significantly expressed in neurons, we carried out RNA sequencing analysis on differentiated Red SNAPR cells (**Supplementary Figure 2F**. A cut-off threshold of counts-per-million (CPM) at 0.25 ($\log_2$CPM = −2) was used (grey bars) and 31 more genes (18 positive, 13 negative) were excluded.

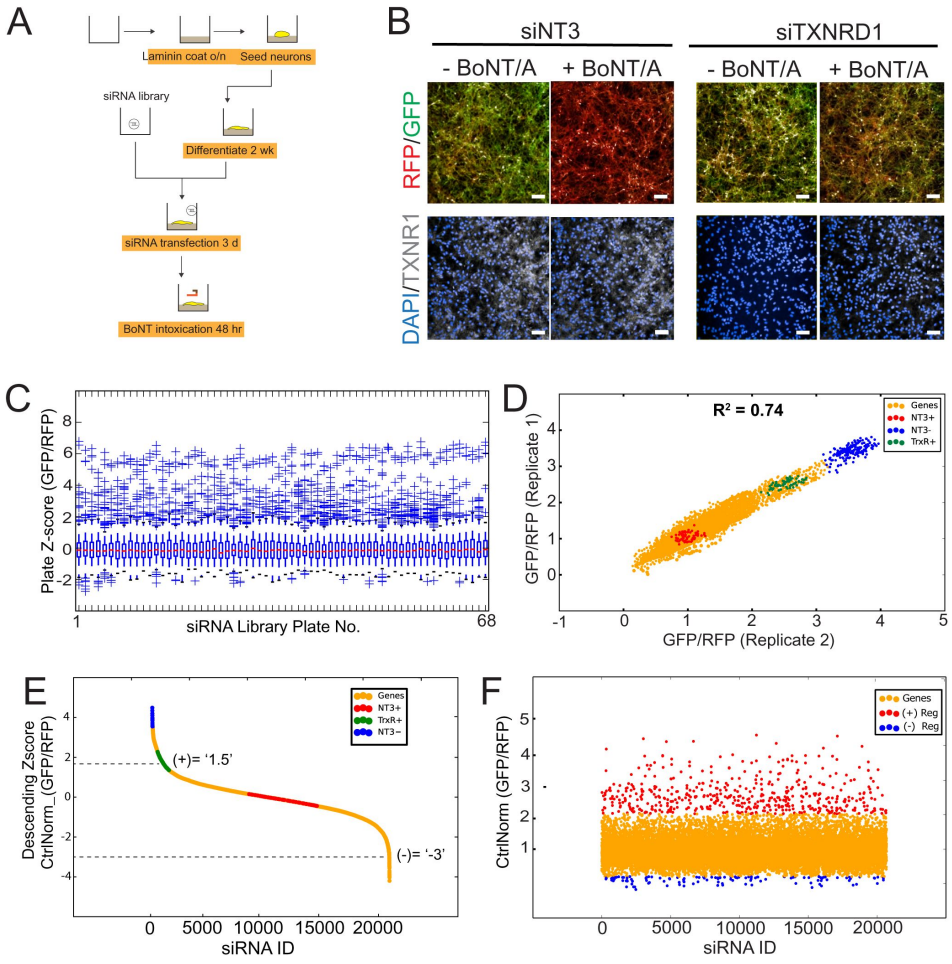
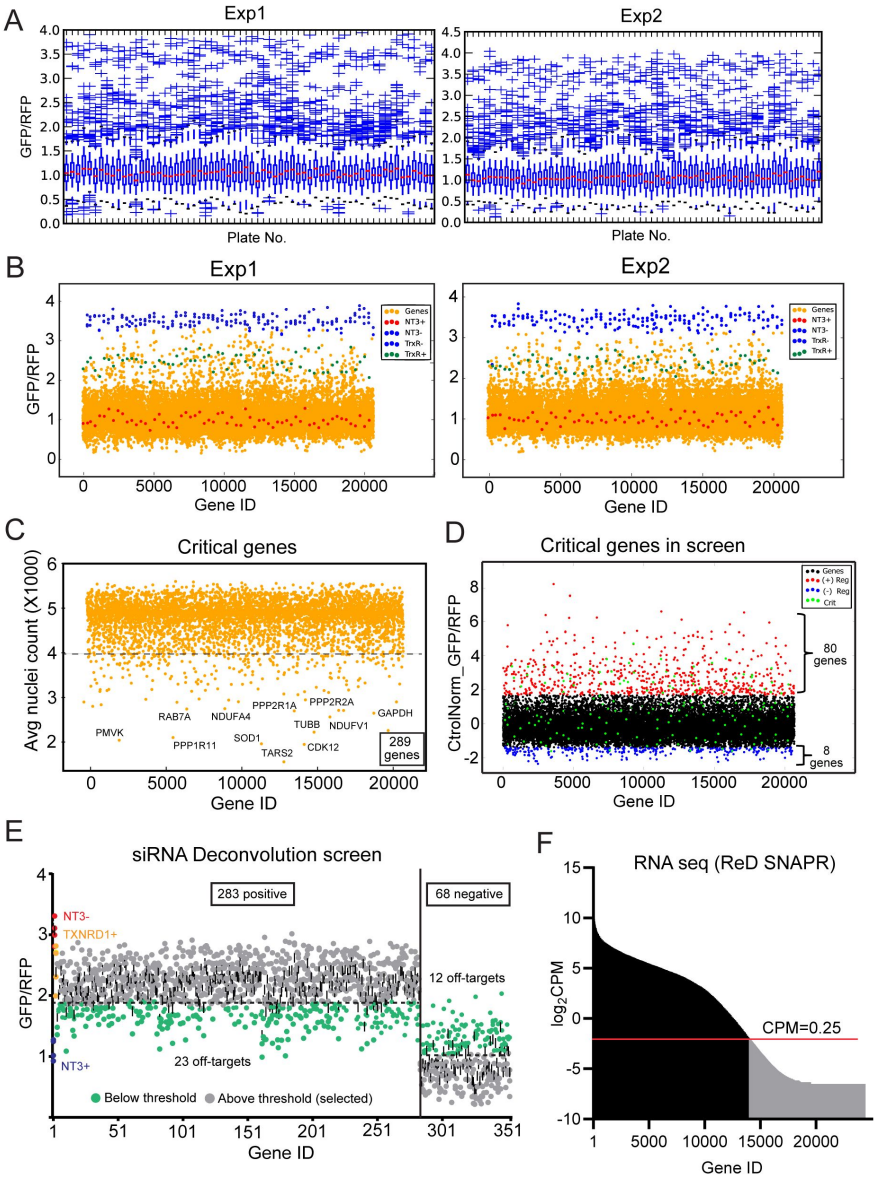


Figure 3.

Genome-wide RNAi screen of BoNT/A-treated Red SNAPR cell line.

(A) Schematic diagram of assay pipeline. **(B)** Cells treated with non-targeting control siRNA (siNT3) and positive control siRNA (siTXNRD1), then incubated with BoNT/A for 48 hours. Cells were stained with DAPI and TXNRD1 antibody post-fixation. Scale bars 50 µm. **(C)** ScreenSifter Z-score analysis of GFP/RFP ratio index of whole genome siRNA library plates. **(D)** R-squared analysis of both genome-wide RNAi screen replicates. Blue dots = No toxin control, Red dots = Toxin control, Green dots = Positive control (siTXNRD1). **(E)** Descending Z-score of control-normalized and averaged GFP/RFP ratio of genome-wide screen to determine cut-off values of +1.5 for positive regulators and −3 for negative regulators. **(F)** Chronological order of genome-wide control-normalized screen reflecting the positive regulators (red) and negative regulators (blue) derived from **(E)**.



Supplementary Figure 2.

Genome-wide RNAi screen data normalization and refinement.

(A) Genome-wide siRNA library plate replicates (Exp1 and Exp2) from **Figure 2C** **(B)** Individual replicates from **Figure 2D**. **(C)** Averaged nuclei count for each siRNA, 289 genes result in cell number below the cut-off of 4000 nuclei per well. Gene targets of siRNA most affecting cell survival are annotated in the graph. **(D)** The 289 genes from C are over-imposed on the SNAPR reporter results, 80 genes fall within the positive regulators and 8 genes with the negative regulators. All 88 genes were excluded from further analysis **(E)** Targeted siRNA deconvolution screen with positive and negative hits and siRNA control either non-toxin treated (NT3-) or toxin-treated (NT3+). Positive controls (TXNRD1+) replicates. The GFP/RFP threshold was set at 1.9 for positive regulators and 1.0 for negative regulators. Green data points are individual siRNAs that do not pass the threshold and do not confirm the pool result. Averaged GFP/RFP ratio for genes below or above threshold lines for positive and negative regulators that were removed from the hit lists. **(F)** RNA sequencing data from ReD SNAPR cells represented as $\log_2$CPM (counts per million) with an exclusion threshold set at CPM = 0.25 ($\log_2$CPM = −2, genes below are deemed as unexpressed).

The surface expression of BoNT/A receptor, SV2, is highly regulated

We next focused on genes influencing BoNT/A cell surface binding by studying the surface expression of SV2, the BoNT/A receptor. We incubated fixed and non-permeabilized cells with an antibody against the extracellular domain of SV2A to quantify cell surface exposure (**Figure 4A**). Depletion of VAMP2, a known regulator of synaptic vesicle fusion and SV2 trafficking (Pennuto et al., 2003), resulted in 60% reduction of surface SV2 levels relative to non-targeting control (siNT3) (**Figure 4B**). By contrast, siTXNRD1 did not affect SV2 surface signal (**Figure 4B**).

We screened a library of hits identified in **Figure 3F** and identified 105 genes that affect SV2, with 38 gene depletions reducing (Repressors, SV2(-) in table) and 67 gene depletions enhancing (Enhancers, SV2(+) in table) surface SV2 (**Figure 4C**, **Supplementary Table 1**).

For instance, depletion of clathrin light chain (siCTLC), a known regulator of endocytosis, increased surface staining of SV2 (Yao et al., 2010). By contrast, depletion of Rab11b decreased surface SV2 which could be due to a block in recycling of SV2 from endosomes to the cell surface (Giorgini and Steinert, 2013). Using STRINGS analysis on 'surface SV2 repressors', we identified a closely-associated network of genes related to clathrin-mediated endocytosis such as AP2M1, CTLC and TDRD1. In addition, distinct subunits of the V-ATPase were identified which can function as adaptins to facilitate endocytosis (Geyer et al., 2002) (**Figure 4D**). The majority of the surface SV2 enhancers are associated with G-protein signaling, which controls neuronal excitation (**Figure 4E**). This agrees with earlier findings (**Figure 1D**) where increased neuronal activity favors intoxication as observed when K^+/Ca^+ are spiked in the medium. Endocytic signaling and membrane trafficking gene families such as Rabs, Arfs and SNAREs were also revealed, congruent with a role in SV2 exocytosis (**Figure 4E**).

Network analysis reveals regulators of signaling, membrane trafficking and thioreductase redox state involved in BoNT/A intoxication

Among the positive regulators of the screen, 135 hits did not influence significantly surface SV2 levels and are thus likely to function in post-endocytic processes (**Supplementary Table 2**). However, we cannot formerly exclude that they could affect binding of BonT to the cell surface independently of SV2. 92 positive regulators (required for intoxication, in red) and 43 negative regulators (reducing intoxication, blue) were mapped to their intracellular localities (**Figure 5A**). Several gene products were localized to endosomes and 16 were associated with the Golgi and ER. At the Golgi, one hit was the glycosylation enzyme B4GALT4, known to be involved in the biosynthesis of the ganglioside co-receptor of BoNT/A, G_{T1b}. Using the STRING database, a protein-protein interaction network of hits was generated (**Figure 5B**) (Szklarczyk et al., 2021). A subnetwork was constituted of heat-shock protein (HSP) chaperones of the HSP70 family (HSPA4 and HSPA14). Another chaperone, HSP90, has been linked to the translocation of the clostridial toxins C2 toxin and BoNT/A into the cytosol (Azarnia Tehran et al., 2017; Haug et al., 2003). As HSP90 and HSP70 act in a sequential cascade for protein folding (Genest et al., 2019), our results suggest that such a chaperone cascade might help BoNT/A LC refold in the cytosol after translocation.

A large subnetwork of signaling and GPCR-related genes was identified, centered on the SRC tyrosine kinase (**Figure 5B**). Depletion of SRC resulted in one of the most stringent intoxication blocks (**Figure 5C**). Interestingly, the tyrosine phosphatase PTPN6 was also one of the strongest negative regulators (Chong and Maiese, 2007) (**Figure 5C**). It has been proposed that SRC directly phosphorylates and regulates BoNT LC catalytic domain (Ferrer-Montiel et al., 1996; Ibañez et al., 2004; Kiris et al., 2015). On the other hand, SRC and its partners are crucial for

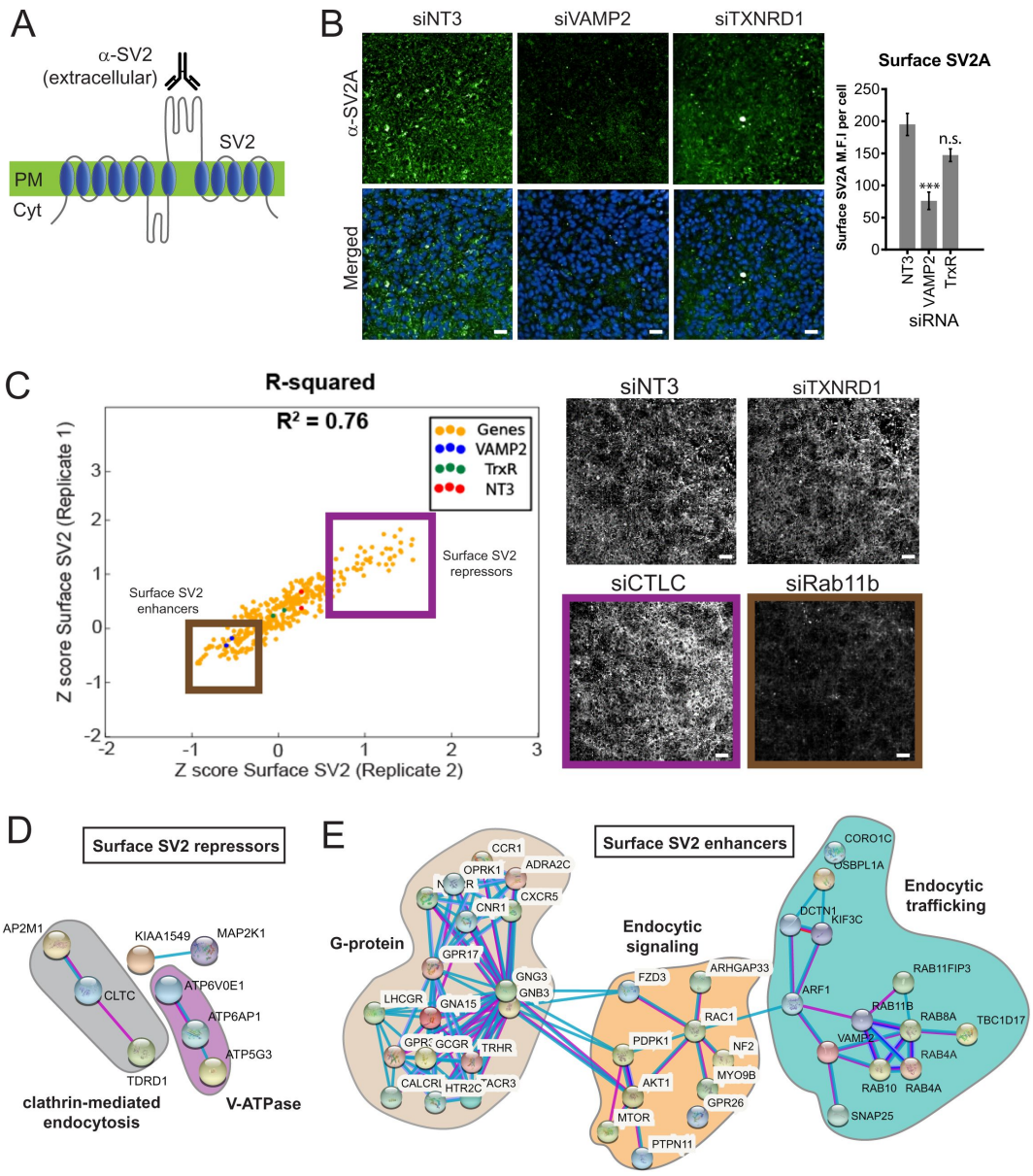


Figure 4.

Surface expression of the BoNT/A receptor SV2 is regulated by endocytic trafficking and signaling genes, forming a cohort of the positive regulators of the genome-wide screen.

(A) Detecting surface SV2 using a specific antibody against the extracellular loop of SV2. **(B)** siVAMP2 as a positive control for surface SV2 expression. ReNcell VM treated with siNT3, siVAMP2 and siTXNRD1 for 3 days and stained with SV2A antibody after fixation without membrane permeabilization. Quantification of surface SV2 per cell where whole image SV2A MFI was divided by total nuclei count (DAPI) from 3 fields of view and 3 independent replicates. Scale bars 50 μm. **(C)** Replicate screen results for surface SV2 regulators from genome-wide positive hits. Purple box = surface SV2 repressors e.g. siCTLC (clathrin light chain); Brown box = surface SV2 enhancers e.g. siRab11b. siNT3 and siTXNRD1 does not regulate surface levels of SV2. Scale bars 50 μm. **(D)** STRINGS analysis of surface SV2 repressors. **(E)** STRINGS analysis of surface SV2 enhancers.

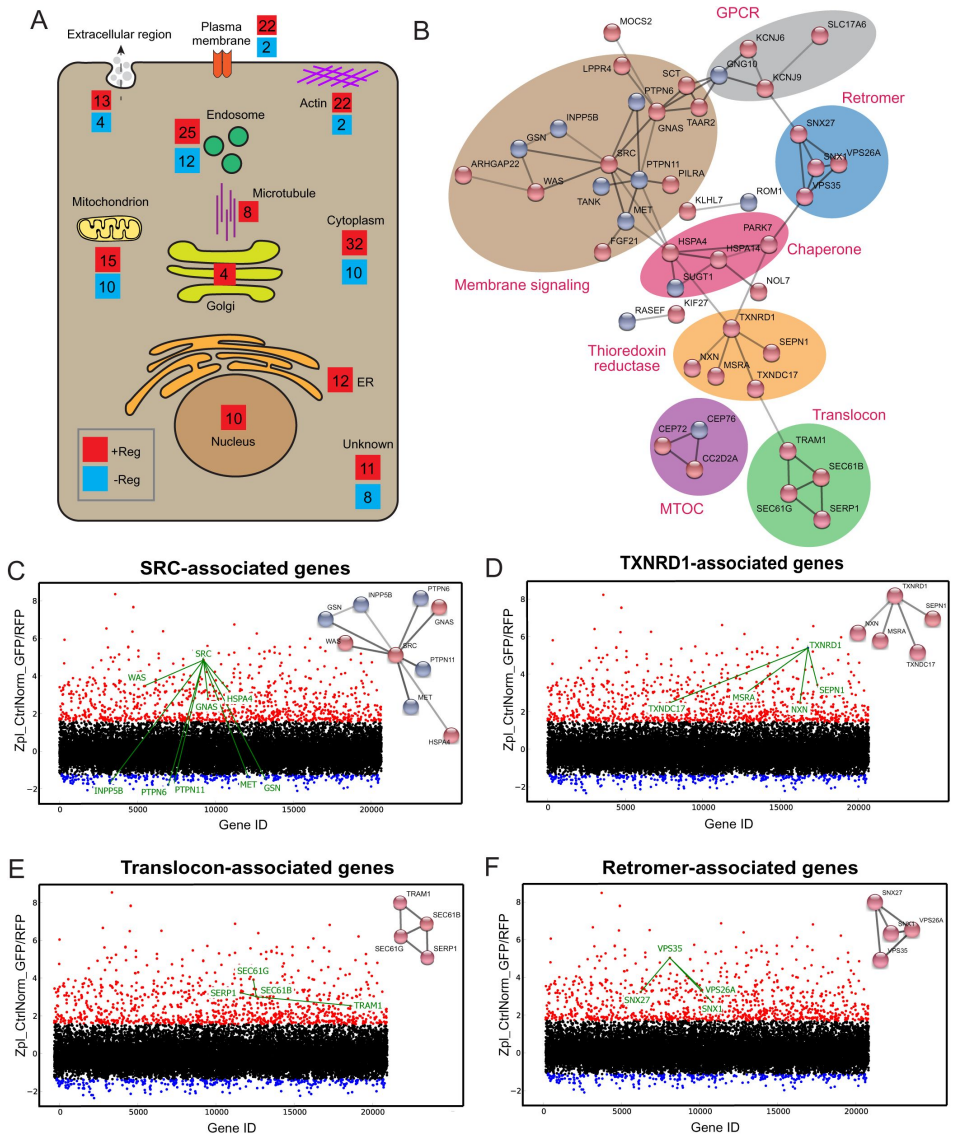


Figure 5.

Protein network analysis of genome-wide hits reveals known and novel regulators of BoNT/A intoxication.

(A) Summary diagram of all positive and negative hits mapped to their intracellular localities. **(B)** STRINGS network analysis of connected hits (non-connected hits are excluded). Associated genes tied to respective cellular molecular complex/processes are bounded and annotated. ScreenSifter analysis revealed **(C)** Src-associated genes **(D)** TXNRD1-associated genes **(E)** Translocon-associated genes **(F)** Retromer-associated genes.

retrograde membrane trafficking and might influence post-endocytic BoNT/A trafficking (Chia et al., 2021; Sandilands and Frame, 2008). The screen identified TXNRD1, consistent with previous literature, but also several genes involved in thioredoxin reduction, including Methionine sulfoxide reductase A (MsrA), Thioredoxin domain-containing protein 17 (TXNDC17), Nucleoredoxin (NXN) and Selenoprotein N (SEPN1) (**Figure 5D**). It suggests that these proteins either interact directly with BoNT/A or are required for TXNRD1 function.

A set of genes linked to the translocon (SEC61G, SEC61B, TRAM1, SERP1) were identified (**Figure 5E**). GO:MF analysis (Molecular Function) indicates high enrichment level of these ER translocon genes (**Supplementary Figure 3A**). The requirement of the translocon suggests its involvement in translocation of the toxin to the cytosol as is the case for other toxins (Moreau et al., 2011; Nowakowska-Gołacka et al., 2019). However, this hypothesis implies that the toxin travels from endocytic vesicles at its site of internalisation to ER membranes where the translocon is localized.

Interestingly, genes for the retromer were also identified, VPS35, VPS26A, SNX1 and SNX27, with a high level of enrichment in gene ontology analysis (**Figure 5F**, **Supplementary Figure 3B**). These genes did not significantly affect surface SV2 levels. The retromer has been linked to endosomes to Golgi traffic, suggesting the toxin might need to traffic between these organelles.

Retro-axonal traffic of the BoNT/A receptor SV2 requires the retromer

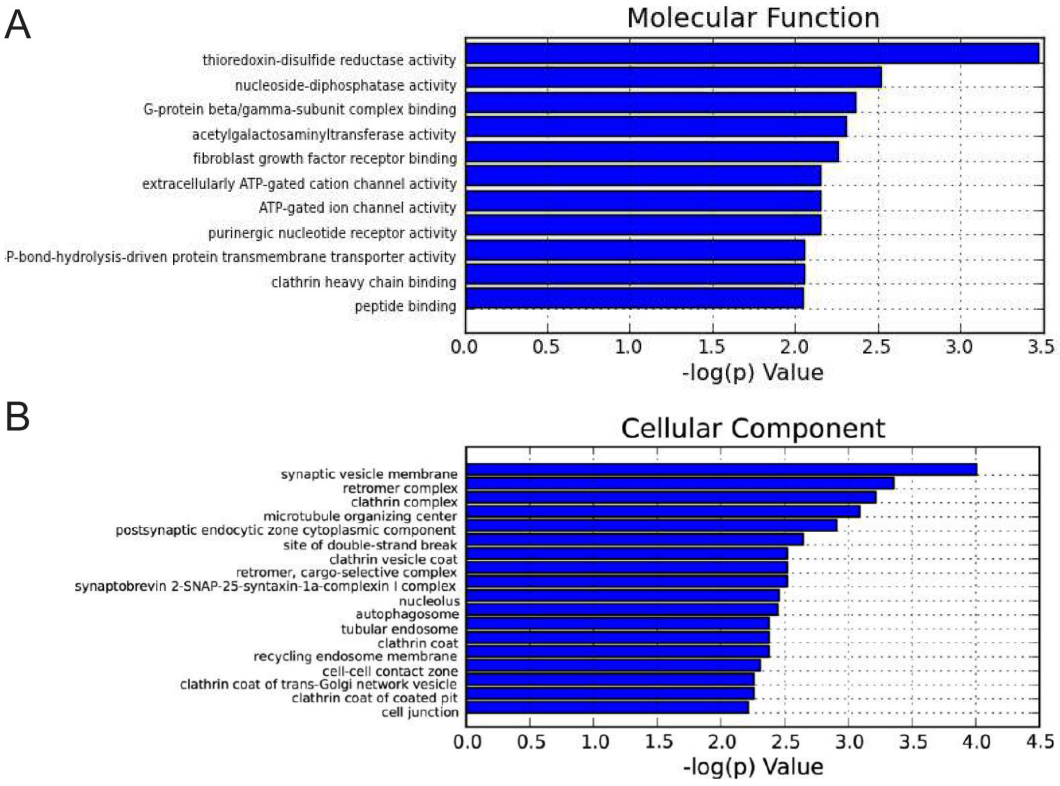
We next analysed the effect of VPS35 depletion on the kinetics of BoNT/A-mNG$_{11}$ arrival in the cytosol. The control siNT3 treated cells showed a progressive increase in signal from 12 to 48h, with a progressive appearance of signal in the neurites (**Supplementary Figure 4A**). By contrast, siVPS35 treated cells only displayed some soma-localised signal after 48h (**Supplementary Figure 4A**).

To test directly the trafficking of BoNT/A intracellularly proved difficult as reagents we tested were not sensitive enough to image the trafficking of the toxin. Since BoNT/A binds to SV2 at the cell surface, we wondered whether the receptor itself was trafficked retrogradely in neurites. We generated a ReNcell VM cell line expressing a chimera of SV2 with the Dendra fluorescent protein. We found that Dendra-SV2 is distributed in the whole neuron, similarly to the endogenous protein (**Supplementary Figure 4B**).

Next, we depleted cells of VPS35 and found that Dendra-SV2 accumulated in bulbous structures at the neurite tips. To quantify this phenomenon, we compared the number and size of SV2 puncta in neurites in control and siVPS35-treated cells. Using ImageJ to threshold and select for particles of interest, we measured the number of particles along every 50µm segment of the neurite, starting from the neurite tips **Supplementary Figure 4C**). In control cells, SV2 punctas were homogeneously spread throughout the neurites. However in VPS35-depleted cells, SV2 punctas were enriched at the neurite tip (1-50µm) and were significantly reduced with the rest of the neurite. These puncta were also much larger than in control cells. This evidence indicates that SV2 is trafficked retro-axonally in a retromer-dependent fashion, thus consistent with the notion of BoNT/A retrogradally to the neuronal body bound to its receptor.

BoNT/A trafficks through the Golgi apparatus

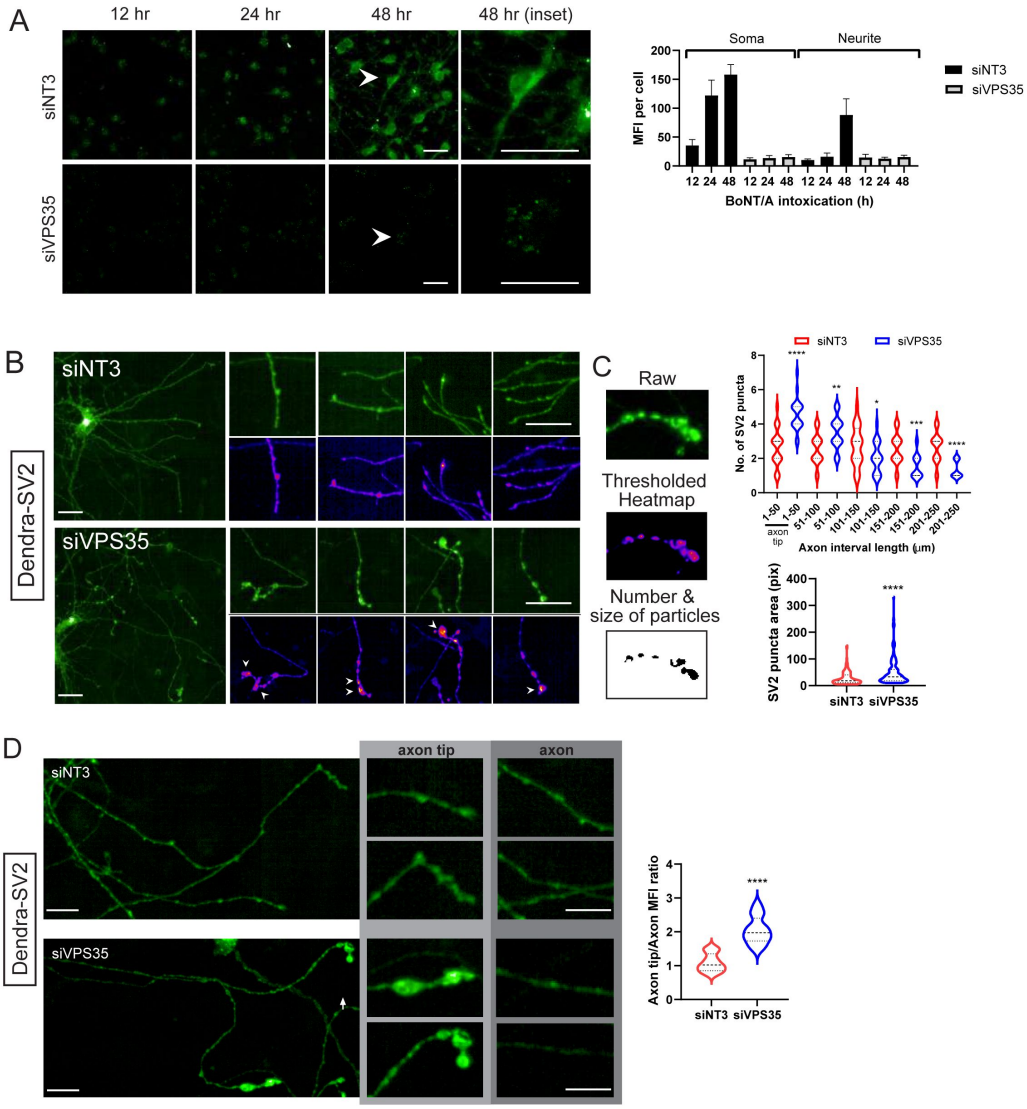
The implication of the retromer suggested that BoNT/A transits through the Golgi apparatus. To test this hypothesis, we sought a method to detect BoNT/A in different intracellular compartments. We decided to exploit the split-monomeric Neon Green (mNG) fluorescence reconstitution approach (Luong et al., 2020). This approach relies on having the 10 beta strands of Neon Green fluorescent protein (mNG$_{1-10}$) and the 11th beta strand in different proteins. When the two proteins can interact, fluorescence is reconstituted. We generated a ReNcell VM cell line stably



Supplementary Figure 3.

Gene ontological analysis of positive hits from genome-wide intoxication screen.

(A) Cellular component **(B)** Molecular function **(C)** Biological Processes



Supplementary Figure 4.

Retromer is required for retro-axonal trafficking of the BoNT/A receptor.

(A) Cytosolic mNG_{1-10} expressing RenVM cells were incubated with 50 nM of BoNT/A-mNG_{11} and fixed at 0, 24, 36 and 48 hour timepoints. The MFI was quantified in 20 cells for each condition and time point, in the soma and neurites. Scale bar 20 µm. **(B)** ReNcell VM stably expressing Dendra-SV2 treated with siVPS35 for 3 days and imaged after fixation. Insets show axons of interest from each condition and arrowheads indicate enlarged SV2 puncta. Scale bar 20 µm and inset shown at 10 µm. **(C)** Method for quantifying number and size of SV2 puncta. tGFP image is background-subtracted, thresholded then analyzed for number and size of particles. Quantification of the number of SV2 puncta along every 50 µm segment of the axon starting from the axon tip from **B.** Quantification of area of SV2 puncta in whole axons from **B. (D)** Comparison of mean fluorescence intensities (MFI) at axons and axon tips from control and siVPS35-treated cells. Quantification of axon tip/axon MFIs. Graphs are obtained from 30 axons across 3 independent experiments. Scale bar 10 µm.

expressing mNG_{1-10} fused with an HA tag and a fragment of β1,4-galactosyltransferase 1 for targeting to the Golgi (Golgi-mNG_{1-10}) (Luong et al., 2020) (**Figure 6A**). We verified by immunofluorescence that the construct was strictly localized at the Golgi by the HA antibody staining and that no GFP fluorescence was detectable (**Figure 6B**).

Upon incubation for 48 h with BoNT/A-mNG_{11}, we observed reconstitution of mNG fluorescence at the Golgi (**Figure 6B**). In addition, there was mNG fluorescence in the cell soma with a reticulated pattern, which could correspond to the ER. Interestingly, the HA pattern was also partially looking like ER in these cells. Thus the reconstituted mNG was not only restricted to the Golgi, which could be due to retrograde trafficking after complementation. Nonetheless, the Golgi pattern clearly indicates that BoNT/A traverses this organelle before translocation. In ReNcell VM cells, the Golgi apparatus is located almost exclusively in the soma of neurons.

BoNT/A trafficks to the ER and translocate through Sec61 complex

To test whether BoNT/A can reach the ER, we next developed a chimera of mNG_{1-10} localized to the ER, based on the soluble, ER-resident BiP protein (ER-mNG_{1-10}) (Luong et al., 2020). As for the Golgi construct, there was no fluorescence in the absence of BonT/A and the construct had the typical pattern of an ER protein (**Figure 6C**). 48h after intoxication with BoNT/A-mNG_{11}, we could detect mNG in the ER (**Figure 6C**). To further confirm that BoNT/A can reach a Sec61-enriched compartment, we also developed ReNcell VM cell lines stably expressing a split-mNG_{1-10} construct fused with the transmembrane protein Sec61G (**Figure 6A**). As for the BiP based construct, the Sec61 staining pattern before addition of the toxin was consistent with the ER. After intoxication, the reconstituted mNG fluorescence had a typical ER pattern (**Figure 6D**). Quantification of fluorescence per cell confirmed that the three reporters were equally accessible to BoNT/A, indicating that the toxin traffics through the Golgi and ER in neurons (**Figure 6E**).

Next, to test whether Sec61 is involved in BoNT/A LC translocation, Golgi-mNG_{1-10}, ER-mNG_{1-10} and Cytosol-mNG_{1-10}-expressing cells were depleted of Sec61G with siRNA (**Figure 6F**). When incubated with BoNT/A-mNG_{11}, the reconstituted mNG fluorescence appeared in Golgi-mNG_{1-10} and ER-mNG_{1-10} cells, while there was a striking reduction of fluorescence in cytosolic mNG_{1-10} expressing cells (**Figure 6G** and **6H**). These results indicate that loss of Sec61G does not prevent BoNT/A uptake nor its trafficking to Golgi and ER compartments, but is instrumental to the translocation of BoNT/A LC into the neuronal cytosol at the soma.

Overall, these reporters reveal the complexity of BoNT/A trafficking in neurons: after internalisation at active synapses in neurites, the toxin is transported retrogradely to the Golgi, then the ER before being translocated to the cytosol.

Discussion

To improve the understanding of BoNT/A intoxication, we designed the engineered BoNT reporter cell line, Red SNAPR. The line is easily maintained, highly sensitive to the toxin and the assay is a direct readout of GFP fluorescence. The assay encompasses all steps of BoNT/A intoxication from binding and endocytosis to light chain translocation and substrate cleavage. While FRET-based reporter assays of BoNT have been established, they tend to be limited by photobleaching and spectral bleedthrough from donor/acceptor fluorescence. Moreover, FRET efficiency is drastically reduced in fixed cells (Anikovsky et al., 2008; Dong et al., 2004). By contrast, SNAPR cleavage is quantified through the ratio of RFP to GFP signal, thanks to the instability of the GFP moiety after cleavage, probably due to the N-terminal arginine residue generated by BoNT/A cleavage at $Q^{197}R$ (Tasaki et al., 2012). As noted by one reviewer, the assay may be sensitive to perturbation in the general rate of protein degradation, a consideration to keep in mind when evaluating the results of

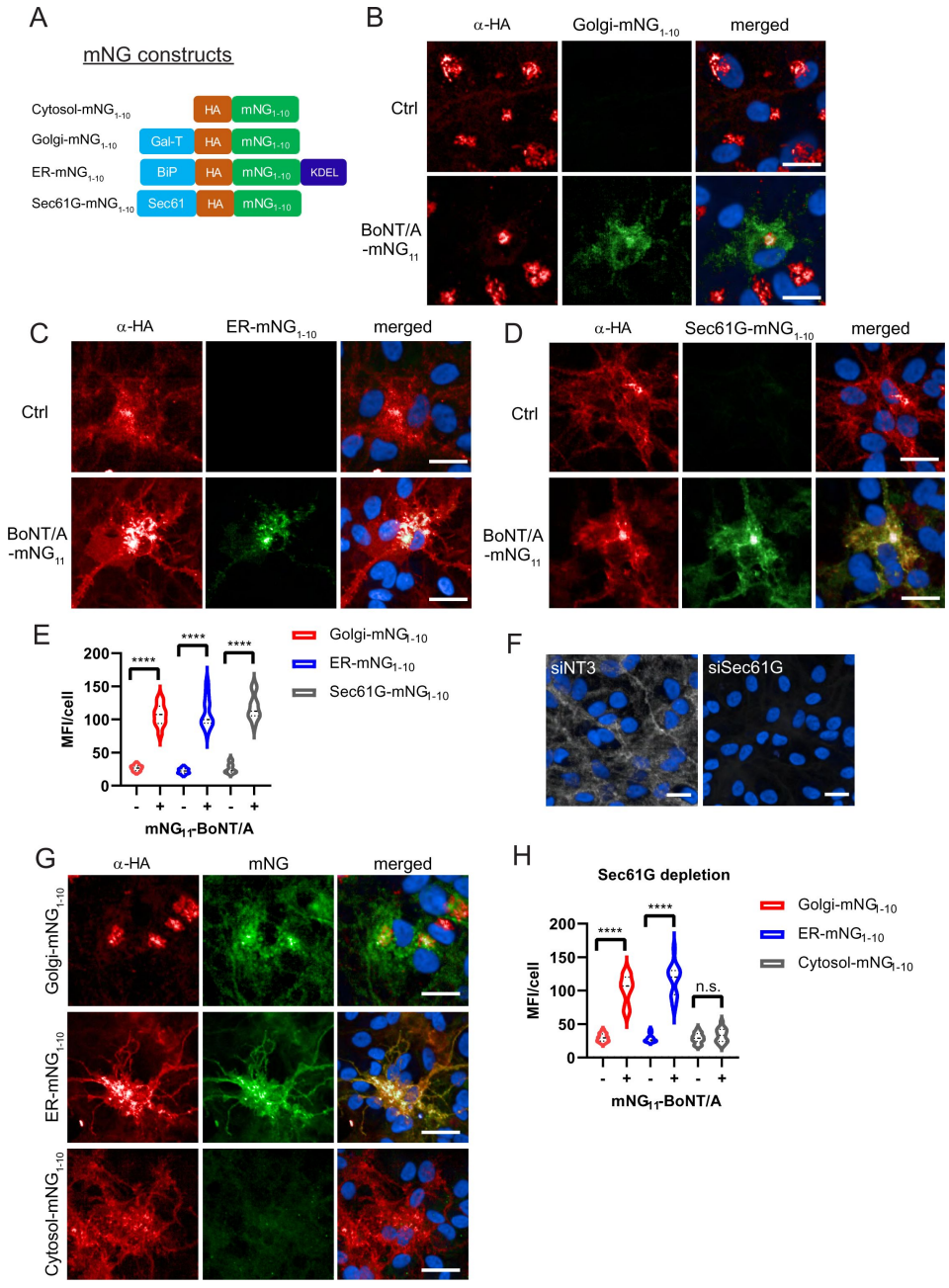


Figure 6.

BoNT/A is retrogradely trafficked through Golgi and ER membranes as revealed by split-GFP reconstitution. (A)

Split-mNG constructs targeted to cytosol, Golgi, ER lumen (KDEL sequence) and ER membranes (Sec61 transmembrane domain) were stably expressed in ReNcell VM and incubated with 50 nM of BoNT/A-mNG$_{11}$. Control cells (without BoNT/A-mNG$_{11}$) are shown at top panels of each condition **(B)** Cells expressing Golgi-mNG$_{1-10}$ and showing reconstituted mNG fluorescence. Overlap of HA antibody and mNG signals in the Golgi **(C)** ER-mNG$_{1-10}$ expressing cells showing mNG fluorescence and distinct overlap of HA antibody and mNG in the ER. **(D)** Sec61G-mNG$_{1-10}$ expressing cells showing mNG fluorescence distinct overlap of HA antibody and Sec61G signals. **(E)** Quantification of MFI/cell from **B-D** showing increased mNG fluorescence in Golgi, ER and Sec61G-expressing cells after addition of BoNT/A-mNG11. **(F)** Representative image of SEC61G depletion in ReNcell VM. **(G)** Golgi-mNG$_{1-10}$, ER-mNG$_{1-10}$ and Cytosol-mNG$_{1-10}$-expressing cells are depleted with siSEC61G and incubated with BoNT/A-mNG$_{11}$ for 48 hours. **(H)** Quantification of MFI/cell from **G**. Graphs are obtained from at least 300 cells across 3 independent experiments. Scale bars 20 μm.

large scale screens. On the other hand, this convenient and simple assay could replace animal-based assays that are used to measure BoNT potency. In addition, SNAPR could be implemented in transgenic mice to study the process of BoNT/A inhibition in vivo.

The sensitivity of the ReNcell VM cells to BoNT/A is highly enhanced by treatments that favor the formation of active synapses, consistent with the fact that BoNT/A binds the synaptic protein SV2, which only becomes exposed at the surface when neurons fire synaptic vesicles. Consistently, a hundred genes regulating BoNT/A intoxication (a quarter of the hits) actually impact cell surface exposure of SV2. The genes identified are involved in endocytic coupling, G protein-coupled receptors and other signaling genes. Thus, it seems highly likely that in our experimental model, BoNT/A is internalised at the tip of neurites (with axonal properties).

After binding to SV2 and internalisation, BoNT/A LC does not appear to translocate in the synaptic bouton as the reporter is unperturbed. The spatiotemporal pattern of BoNT/A activity and the cytosolic detection of the protein both indicate that the toxin appears in the soma of neurons about 12h after the start of incubation. Presumably in our cell culture system, these 12 hours are required for BoNT/A trafficking from synapses to the soma's cytosol. Progression of cleavage of the SNAPR reporter from the soma to the neurite's terminal requires an additional 36 h. The split-GFP organellar reporters indicate that BoNT/A traffics from endosomes to Golgi and then to the ER.

The genetic signature of BoNT/A intoxication requirements is consistent with this trafficking route. For instance, a significant positive regulator is the tyrosine kinase SRC. We and others have reported on the role of SRC at the Golgi/ER interface (Pulvirenti et al., 2008; Weller et al., 2010). We previously reported SRC's role in regulating Pseudomonas Exotoxin A (PE) trafficking between Golgi and ER (Bard et al., 2003). More recently, we have shown the role of Src in directly controlling GBF1, a GTP Exchange Factorinvolved in Golgi to ER traffic (Chia et al., 2021). Interestingly, the counteracting tyrosine phosphatases PTPN6 and PTPN11 are negative regulators of BoNT/A and their depletion favors intoxication (Frank et al., 2004; Somani et al., 1997). To note, SRC has also been proposed to act directly on the toxin to activate it (Ferrer-Montiel et al., 1996; Ibañez et al., 2004; Kiris et al., 2015).

These data suggests that BonT/A effects could be counteracted clinically by Src targeting drugs. This is important as serious or even lethal botulinic intoxications, while rare, still occur in developed countries like France ("Cas de botulisme alimentaire à Bordeaux : 15 cas recensés, dont 10 hospitalisés et 1 décès. Point de situation au 14 septembre 2023," n.d.).

Another four genes are linked to the retromer complex, which controls endosomes to Golgi traffic. For BoNT/A, traffic from endocytic vesicle to Golgi requires retro-axonal transport, which is supported by the requirement for dynein. Previous studies had shown retro-axonal transport for BoNT/A (Harper et al., 2016; Restani et al., 2012). By extension, these results suggest that SV2 is also transported retro-axonally (Lund et al., 2021; Tanner et al., 1996). Consistently, we found that SV2 accumulates at neurite tips when the retromer complex is silenced. SV2 is implicated in epilepsy and various neurodegenerative diseases such as Alzheimer's and Parkinson's (Ciruelas et al., 2019; Stout et al., 2019).

Another complex required is the translocon. When Sec61G is depleted, mNG-complementing BoNT/A still accumulates in the ER but is unable to reach the cytosol, indicating that the Sec61 complex is involved in retrotranslocation. While the best-described function is the co-translational insertion of proteins in the ER, Sec61 has also been implicated in retrotranslocation linked to ER-associated degradation (ERAD) (Römisch, 2017). Sec61 thus likely functions as a bidirectional channel for proteins (Römisch, 2017).

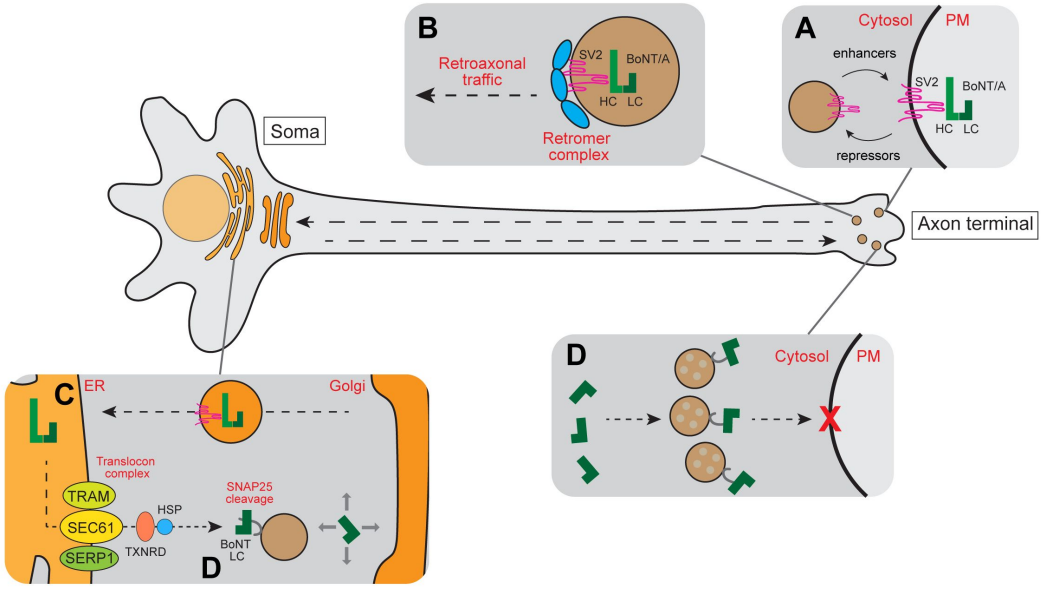
Overall, we propose that the BoNT/A-SV2 complex is internalised by ReNcell VM cells at the neurite tip where SV2 is exposed. While we did not demonstrate this point formally in RenCell VM cells, it is well established in other neurons and it is consistent with the increased toxin sensitivity after neuronal stimulation **(Supplementary Figure 5A)**. After internalisation, instead of being translocated from endocytic vesicles, the toxin is retrogradely trafficked to the soma-located Golgi in a retromer-dependent fashion **(Supplementary Figure 5B)**. After trafficking through the Golgi, the toxin is transported to the ER **(Supplementary Figure 5C)**. There, BoNT/A LC translocates via the Sec61 translocon into the cytosol. It is likely that LC detachment from ER membranes requires the separation of heavy and light chains through the activity of thioredoxin reductases and chaperones **(Supplementary Figure 5C)**. Thus, in neurons in culture, BoNT/A LC likely cleaves SNAP25 first in the soma before reaching the neurite terminals where it blocks fusion of synaptic vesicles at the plasma membrane **(Supplementary Figure 5D)**. The location of the Golgi apparatus virtually exclusively in the soma of differentiated Ren-VM cells explains why the toxin has to traffic to the soma. This trafficking in turn explains the 48h between addition of the toxin and full cleavage of SNAPR.

Our study contradicts the long-established model of BoNT intoxication, which is described in several reviews specifically dedicated to the subject (Dong et al., 2019; Pirazzini et al., 2017, 2016; Rossetto et al., 2021). In short, these reviews support the notion that BoNT are molecular machines able to mediate their own translocation across membranes. This notion has convinced some cell biologists interested in toxins and retrograde membrane traffic, who follows this model of BoNT mode of translocation in their reviews (Mesquita et al., 2020; Williams and Tsai, 2016).

But is this notion well supported by data? A careful examination of the primary literature reveals that early studies indeed report that BonTs form ion channels at low pH values (Donovan and Middlebrook, 1986; Hoch et al., 1985). These studies have been extended by the use of patch-clamp (Fischer et al., 2009; Fischer and Montal, 2007a). These works and others lead to various suppositions on how the toxin forms a channel and translocate the LC (Fischer and Montal, 2007b; Pirazzini et al., 2016).

However, only a single study claims to reconstitute in vitro the translocation of BonT LC across membranes (Koriazova and Montal, 2003). In this paper, the authors report one key experiment using a system of artificial membranes separating two aqueous compartments. They load the toxin in the cis compartment and measure the protease activity in the trans compartment after incubation. However, when the experimental conditions described are actually converted in terms of molarity, it appears that the cis compartment was loaded at 10^{e-8}M BonT and that the reported translocated protease activity is equivalent to 10^{e-17} M (**Figure 3D**, (Koriazova and Montal, 2003)). Thus, in this experiment, about 1 LC molecule in 100 millions has crossed the membrane. Such extremely low transfert rate does not tally with the extreme efficiency of intoxication in vivo, even while taking into account the difference between artificial and biological membranes.

In sum, a careful analysis of the primary literature indicate that while there is ample evidence that BoNTs have the ability to affect membranes and possibly create ion channels, there is actually no credible evidence that these channels can mediate the translocation of the LC. As mentioned earlier, it is unclear how such a self-translocation mechanism would function thermodynamically. It is worth noting that a similar self-translocation model was proposed for other protein toxins such as Pseudomonas exotoxin, which have similar molecular organisation as BonT (Taupiac et al., 1999). However, it has since been demonstrated that the PE toxins require cellular machinery, in particular in the ER, for intoxication (Bassik et al., 2013; Moreau et al., 2011; Schäuble et al., 2014).



Supplementary Figure 5.

Schematic model for intracellular trafficking of BoNT/A.

(A) BoNT/A HC binds to its cognate receptor SV2. Surface expression of SV2 is regulated by a cohort of enhancers and repressors. **(B)** BoNT/A-containing endosomes are retro-axonally trafficked to the soma through the retrograde function of the retromer complex. **(C)** BoNT/A eventually traffics to the translocation-competent ER via the Golgi. **(D)** The ER SEC61 translocon complex facilitates LC translocation of BoNT/A from the ER lumen into the cytosol where the TXNRD1 and HSP complexes release and refold BoNT/A LC. The LC diffuses and cleaves SNAP25 in the soma. **(E)** Progressive diffusion of LC into the axons and axon terminals ultimately cleaves SNAP25 on neurotransmitter-containing vesicles, blocking neurotransmission at the synapse.

By contrast, our model proposes a mechanism without a thermodynamic problem, is consistent with current knowledge about other protein toxins, such as PE, Shiga and Ricin, and can help explain previously puzzling features of BonT effects.

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Methods

Chemical reagents

The reagents used in this study are summarized in **Supplementary Figure 6A**. BoNT/A was synthesized as previously described (Stancombe et al., 2012).

Constructs

Constructs used in this study are depicted in **Supplementary Figure 6B** were generated using gene synthesis and cloned into pDONR221 entry vector (GeneArt, ThermoFisher Scientific). The entry clones were subcloned into pLenti6.3-V5 destination vectors using gateway LR cloning (ThermoFisher Scientific). In the split-mNG fluorescence reconstitution system, the first 10 β-barrel helices of monomeric Neon Green (mNG) are fused with or without target proteins while the last beta-strand is fused with the protein of interest. Interaction of the two partners will reconstitute fluorescence (Luong et al., 2020). BoNT/A-mNG_{11x3} was synthesized by addition of 3 mNG_{11} tags flanked with GSGSG (Gly-Ser) linkers at the N-termini of BoNT/A. siGENOME Human siRNA libraries were purchased from Dharmacon (Horizon Discovery).

Cell lines

ReNcell®VM human neural progenitor cell line (Sigma Aldrich) were maintained in ReNcell ®NSC Maintenance medium supplemented with 20ng/mL of epidermal growth factor, EGF and basic fibroblast growth factor, bFGF at 37°C with 5% CO_2. Cells were differentiated in differentiation medium (maintenance medium without EGF and bFGF; and addition of 10ng/mL of both glial-derived nerve factor (GDNF) and brain-derived nerve factor (BDNF) for two weeks with a change of media every 3 days. Cells were seeded on culture flasks or plates precoated overnight at 4°C with 20ug/mL laminin in DPBS. The cells were subcultured approximately every five days (90% confluence) by detaching them with Accutase.

Stable cell line generation

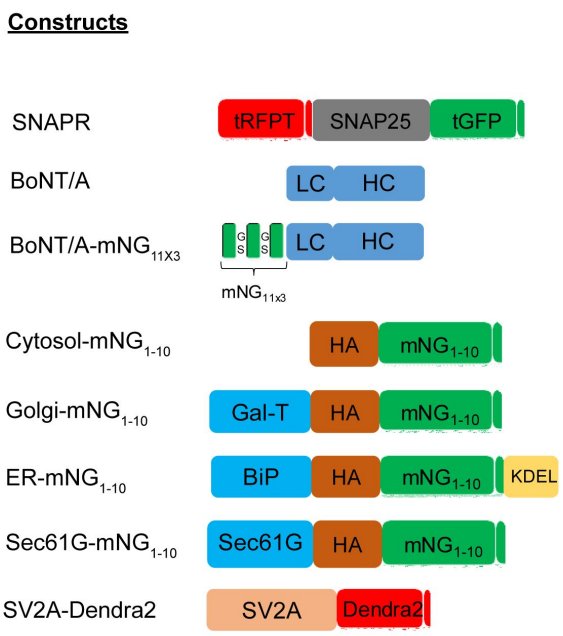
Stable cell lines were generated using ViraPower Lentiviral Packaging Mix together with the following lentiviral constructs containing SNAP25 were cotransfected into HEK293FT cells using Lipofectamine 3000 reagent. Following incubation of cells, supernatant containing lentivirus was harvested and cellular debris was removed by centrifugation. The virus was transduced in ReNcell® VM with polybrene reagent (8mg/ml) and removed after 24 hours. Fresh complete growth medium was added and transduced cells were sorted using fluorescence-activated cell sorting using tGFP signal and selected using 10ug/mL blasticidin in maintenance medium.

Supplementary Figure 6.

A

Reagent	Cat ID	Source
siGENOME pooled siRNA		Dharmacon, USA
Deconvoluted siRNA		Dharmacon, USA
Lipofectamine RNAimax	13778150	Invitrogen, USA
Lipofectamine 3000	L3000015	Invitrogen, USA
Human Epidermal Growth Factor	PHG0311	Gibco, USA
Human Fibroblast Growth Factor-basic	PHG0261	Gibco, USA
Human Nerve Growth Factor	A42578	Invitrogen, USA
Human Brain-Derived Neurotrophic Factor	PHC7074	Gibco, USA
ReNcell VM NSC Maintenance Medium	SCM005	Sigma-Aldrich, USA
ReNcell NSC Freezing Medium	SCM007	Sigma-Aldrich, USA
Laminin Mouse Protein	23017015	Gibco, USA
Accutase	A6964	Sigma-Aldrich, USA
Potassium Chloride	P9541	Sigma-Aldrich, USA
Calcium Chloride	C8106	Sigma-Aldrich, USA
PBS, DPBS, Sterile water		Gibco, USA
ViraPower Lentiviral Packaging Mix	A11146	Invitrogen, USA
Polybrene	TR-1003	Sigma-Aldrich, USA
Neuronal Marker IF Antibody Sampler Kit	8752	Cell Signaling, USA
SNAP25 antibody	5308	Cell Signaling, USA
tagRFPT antibody	AB233	Evrogen, Russia
tagGFP antibody	AB121	Evrogen, Russia
Thioredoxin Reductase 1 antibody	ab124954	Abcam
SV2A antibody	66724	Cell Signaling, USA
SEC61G antibody	HPA053196	Sigma-Aldrich, USA
Hoechst 33342	H3570	Invitrogen, USA

B



High-throughput siRNA and BoNT/A intoxication screen assay

The siRNA library was spotted in 384-well black µClear plates (PerkinElmer). siRNAs were transiently transfected at a final concentration of 25 nM per well using 0.25 µl Lipofectamine RNAiMAX reagent in 7.25 µl of OptiMEM medium, according to the manufacturer's protocol. siNT3 (Non-targeting 3) was chosen as a control siRNA due to its least toxic properties in ReNcell VM. After 20 min of complex formation, complexes were dispensed to the differentiated cells per well using a Multidrop Combi dispenser (Thermo-Fisher Scientific). Medium was removed at 72 h post-transfection using Integra VIAFLO 384 (Integra Biosciences AG, Switzerland), and cells were washed with phosphate-buffered saline (PBS) twice before incubated with 6nM BoNT/A in differentiation medium with 58 mM KCl and 2.2 mM CaCl2 for 48 h. siRNA screening was performed in duplicate.

Immunofluoresence

After 48 hours of BoNT/A intoxication, cells were fixed with 4% paraformaldehyde and 2% sucrose in PBS for 30 minutes and permeabilized with 0.2% Triton X-100 for a further 10 minutes. The cells were then stained with primary antibody diluted in 2%FBS in PBS for 2 hours. Cells were subsequently washed three times for 5 minutes with 2%FBS in PBS and stained for 1 hour with secondary antibody conjugated with fluorophore and Hoechst 33342 diluted in 2% FBS in PBS. The cells were then washed three times for 5 minutes with PBS before imaging.

Imaging and image analysis

High-throughput imaging was carried out using an automation-enabled Opera Phenix system with 20X objective (PerkinElmer). GFP, RFP and Hoechst channels were imaged and image analysis was done using Harmony and Columbus software (PerkinElmer). In brief, nuclei counts were generated using the Hoechst channel while cell masks were obtained using the RFP channel. The cell-masked GFP channel was measured and GFP/RFP ratio readout was obtained.

Data formatting and normalization

Genome-wide RNAi screen data was imported and analyzed in ScreenSifter software as previously described (Kumar et al., 2013). GFP/RFP ratio was normalized to controls and Z-score graphs were plotted.

Western blot analysis

Rencell VM cells were transfected with siRNAs in a 10 cm dish for 3 days. On the third day, Cells were washed twice using ice-cold PBS before scraping in PBS. Cells were centrifuged at 300g for 5 min at 4°C and were lysed with ice-cold lysis buffer (50 mM Tris [pH 8.0, 4°C], 200 mM NaCl, 0.5% NP-40 alternative, 1mM DTT, and Complete Protease Inhibitor (Roche) for 30 min with gradual agitation before clarification of samples by centrifugation at 10,000 g for 10 min at 4°C. Samples were diluted in lysis buffer with 2X SDS loading buffer and boiled at 95°C for 2 min. They were then resolved by SDS-PAGE electrophoresis using bis-tris NuPage gels as per manufacturer's instructions (Invitrogen) and transferred to PVDF membranes which was blocked using 3% BSA dissolved in TBST (50 mM Tris [pH8.0, 4°C], 150 mM NaCl, and 0.1% Tween 20) at room temperature for 1 hour before incubation with antibodies as manufacturer's instructions.

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Reviewer #1 (Public Review):

As outlined in my previous public review, Yeo et al. revised the current neuronal intoxication model, common to all serotypes of botulinum neurotoxins. Using a combination of genetic and imaging approaches, they demonstrate that upon internalization, BoNT/A-containing endosomes undergo retro-axonally trafficking to the neuronal soma. Within the soma, this particular serotype then traffics to the endoplasmic reticulum (ER) via the Golgi apparatus. At the ER, the SEC61 translocon complex facilitates the translocation of BoNT/A's metalloprotease domain (light chain, LC) from the ER lumen into the cytosol, where the thioredoxin reductase/thioredoxin system and HSP complexes release and refold the catalytic LC. Subsequently, the LC diffuses and cleaves SNAP25 first in the soma before reaching neurites and synapses.

Although I still acknowledge the well-executed and thoroughly analyzed genome-wide RNAi screen, I must once again highlight significant pitfalls and weaknesses in the paper due to the lack of essential controls and validations. Consequently, I suggest readers to approach the authors' findings with caution, as they may be limited to the combination of one specific cellular model and genetic engineering tools. During the revision process, authors declined to

conduct additional experiments that could have strengthened their main conclusions. These include, but are not limited to:

(1) Investigating weather in the newly generated cell line Red-SNAPR, the GFP fragment produced upon toxin cleavage degrades more rapidly in the soma compared to axon terminals, possibly due to differences in proteasome activity in these two compartments.

(2) Validating toxin cleavage activity in the soma before reaching synapses by conducting an additional and more physiological approach, a time course experiment using native BoNT/A and staining BoNT/A-cleaved SNAP25 with specific antibodies.

(3) Assessing whether the addition of mNG1-11 to the LC affects the translocation process itself and quantifying the mean fluorescence intensity (MFI) per cell, taking into consideration the amount of HA-tagged Cyt-mG1-10, which appears predominantly expressed in the cytosol and less detected in neurites. This raises the question of potential bias toward the cell soma in this assay.

(4) Validating major hits (e.g., VPS34 and Sec61) by performing WB or IF analysis to test the cleavage of endogenous SNAP25.

Additionally, during the revision process, the authors raised concerns about the level of scrutiny applied by this reviewer, particularly in comparison to the seminal study of Lilia K. Koriazova & Mauricio Montal published in Nature Structural Biology (PMID: 12459720). In this 2003 paper, Montal's lab pioneered the use of single-channel recordings and substrate proteolysis analysis to reconstitute the translocation of BoNT/A light chain protease across an artificial lipid bilayer via the channel formed by its heavy chain. The authors highlighted that, when converting the experimental conditions from the aforementioned paper into molarity, it appears that the cis compartment was loaded with 10−8 M BoNT/A, and the reported translocated protease activity (measured by substrate cleavage) is equivalent to 10−17 M. This implies that only about 1 LC molecule in 100 million has crossed the membrane. The calculation performed by authors is indeed accurate. However, readers should be informed about another piece of information present in the same paper that might help them to clarify this important point. Koriazova & Montal, by discussing this experiment, have pointed out that this value (10−17 M) corresponds to ≈3600 LC molecules, a number closed to the maximum number of channels that can be formed under the used experimental conditions. Indeed, from the same paper, quotation: 'This number is in close agreement with the maximum number of channels inserted in the bilayer under the assay condition, ≈2000 (Fig. 3a), as estimated from macroscopic membrane conductance ~1 × 105 pS and γ = 50 pS measured in 0.1 M KCl'. Another aspect that Yeo et al. forgot to mention in their rebuttal letter is that the system used by Koriazova & Montal lacks any chaperones in the trans compartment. Nowadays, we know that upon translocation, the refolding of the L chain is aided by Hsp90 (Azarnia Tehran et al., Cellular microbiology, 2017). Keeping this in mind, is not unrealistic to hypothesize that the number of LC molecules calculated more than 22 years ago by Koriazova & Montal (in an indirect way by checking SNAP25 cleavage using an ELISA-based assay) might be an underestimation. Indeed, the addition of Hsp90 in their system might aid in the refolding of LC molecules that, even if they have successfully be translocated, might not cleave the substrate due to their unfolded state.

As active scientist, I understand the challenges of peer review and publication, which can often be slow and frustrating involving seemingly endless rounds of review. Therefore, I am in favor of the new eLife publishing model. Indeed, this paper has already been published as Reviewed Preprints and will soon be declared as the final Version of Record, accompanied by this public review. Having said that, I hope that the readers of this journal and future scientists will prove me wrong. I hope they will engage with this paper, providing comments, validations (which are currently missing), and citations as frequently as they did for the seminal works of Koriazova & Montal.

https://doi.org/10.7554/eLife.92806.2.sa2

Reviewer #2 (Public Review):

Summary:

The study by Yeo and co-authors addresses a long-lasting issue about botulinum neurotoxin (BoNT) intoxication. The current view is that the toxin binds to its receptors at the axon terminus by its HCc domain and is internalized in recycled neuromediator vesicles just after release of the neuromediators. Then, the HCn domain assists the translocation of the catalytic light chain (LC) of the toxin through the membrane of these endocytic vesicles into the cytosol of the axon terminus. There, the LC cleaves its SNARE substrate and blocks neurosecretion. However, other views involving kinetic aspects of intoxication suggest that the toxin follows the retrograde axonal transport up to the nerve cell body and then back to the nerve terminus before cleaving its substrate.

In the current study, the authors claim that the BoNT/A (isotype A of BoNT) not only progresses to the cell body but once there, follows the retrograde transport trafficking pathway in a retromer-dependent fashion, through the Golgi apparatus, until reaching the endoplasmic reticulum. Next, the LC dissociates from the HC (a process not studied here) and uses the translocon Sec61 machinery to retro-translocate into the cytosol. Only then, the LC traffics back to the nerve terminus following the anterograde axonal transport. Once there, LC cleaves its SNARE substrate (SNAP25 in the case of BoTN/A) and blocks neurosecretion.

To reach their conclusion, Yeo and co-authors use a combination of engineered tools: a cell line able to differentiate into neurons (ReNcell VN), a reporter dual fluorescent protein derived from SNAP25, the substrate of BoNT/A (called SNAPR), the use of either native BoNT/A or a toxin to which three fragment 11 of the reporter fluorescent protein Neon Green (mNG) are fused to the N-terminus of the LC (BoNT/A-mNG11x3), and finally ReNcell VN transfected with mNG1-10 (a protein consisting of the first 10 beta strands of the mNG).

SNAPR is stably expressed all over in the ReNcell VN. SNAPR is yellow (red and green) when intact and becomes red only when cleaved by BoNT/A LC, the green tip being degraded by the cell. When the LC of BoNT/A-mNG11x3 reaches the cytosol in ReNcell VN transfected by mNG1-10, the complete mNG is reconstituted and emits a green fluorescence.

In the first experiment, the authors show that the catalytic activity of the LC appears first in the cell body of neurons where SNAPR is cleaved first. This phenomenon starts 24 h after intoxication and progresses along the axon towards the nerve terminus during an additional 24 h. In a second experiment, the authors intoxicate the ReNcell VN transfected by mNG1-10 using the BoNT/A-mNG11x3. The fluorescence appears also first in the soma of neurons, then diffuses in the neurites in 48 h. The conclusion of these two experiments is that translocation occurs first in the cell body and that the LC diffuses in the cytosol of the axon in an anterograde fashion.

In the second part of the study, the authors perform a siRNA screen to identify regulators of BoNT/A intoxication. Their aim is to identify genes involved in intracellular trafficking of the toxin and translocation of the LC. Interestingly, they found positive and negative regulators of intoxication. Regulators could be regrouped according to the sequential events of intoxication. Genes affecting binding to the cell-surface receptor (SV2) and internalization. Genes involved in intracellular trafficking. Genes involved in translocation such as reduction of the disulfide bond linking the LC to the HC and refolding in the cytosol. Genes involved in signaling such as tyrosine kinases and phosphatases. All these groups of genes may be consistent with the current view of BoNT intoxication within the nerve terminus. However, two sets of genes were particularly significant to reach the main conclusion of the work and

definitely constitute an original finding important to the field. One set of genes consists in those of the retromer, the other relates to the Sec61 translocon. This should indicate that once endocytosed, the BoNT traffics from the endosomes to Golgi apparatus, then to the ER. Ultimately, the LC should translocate from the ER lumen to the cytosol using the Sec61 translocon. The authors further control that the SV2 receptor for the BoNT/A traffics along the axon in a retromer-dependent fashion and that BoNT/A-mNG11x3 traverses the Golgi apparatus by fusing the mNG1-10 to a Golgi resident protein.

Strengths:

The findings in this work are convincing. The experiments are carefully done and are properly controlled. In the first part of the study, both the activity of the LC is monitored together with the physical presence of the toxin. In the second part of the work, the most relevant genes that came out of the siRNA screen are checked individually in the ReNcell VN / BoNT/A reporter system to confirm their role in BoNT/A trafficking and retro-translocation. These findings are important to the fields of toxinology and medical treatment of neuromuscular diseases by BoNTs. They may explain some aspects of intoxication such as slow symptom onset, aggravation and appearance of central effects.

Weaknesses:

The findings antagonize the current view of the intoxication pathway that is sustained by a vast amount of observations. The findings are certainly valid, but their generalization as the sole mechanism of BoNT intoxication should be tempered. These observations are restricted to one particular neuronal model and engineered protein tools. Other models such as isolated nerve/muscle preparations display nerve terminus paralysis within minutes rather than days. Also, the tetanus neurotoxin (TeNT), which mechanism of action involving axonal transport to the posterior ganglia in the spinal cord is well described, takes between 5 and 15 days. It is thus possible that different intoxication mechanisms co-exist for BoNTs or even vary depending on the type of neurons.

Although the siRNA experiments are convincing, it would be nice to reach the same observations with drugs affecting the endocytic to Golgi to ER transport (such as Retro-2, golgicide or brefeldin A) and the Sec61 retrotranslocation (such as mycolactone). Then, it would be nice to check other neuronal systems for the same observations.

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Reviewer #3 (Public Review):

Summary:

The manuscript by Yeo et al. investigates the intracellular trafficking of Botulinum neurotoxin A (BoNT/A), a potent toxin used in clinical and cosmetic applications. Contrary to the prevailing understanding of BoNT/A translocation into the cytosol, the study suggests a retrograde migration from the synapse to the soma-localized Golgi in neurons. Using a genome-wide siRNA screen in genetically engineered neurons, the researchers identify over three hundred genes involved in this process. The study employs organelle-specific split-mNG complementation, revealing that BoNT/A traffics through the Golgi in a retromer-dependent manner before moving to the endoplasmic reticulum (ER). The Sec61 complex is implicated in the retro-translocation of BoNT/A from the ER to the cytosol. Overall, the research challenges the conventional model of BoNT/A translocation, uncovering a complex route from synapse to cytosol for efficient intoxication. The findings are based on a comprehensive approach, including the introduction of a fluorescent reporter for BoNT/A catalytic activity and genetic manipulations in neuronal cell lines. The conclusions highlight the importance of retrograde

trafficking and the involvement of specific genes and cellular processes in BoNT/A intoxication.

Strengths:

The major part of the experiments are convincing. They are well-controlled and the interpretation of their results is balanced and sensitive.

Weaknesses:

To my opinion, the main weakness of the paper is that all experiments are performed using a single cellular system (RenVM neurons), as stated in the title. It is therefore unclear at the moment to what extent the findings in this paper can be generalized to other neuronal cell models / in vivo situation.

https://doi.org/10.7554/eLife.92806.2.sa0

Author response:

The following is the authors' response to the original reviews.

> ***Public Reviews:***
>
> ***Reviewer #1 (Public Review):***
>
> *Summary:*
>
> *During the last decades, extensive studies (mostly neglected by the authors), using in vitro and in vivo models, have elucidated the five-step mechanism of intoxication of botulinum neurotoxins (BoNTs). The binding domain (H chain) of all serotypes of BoNTs binds polysialogangliosides and the luminal domain of a synaptic vesicle protein (which varies among serotypes). When bound to the synaptic membrane of neurons, BoNTs are rapidly internalized by synaptic vesicles (SVs) via endocytosis. Subsequently, the catalytic domain (L chain) translocates, a process triggered by the acidification of these organelles. Following translocation, the disulfide bridge connecting the H chain with the L chain is reduced by the thioredoxin reductase/thioredoxin system, and it is refolded by the chaperone Hsp90 on SV's surface. Once released into the cytosol, the L chains of different serotypes cleave distinct peptide bonds of specific SNARE proteins, thereby disrupting neurotransmission. In this study, Yeo et al. extensively revise the neuronal intoxication model, suggesting that BoNT/A follows a more complex intracellular route than previously thought. The authors propose that upon internalization, BoNT/A-containing endosomes are retro-axonally trafficked to the soma. At the level of the neuronal soma, this serotype then traffics to the endoplasmic reticulum (ER) via the Golgi apparatus. The ER SEC61 translocon complex facilitates the translocation of BoNT/A's LC from the ER lumen into the cytosol, where the thioredoxin reductase/thioredoxin system and HSP complexes release and refold the catalytic L chain. Subsequently, the L chain diffuses and cleaves SNAP25 first in the soma before reaching neurites and synapses.*
> *Strengths:*
>
> *I appreciate the authors' efforts to confirm that the newly established methods somehow recapitulate aspects of the BoNTs mechanism of action, such as toxin binding and uptake occurring at the level of active synapses. Furthermore, even though I consider the SNAPR approach inadequate, the genome-wide RNAi screen has been well executed and thoroughly analyzed. It includes well-established positive and negative controls, making it a comprehensive resource not only for scientists working in the field of botulinum neurotoxins but also for cell biologists studying endocytosis more broadly. Weaknesses:*

> *I have several concerns about the authors' main conclusions, primarily due to the lack of essential controls and validation for the newly developed methods used to assess toxin cleavage and trafficking into neurons. Furthermore, there is a significant discrepancy between the proposed intoxication model and existing studies conducted in more physiological settings. In my opinion, the authors have omitted over 20 years of work done in several labs worldwide (Montecucco, Montal, Schiavo, Rummel, Binz, etc.). I want to emphasize that I support changes in biological dogma only when these changes are supported by compelling experimental evidence, which I could not find in the present manuscript.*

We thank the reviewer for his reading and comments and for pointing out the discrepancy between our proposed model and the existing model. However, we respectfully disagree with the phrase of “extensive studies have elucidated the five-steps mechanism of intoxication…”. This sentence and the following imply that the model is well-established and demonstrated. It also highlights how the reviewer is convinced about this previous model.

We contest this model for theoretical reasons and contest the strength of evidences that support it. We previously included references to previous work showing that the model is also being challenged by others. In light of the reviewer’s comments, we incluced more references in the introduction and we also explicit our main theoretical concern in the introduction:

“Arguably, the main problem of the model is its failure to propose a thermodynamically consistent explanation for the directional translocation of a polypeptidic chain across a biologial membrane. Other known instances of polypeptide membrane translocation such as the co-translational translocation into the ER indicate that it is an unfavorable process, which consumes significant energy (Alder and Theg 2003). ”

We also added the following text in the Discussion to address with the reviewer’s concerns: “Our study contradicts the long-established model of BoNT intoxication, which is described in several reviews specifically dedicated to the subject 1–4. In short, these reviews support the notion that BoNT are molecular machines able to mediate their own translocation across membranes; this notion has convinced some cell biologists interested in toxins and retrograde traffic, who describe BoNT mode of translocation in their reviews 5,6.

But is this notion well supported by data? A careful examination of the primary literature reveals that early studies indeed report that BonTs form ion channels at low pH values 7,8. These studies have been extended by the use of patch-clamp 9,10. These works and others lead to various suppositions on how the toxin forms a channel and translocate the LC 1,11 .

However, only a single study claims to reconstitute in vitro the translocation of BonT LC across membranes 12. In this paper, the authors report using a system of artificial membranes separating two aqueous compartments. They load the toxin in the cis compartment and measure the protease activity in the trans compartment after incubation. However, when the experimental conditions described are actually converted in terms of molarity, it appears that the cis compartment was loaded at 10e-8M BonT and that the reported translocated protease activity is equivalent to 10e-17 M (Figure 3D, 12). Thus, in this experiment, about 1 LC molecule in 100 millions has crossed the membrane. Such extremely low transfert rate does not tally with the extreme efficiency of intoxication in vivo, even while taking into account the difference between artificial and biological membranes.

In sum, a careful analysis of the primary literature indicate that while there is ample evidence that BoNTs have the ability to affect membranes and possibly create ion channels, there is actually no credible evidence that these channels mediate translocation of the LC. As mentioned earlier, it is not clear how such a self-translocation mechanism would function

thermodynamically. By contrast, our model proposes a mechanism without a thermodynamic problem, is consistent with current knowledge about other protein toxins, such as PE, Shiga and Ricin, and can help explain previously puzzling features of BonT effects. It is worth noting that a similar self-translocation model was proposed for other protein toxins such as Pseudomonas exotoxin, which have similar molecular organisation as BonT (68). However, it has since been demonstrated that the PE toxins require cellular machinery, in particular in the ER, for intoxication (21,69,70)."

> ***Reviewer #2 (Public Review):***
>
> *Summary:*
>
> *The study by Yeo and co-authors addresses a long-lasting issue about botulinum neurotoxin (BoNT) intoxication. The current view is that the toxin binds to its receptors at the axon terminus by its HCc domain and is internalized in recycled neuromediator vesicles just after the release of the neuromediators. Then, the HCn domain assists the translocation of the catalytic light chain (LC) of the toxin through the membrane of these endocytic vesicles into the cytosol of the axon terminus. There, the LC cleaves its SNARE substrate and blocks neurosecretion. However, other views involving kinetic aspects of intoxication suggest that the toxin follows the retrograde axonal transport up to the nerve cell body and then back to the nerve terminus before cleaving its substrate.*
>
> *In the current study, the authors claim that the BoNT/A (isotype A of BoNT) not only progresses to the cell body but once there, follows the retrograde transport trafficking pathway in a retromer-dependent fashion, through the Golgi apparatus, until reaching the endoplasmic reticulum. Next, the LC dissociates from the HC (a process not studied here) and uses the translocon Sec61 machinery to retro-translocate into the cytosol. Only then, does the LC traffic back to the nerve terminus following the anterograde axonal transport. Once there, LC cleaves its SNARE substrate (SNAP25 in the case of BoTN/A) and blocks neurosecretion.*
>
> *To reach their conclusion, Yeo and co-authors use a combination of engineered tools: a cell line able to differentiate into neurons (ReNcell VN), a reporter dual fluorescent protein derived from SNAP25, the substrate of BoNT/A (called SNAPR), the use of either native BoNT/A or a toxin to which three fragment 11 of the reporter fluorescent protein Neon Green (mNG) are fused to the N-terminus of the LC (BoNT/A-mNG11x3), and finally ReNcell VN transfected with mNG1-10 (a protein consisting of the first 10 beta strands of the mNG).*
>
> *SNAPR is stably expressed all over in the ReNcell VN. SNAPR is yellow (red and green) when intact and becomes red only when cleaved by BoNT/A LC, the green tip being degraded by the cell. When the LC of BoNT/A-mNG11x3 reaches the cytosol in ReNcell VN transfected by mNG1-10, the complete mNG is reconstituted and emits a green fluorescence.*
>
> *In the first experiment, the authors show that the catalytic activity of the LC appears first in the cell body of neurons where SNAPR is cleaved first. This phenomenon starts 24 hours after intoxication and progresses along the axon towards the nerve terminus during an additional 24 hours. In a second experiment, the authors intoxicate the ReNcell VN transfected by mNG1-10 using the BoNT/A-mNG11x3. The fluorescence appears also first in the soma of neurons, then diffuses in the neurites in 48 hours. The conclusion of these two experiments is that translocation occurs first in the cell body and that the LC diffuses in the cytosol of the axon in an anterograde fashion.*
>
> *In the second part of the study, the authors perform a siRNA screen to identify regulators of BoNT/A intoxication. Their aim is to identify genes involved in intracellular trafficking*

of the toxin and translocation of the LC. Interestingly, they found positive and negative regulators of intoxication. Regulators could be regrouped according to the sequential events of intoxication.

Genes affecting binding to the cell-surface receptor (SV2) and internalization. Genes involved in intracellular trafficking. Genes involved in translocation such as reduction of the disulfide bond linking the LC to the HC and refolding in the cytosol. Genes involved in signaling such as tyrosine kinases and phosphatases. All these groups of genes may be consistent with the current view of BoNT intoxication within the nerve terminus. However, two sets of genes were particularly significant to reach the main conclusion of the work and definitely constitute an original finding important to the field. One set of genes consists of those of the retromer, and the other relates to the Sec61 translocon. This should indicate that once endocytosed, the BoNT traffics from the endosomes to the Golgi apparatus, and then to the ER. Ultimately, the LC should translocate from the ER lumen to the cytosol using the Sec61 translocon. The authors further control that the SV2 receptor for the BoNT/A traffics along the axon in a retromer-dependent fashion and that BoNT/A-mNG11x3 traverses the Golgi apparatus by fusing the mNG1-10 to a Golgi resident protein.

Strengths:

The findings in this work are convincing. The experiments are carefully done and are properly controlled. In the first part of the study, both the activity of the LC is monitored together with the physical presence of the toxin. In the second part of the work, the most relevant genes that came out of the siRNA screen are checked individually in the ReNcell VN / BoNT/A reporter system to confirm their role in BoNT/A trafficking and retro-translocation.

These findings are important to the fields of toxinology and medical treatment of neuromuscular diseases by BoNTs. They may explain some aspects of intoxication such as slow symptom onset, aggravation, and appearance of central effects.

Weaknesses:

The findings antagonize the current view of the intoxication pathway that is sustained by a vast amount of observations. The findings are certainly valid, but their generalization as the sole mechanism of BoNT intoxication should be tempered. These observations are restricted to one particular neuronal model and engineered protein tools. Other models such as isolated nerve/muscle preparations display nerve terminus paralysis within minutes rather than days. Also, the tetanus neurotoxin (TeNT), whose mechanism of action involving axonal transport to the posterior ganglia in the spinal cord is well described, takes between 5 and 15 days. It is thus possible that different intoxication mechanisms co-exist for BoNTs or even vary depending on the type of neurons.

Although the siRNA experiments are convincing, it would be nice to reach the same observations with drugs affecting the endocytic to Golgi to ER transport (such as Retro-2, golgicide or brefeldin A) and the Sec61 retrotranslocation (such as mycolactone). Then, it would be nice to check other neuronal systems for the same observations.

We thank the reviewer for the careful reading and comments of our manuscript. The reference to “a vast amount of observation” is a similar argument to the Reviewer 1 and used to suggest that our study may not be applicable as a general mechanism.

We respectfully disagree as described above and posit on the contrary that the model we propose is much more likely to be general than the model presented in current reviews for the several reasons cited (see added text in Introduction and Discussion). While we agree that

more work is needed to confirm the proposed mechanisms of BonT translocation in other models, these experiments fall outside the perimeter of our study.

The fact that nerve/muscle preparations of BonT activity have relatively fast kinetics does not pose a contradiction to our model. Our model reveals primarily the requirement for trafficking to the ER membranes. This ER targeting requires trafficking through the Golgi complex, in turn explaining the requirement for trafficking to the soma of neurons in the experimental system we used. However, in neuronal cells in vivo, Golgi bodies can be found along the lenght of the axon, thus BonT may not always require trafficking to the soma of the affected cells. The time required for intoxication could thus vary greatly depending on the neuronal structural organisation.

TenT is proposed to transfer from excitatory neurons into inhibitory neurons before exerting its action. While the detailed mechanism of this fascinating mechanism remain to be explored, it clearly falls beyond the purview of this manuscript.

Regarding the use of drugs, we agree that it would be a nice addition; unfortunately we are unable to perform such experiments at this stage. Setting up a large scale siRNA screen for BonT mechanism of action is challenging as it requires a special facility with controlled access and police authorisation (in Singapore) given the high toxicity of this molecule. Unfortunately, the authorisations have now lapsed.

> ***Reviewer #3 (Public Review):*** *Summary:*
>
> *The manuscript by Yao et al. investigates the intracellular trafficking of Botulinum neurotoxin A (BoNT/A), a potent toxin used in clinical and cosmetic applications. Contrary to the prevailing understanding of BoNT/A translocation into the cytosol, the study suggests a retrograde migration from the synapse to the soma-localized Golgi in neurons. Using a genome-wide siRNA screen in genetically engineered neurons, the researchers identified over three hundred genes involved in this process. The study employs organelle-specific split-mNG complementation, revealing that BoNT/A traffics through the Golgi in a retromer-dependent manner before moving to the endoplasmic reticulum (ER). The Sec61 complex is implicated in the retro-translocation of BoNT/A from the ER to the cytosol. Overall, the research challenges the conventional model of BoNT/A translocation, uncovering a complex route from synapse to cytosol for efficient intoxication. The findings are based on a comprehensive approach, including the introduction of a fluorescent reporter for BoNT/A catalytic activity and genetic manipulations in neuronal cell lines. The conclusions highlight the importance of retrograde trafficking and the involvement of specific genes and cellular processes in BoNT/A intoxication.*
>
> *Strengths:*
>
> *The major part of the experiments are convincing. They are well-controlled and the interpretation of their results is balanced and sensitive.*
>
> *Weaknesses:*
>
> *To my opinion, the main weakness of the paper is in the interpretation of the data equating loss of tGFP signal (when using the Red SNAPR assay) with proteolytic cleavage by the toxin. Indeed, the first step for loss of tGFP signal by degradation of the cleaved part is the actual cleavage. However, this needs to be degraded (by the proteasome, I presume), a process that could in principle be affected (in speed or extent) by the toxin.*

We thank the reviewer for his comments and careful reading of our manuscript.

Regarding the read-out of the assay, we agree that the assay could be sensitive to alteration in the protein degradation pathway. We have added the following sentence in the Discussion to take it into account:

“As noted by one reviewer, the assay may be sensitive to perturbation in the general rate of protein degradation, a consideration to keep in mind when evaluating the results of large scale screens.”

While this may be valid for some hits in the general list, it is important to note that the main hits have been shown to affect toxin trafficking by an independent, orthogonal assay based on the split GFP reconstitution.

> ***Recommendations to authors:***
>
> ***Reviewer #1 (Recommendations For The Authors):***
>
> *(1) To assess the activity of BoNT/A in neurons, Yeo et al. have generated a neuronal stem line referred to as SNAPR. This cell line stably expresses a chimeric reporter protein that consists of SNAP25 flanked at its N-terminus with a tagRFPT and at its C-terminus with a tagGFP. After exposure to BoNT/A, SNAP25 is cleaved and, the C-terminal tGFP-containing moiety is rapidly degraded. I have many doubts about the validity of the described method. Indeed, BoNT/A activity is analysed in an indirect way by quantifying the degradation of the GFP moiety generated after toxin cleavage (Fig. 2). In this regard, the authors should consider that their approach is dependent, not only on the toxin's metalloprotease activity but also on the functionality of the proteasome in neurons. Therefore, considering the current dataset, it is impossible to rule out the possibility that the progression of GFP signal loss from the soma to the neurite terminals may be attributed to the different proteasome activity in these compartments. Is it conceivable that the GFP fragment generated upon toxin cleavage degrades more rapidly in the soma in comparison to axonal terminals? This alternative explanation could challenge the conclusion drawn in Fig. 2.*

The reviewer’s alternative explanation disregards the experiments performed with the split-GFP complementation approach, which indicate translocation in the soma first. The split GFP reporter is not dependent on the proteasome activity. It also disregard the genetic data implicating many genes involved in membrane retrograde traffic, which are also not consistent with the hypothesis of the reviewer. These genes depletions not only affect SNAPR degradation but also BoNT/A-mNG11 trafficking: thus, their effect cannot be attributed to an completely hypothetical spatial heterogeneous distribution of the proteasome.

> *For this reason, I strongly suggest using a more physiological approach that does not depend on proteasomal degradation or on the expression of the sensor in neurons. The authors should consider performing a time course experiment following intoxication and staining BoNT/A-cleaved SNAP25 by using specific antibodies (see Antonucci F. et al., Journal of Neuroscience, 2008 or Rheaume C. et al., Toxins 2015).*

For the above reason, we do not agree with the pressing importance of confirming by a third method using specific antibodies; especially considering that BonT is very difficult to detect in cells when incubated at physiological levels. By the way, the cited paper, by Antonucci F; et al. documents long distance retrograde traffic of BonT/A, which is in line with our data.

> *An alternative approach could involve the use of microfluidic devices that physically separate axons from cell bodies. Such a separation will allow us to test the authors' primary conclusion that SNAP25 is initially cleaved in the soma. The suggested experiments will also rule out potential overexpression artifacts that could influence the*

> *authors' conclusions when using the newly developed SNAPR approach. Without these additional experiments, the authors' main conclusion that SNAP25 is cleaved first in the neuronal soma rather than at the nerve terminal is inadequate.*

As discussed above we disagree about the doubts raised by the reviewer: we present three types of evidences (SNAPR, split GFP and genetic hits) and they all point in the same direction. Thus, we respectfully doubt that a fourth approach would convince this reviewer. To note, we have attempted to use microfluidics devices as suggested by the reviewer, however, the Ren-VM neurons were not able to extend axons long enough across the device.

> *(2) To detect BoNT/A translocation into the cytosol, the authors have used a complementation assay by intoxicating ReNcell VM cell expressing a cytosolic HA-tagged split monomeric NeonGreen (Cyt-mNG1-10) with an engineered BoNT/A, where the catalytic domain (LC) was fused to mNG1-11. When drawing conclusions regarding the detection of cytosolic LC in the neuronal soma, the authors should highlight the limitations of this assay and explicitly describe them to the readers. Firstly, the authors need to investigate whether the addition of mNG1-11 to the LC affects the translocation process itself (by comparing with a WT, not tagged, LC).*
>
> *Additionally, from the data shown in Fig. 2C, it is evident that the Cyt-mNG1-10 is predominantly expressed in the cytosol and less detected in neurites. This raises the question of whether there might be a bias for the cell soma in this assay. To address this important concern, I suggest quantifying MFI per cell (Fig. 2D) taking into consideration the amount of HA-tagged Cyt-mNG1-10. Furthermore, I strongly suggest targeting mNG1-10 to synapses and performing a similar time course experiment to observe when LC translocation occurs at nerve terminals. Alternative experiments, to prove that BoNT/A requires retrograde trafficking before it can translocate, may be done to repeat the experiments shown in Fig. 2D in the presence of inhibitors (or by KD some of the hits identified as microtubule stabilizers) that should interfere with BoNT/A trafficking to the neuronal somata. Without these additional experiments, the authors' main conclusion that the BoNT/A catalytic domain is first detected in the neuronal soma rather than at the nerve terminal is very preliminary.*

Similarly as for the SNAPR assay, the reviewer is raising the level of doubt to very high levels. We respect his thoroughness and eagerness to question the new model. However, we note that a similar level of scrutiny does not apply to the prevalent competitive model. Indeed, the data supporting the self-translocation model is based on a single in vitro experiment published in one panel as we have explain din the discussion (see above).

> *(3) In the genome-wide RNAi screening, rather than solely assessing SV2 surface levels, it would have been beneficial to directly investigate BoNT/A binding to the neuronal membrane. For instance, this could have been achieved by using a GFP-tagged HC domain of BoNT/A. At present, the authors cannot exclude the possibility that among the 135 hits that did not affect SV2 levels, some might still inhibit BoNT/A binding to the neuronal surface. These concerns, already exemplified by B4CALT4 (which is known to be involved in the synthesis of GT1b), should be explicitly addressed in the main text.*

We agree with the reviewer that perturbation of binding of BonT is possible. We added the following text:

“Network analysis reveals regulators of signaling, membrane trafficking and thioreductase redox state involved in BoNT/A intoxication

Among the positive regulators of the screen, 135 hits did not influence significantly surface SV2 levels and are thus likely to function in post-endocytic processes (Supplementary Table

2). However, we cannot formerly exclude that they could affect binding of BonT to the cell surface independently of SV2."

> *(4) The authors should clearly state which reagents they have tried to use in order to explain the challenges they faced when directly testing the trafficking of BoNT/A. The accumulation of Dendra-SV2 bulbous structures at the neurite tips in VPS35-depleted cells could be interpreted as a sign of neuronal stress/death. Have the authors investigated other proteins that do not undergo retro-axonal trafficking in a retromer-dependent manner? This control is essential. In this regard, the use of a GFP-tagged HC domain of BoNT/A could prove to be quite helpful.*

We tried multiple commercially available antibodies against BonT but we could not get a very good signal. The postdoc in charge of this project has now gone to greener pastures and we are not in the capacity to provide the details corresponding to these antibodies. We di dnot observe significant cell death after VPS-35 knockdown at the time of the experiment, however longe rterm treatment might result in toxicity indeed.

> *(5) Considering my concerns related to the SNAPR system and the complementation assay to study SNAP25 cleavage and BoNT/A trafficking, I suggest validating some of their major hits (ex. VPS34 and Sec61) by performing WB or IF analysis to examine the cleavage of endogenous SNAP25. Furthermore, the authors should test VPS35 depletion in the context of the experiments performed in Fig. 6G-H, by validating that this protein is essential for BoNT/A retrograde trafficking.*

The reviewer concerns are well noted but as discussed above, the two systems we used are completely orthogonal. Thus, for the reviewer's concerns to be valid, it would have to be two completely independent artefacts giving rise to the same result. The alternative explanation is that BonT/A translocates in the soma. The Ockham razor principle dictates that the simplest explanation is the likeliest.

> *(6) The introduction and the discussion section of this paper completely disregard more than 20 years of research conducted by several labs worldwide (Montecucco, Montal, Schiavo, Rummel, Binz, etc). The authors should make an effort to contextualize their data within the framework of these studies and address the significant discrepancies between their proposed intoxication model and existing research that clearly demonstrates BoNTs translocating upon the endocytic retrieval of SVs at presynaptic sites. Nevertheless, even assuming that the model proposed by the authors is accurate, numerous questions emerge. One such question is: How can the authors explain the exceptional toxicity of botulinum neurotoxin in an ex vivo neuromuscular junction preparation devoid of neuronal cell bodies (see Cesare Montecucco and Andreas Rummel's seminal studies)?*

Please see above in the answer to public reviews.

> *(7) Scale bars should be added to all representative pictures.*

This has been done. Thank you for the thorough reading of our manuscript.

> Reviewer #2(Recommendations For The Authors):*
>
> *(1) The title overstates the results. It may be indicated "in differenciated ReNcell VM".*

Title changed to: "Botulinum toxin intoxication requires retrograde transport and membrane translocation at the ER in RenVM neurons"

> *(2) In the provided manuscript there are two Figure 2 and no Figure 3. This made the reading and understanding extremely difficult and should be corrected. As a result, the Figure legends do not fit the numbering. There are also discrepancies between some Figure panels (A, B, C, etc), the text, and the Legends. All this needs to be carefully checked.*

We apologize for the confusion as the manuscript as followed multiple rounds of revisions. We have carefully verified labels and legends.

> *(3) The BoNT/A-mNG11x3 may introduce some bias that could be discussed. Would these additional peptides block LC translocation from synaptic vesicles in the nerve termini? In addition, the mNG peptides that are unfolded before complementation may direct LC towards Sec61. These aspects should be discussed.*

The comment would be valid if BoNT/A-mNG11x3 was the only approach used in the paper, however the SNAPR reporter is used with native BonT and shows data consistent with the split GFP approach.

> *(4) In the Figure about SV2 (Fig 3 or 4): The authors did not locate SV2. The cells seem not to have the same differentiated phenotype as in Figure 1 and Figure 2/3A.*

We apologized above for the mislabeling. It is not clear what is the question here.

> *(5) The authors should check whether BoNT/A wt cleaves the endogeneous SNAP25 by western blot for instance in the original ReNcell VN before SNAPR engineering. This should be compared with wt SNAP25 cleavage by the BoNT/A-LC-mNG.*

It is likely that BoNT/A-LC-mNG11 should have similar activity as it is only adding a small peptide at the end of the LC. At any rate, it is not clear why this is so important since both molecules translocate in the cytosol, with the same kinetics and in the same subcellular locale.

> *(6) Perhaps I did not understand. How can the authors exclude that what is observed is the kinetic overproduction of the reporter substrate SNAPR?*

The authors could use SLO toxin (PNAS 98, 3185-3190, 2001) to permeabilize the cells all along their body and axon to introduce BoNT/A or LC (wt) and observe synchronized SNAPR cleavage throughout the cells.

The concept mentioned here is not very clear to us. The reviewer is proposing that the SNAPR is produced much more efficiently at the tips of the neurites and thus its cleavage takes longer to be detected and is apparent first in the soma?? With all due respect, this is a strange hypothesis, at odds with what we know of protein dynamics in the neurons (i.e. most proteins are largely made in the soma and transported or diffuse into the neurites).

Again, the two orthogonal approaches: split GFP and SNAPR reporter use different constructs and methods, yet converge on similar results. Perhaps, the incredulity of the reviewer might be more productively directed at the current data “demonstrating” the translocation of LC in the synaptic button?

> *(7) The authors could also use an essay on neurotransmitter release monitoring by electrophysiology measurements to check the functional consequences of the kinetic diffusion of LC activity along the axon. Can the authors exclude that some toxin*

> *molecules translocate from the endocytic vesicles and block neurotransmission within minutes or a few hours?*

It is well established that inhibition of neurotransmission does not occur within minutes in vivo and in vitro, but rather within hours or even days. This kinetic delay is experienced by many patients and is one of the key argument against the current model of self-translocation at the synaptic vesicle level.

> *Minor remarks*

Thank you for pointing out all these.

> *(1) Please check typos. There are many. Check space before the parenthesis, between numbers and h (hours), reference style etc.*

Thank you. We have reviewed the text and try to eliminate all these instances.

> *(2) Line 90: The C of HC should be capitalized.*

Fixed

> *(3) Line 107: add space between "neurons(Donato".*

Fixed

> *(4) Line 109: space "72 h".*

Fixed

> *(5) Line 115: a word is missing ? ...to show retro-axonal... ? Please clarify this sentence.*

Fixed

> *(6) Figure 1E: does nm refer to nM (nanomolar)? Please correct. No mention of panel F.*

Fixed

> *(7) Line 161: do you mean ~16 µm/h? Please correct.*

Fixed

> *(8) Line 168, words are missing.*

Fixed, thank you

We verified that Cyt-mNG1-10 was expressed using the HA tag, the expression was homogeneously distributed in differentiated neurons and we observed no GFP signal (Figure2C).

> *(9) Line 171: Isn't mNG 11 the eleventh beta strand of the neon green fluorescent protein, not alpha helix? Otherwise, can the authors confirm it acquires the shape of an alpha helix? Same at line 326.*

We have corrected the mistake; thanks for pointing it out.

> *(10) Figure 2 is doubled. The legend of Fig 2 refers to Figure 3. There is no legend for Figure 2. Then, some figures are shifted in their numbering.*

Fixed

> *(11) The fluorescence in the cell body must appear before the fluorescence in the axon due to higher volume. Please discuss.*

The fluorescence progresses in the neurites extensions in a centripetal fashion. The volume of the neurite near the cell body is not significantly different from the end of the neurite. Thus the fluorescence data is consistent with translocation in soma and not with an effect due to higher volume in the soma.

> *(12) Figure 2D, right: the term intoxication is improper for this experiment. Rather, it is the presence of the BoNT/A-mNG11 that is detected. I believe the authors should be particularly careful about the use of terms: intoxication means blockade of neurosecretion, SNAPR cleavage means activity etc.*

While the reviewer is correct that it is the presence of BoNT/A-mNG11 that is detected, it remains that it is an active toxin, so the neurons are effectively intoxicated; as they are when we use the wild type toxin. We do not imply that we are measuring intoxication, but simply that the neurons are put into contact with a toxin.

> *(13) Line 196: Should we read TXNRD1 is required for BoNT/A LC translocation? TXNRD1 in the current model of translocation is located in the cytoplasm and is supposed to play a role in the cleavage of the disulfide bond linking LC to HC. In the model proposed by this study, LC is translocated through the Sec61 translocon. In this case, I would assume that the protein disulfide isomerase (PDI) in the endoplasmic reticulum would reduce the LC-HC disulfide bond. In that case, TXNRD1 would not be required anymore. Please discuss.*

Why should we assume that a PDI is involved in the reduction of the LC-HC disulfide bond? In our previous studies on A-B toxins (PE and Ricin), different reduction systems seemed to be at play. There is no conceptual imperative to assume reduction in the ER because the Sec61 translocon is implicated. Reduction might occur on the cytosolic side by TXNRD1 or the effect of this reductase could be indirect.

> *(14) The legend of Figure 4 (in principle Figure 5?) is not matching with the panels and panel entries are missing (Figure 4F in particular).*

Fixed

> *(15) Figure 6 panels E and H, please match colors with legend (grey and another color).*

Not clear

> *(16) Please indicate BoNT/A construct concentrations in all Figure legends.*

Done

> *(17) Line 416: isn't SV2 also involved in epilepsy?*

Yes it is.

> *(18) Line 433: as above, shouldn't the disulfide bond linking LC to HC be cleaved by PDI in the ER in this model (as for other translocating bacterial toxins) rather than by thioredoxin reductases in the cytoplasm? Please discuss.*

See above

> *(19) Identification of vATPase in the screen could be consistent with the endocytic vesicle acidification model of translocation.*

Yes

> *(20) Did the authors add KCl in screening controls without toxins? This should be detailed in the Materials and Methods. Could there be a KCl effect on the cells? KCl exposure for 48 hours may be highly stressful for cells. The KCl exposure should last only several minutes for toxin entry.*

We did not observe significant cell detah with the cell culture conditions used. Cell viability was controlled at multiple stages using nuclei number for instance

> ***Reviewer #3 (Recommendations For The Authors):***
>
> *Main comments: (1) In Figure 1B: could you devise a means to prevent proteosomal degradation of the tGFP cleaved part to assess whether this is formed?*

We have also used a FRET assay after tintoxication and obtained similar results

> *(2) Line 152: Where it reads "was not surprising", maybe I missed something, but to me, this is indeed surprising. If the toxin is rapidly internalized and translocated (therefore, it is able to cleave SNAP25), the fact that tGFP requires 48 hours to be degraded seems surprising to me. Or does it mean that the toxin also slows down the degradation of the tGFP fragment? So, how can you differentiate between the effect being on cleavage of the fragment or in tGFP degradation?*

The reviewer is correct, the “not” was a typo due to re-writting; the long delay between adding the toxin and observing cleavage was suprising indeed. Our interpretation is that it is trafficking that takes time, indeed, the split-GFP data kinetics indicates that the toxin takes about 48h to fill up the entire cytosol (Fig. 2D).

> *(3) Regarding the effect of Sec61G knockdown, is it possible that the observed effects are indirect and not due to the translocon being directly responsible for translocating the protein?*

As discussed in the last part of the results,Sec61 knock-down results in block of intoxication, but does not prevent BonT from reaching the lumen of the ER (Figure 6G,H). Thus, Sec61 is “is instrumental to the translocation of BoNT/A LC into the neuronal cytosol at the soma.”

Minor comments:

> *(1) Fig. 3E: in the legend I think one of the NT3+ should be NT3-.*

Yes, thanks for spotting it

> *(2) Would you consider adding Figure S4 as a main figure?*

Thanks for the suggestion

> *(3) Please, check that all microscopy image panels have scale bars.*

Done

> *(4) Figure 6B (bottom panes): why does it seem that there is a lot of mNeonGreen positive signal in regions that are not positive for HA? Shouldn't complementation keep HA in the complemented protein.*

Our assumption i sthat there is an excess of receptor protein (HA tag) over reconstituted protein (GFP protein) given the relatively low concentration of toxin being internalized and translocated Refs: (1) Pirazzini M, Azarnia Tehran D, Leka O, Zanetti G, Rossetto O, Montecucco C. On the translocation of botulinum and tetanus neurotoxins across the membrane of acidic intracellular compartments. Biochim Biophys Acta. 2016 Mar;1858(3):467–474. PMID: 26307528

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(15) Tian S, Muneeruddin K, Choi MY, Tao L, Bhuiyan RH, Ohmi Y, Furukawa K, Furukawa K, Boland S, Shaffer SA, Adam RM, Dong M. Genome-wide CRISPR screens for Shiga toxins and ricin reveal Golgi proteins critical for glycosylation. PLoS Biol. 2018 Nov;16(11):e2006951. PMCID: PMC6258472