Published in final edited form as:
Biochem Biophys Res Commun. 2011 February 25; 405(4): 673–677. doi:10.1016/j.bbrc.2011.01.093.

Neuronal Targeting, Internalization, and Biological Activity of a Recombinant Atoxic Derivative of Botulinum Neurotoxin A

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Abstract

Non-toxic derivatives of Botulinum neurotoxin A (BoNT/A) have potential use as neuron-targeting delivery vehicles, and as reagents to study intracellular trafficking. We have designed and expressed an *a*toxic *d*erivative of BoNT/A (BoNT/A *ad*) as a full-length 150kDa molecule consisting of a 50 kDa light chain (LC) and a 100 kDa heavy chain (HC) joined by a disulfide bond and rendered atoxic through the introduction of metalloprotease-inactivating point mutations in the light chain. Studies in neuronal cultures demonstrated that BoNT/A *ad* cannot cleave synaptosomal-associated protein 25 (SNAP25), the substrate of *wt* BoNT/A, and that it effectively competes with *wt* BoNT/A for binding to endogenous neuronal receptors. *In vitro* and *in vivo* studies indicate accumulation of BoNT/A *ad* at the neuromuscular junction of the mouse diaphragm. Immunoprecipitation studies indicate that the LC of BoNT/A *ad* forms a complex with SNAP25 present in the neuronal cytosolic fraction, demonstrating that the atoxic LC retains the SNAP25 binding capability of the *wt* toxin. Toxicity of BoNT/A *ad* was found to be reduced approximately 100,000-fold relative to *wt* BoNT/A.

Keywords

Clostridium botulinum; Botulinum neurotoxin A (BoNT/A); spinal cord cells; trafficking; atoxic; delivery vehicle

Introduction

Clostridium botulinum neurotoxins (BoNTs) are the most poisonous biological toxins known [1]. The murine LD_{50} of BoNT serotype A (BoNT/A), which typifies the major structural features among the seven known BoNT serotypes [2,3], and which is widely used in pharmaceutical formulations (e.g. BOTOX®, Dysport®), is approximately 0.5 ng per kg body weight. BoNTs target neuromuscular junctions, inhibiting neurotransmitter release and

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causing peripheral neuromuscular blockade, ultimately resulting in respiratory paralysis [4]. BoNT/A is synthesized as a single chain protein, Mr ~150,000, which is proteolytically processed by an endogenous clostridial protease generating a dichain molecule consisting of a light chain (LC, Mr ~50,000) and a heavy chain (HC, Mr ~100,000) linked by a disulfide bond [3]. The dichain BoNT/A contains three major functional domains. The LC comprises the catalytic (toxic) domain. The HC contains both the translocation domain within the N-terminal half (HC_N), which is responsible for delivering the LC to the cytosol, as well as the receptor-binding domain within the C-terminal half (HC_C) [3–5].

The toxicity of BoNT/A is a consequence of a multi-step mechanism culminating in a LC-mediated proteolytic event that disrupts the neuronal machinery for synaptic vesicle exocytosis. After specific binding to neurons *via* gangliosides and synaptic vesicle protein 2 (SV2) co-receptors [3–6], BoNT/A is endocytosed and a conformational change in the acidic endosomal compartment enables HC-mediated translocation of the LC into the neuronal cytoplasm [7,8]. This results in LC zinc-endopeptidase mediated catalytic cleavage of SNAP25, a SNARE protein required for synaptic vesicle exocytosis [9].

To create a molecular vehicle that can deliver therapeutic agents to the neuronal cytoplasm, we have previously developed a recombinant full-length, disulfide-bonded *a*toxic *d*erivative of BoNT/A, BoNT/A *ad* [10]. This derivative was rendered atoxic by a double-point mutation to the active site of the LC protease, but was specifically engineered to retain the characteristics required for neuronal targeting. In this report we present *in vivo* and *in vitro* data for BoNT/A *ad*, documenting its low toxicity relative to *wt* BoNT/A, and its retention of native BoNT/A trafficking properties.

Materials and Methods

Materials

Unless otherwise stated, molecular biology grade chemicals and reagents were obtained from Sigma-Aldrich Co. Tissue culture media, reagents, and supplements were from Invitrogen. Sprague-Dawley rat embryonic-day-18 spinal cords were either isolated from pregnant animals [11] or purchased from BrainBits, LLC and handled as described by the supplier. The *wt* BoNT/A was purified as previously described from *C. botulinum* strain Hall A-*hyper* [12]. The specific activity was determined *via* mouse bioassay [13,14] to be about 1.3×10^8 mouse LD_{50} Units/mg. The full-length BoNT/A *ad* (atoxic derivative) DNA and proteins were generated as described [10].

Mouse bioassay

Recombinant BoNT/A *ad* was tested for toxicity in both the single and dichain forms by mouse bioassay [13,14]. The single-chain BoNT/A *ad* was converted to the dichain form by treatment with enterokinase or TEV protease as described [10]. All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee.

Diaphragm nerve-muscle preparation

Diaphragm nerve-muscle preparations were dissected from 6 week old mice, stretched, and pinned to a Sylgard-coated tissue culture plate. Preparations were bathed in Krebs-Ringer resting (low potassium) bicarbonate buffer with glucose, aerated with 95% O_2 and 5% CO_2 to maintain pH ~7.2. For evaluation of recombinant protein uptake, 15 nM (~2.25 μg/mL) BoNT/A *ad* was added for 30 min at 25°C. After incubation, the preparations were washed with ice-cold Krebs Ringer resting bicarbonate buffer and three times with ice-cold PBS prior to further processing.

Whole-mount diaphragm staining

Pinned nerve-muscle diaphragm preparations were fixed in 1% formaldehyde/PBS for 4 hours at 4°C, followed by a brief rinse and 10-min triplicate washes with PBS. To block free aldehydes, tissue was incubated for 5 minutes in 100 mM glycine, pH 7.3, followed by three washes with PBS. Tissue was permeabilized and blocked against non-specific binding by incubation in PBS supplemented with 0.5% Triton X-100, 2% BSA, and 4% normal goat serum (PBT) for 2 h at room temperature. Overlying connective tissue was removed to allow adequate access of the antibodies into the muscle. The tissue was incubated overnight at 4°C with anti-LC monoclonal antibody F1-40 [15] (10 μg/mL) in PBT buffer. Antibody solution was forcefully pipetted onto the tissue, to provide good access of the antibody. After incubation, the tissue was rinsed with PBT and washed six times for 10 min with PBS at room temperature. Tissue was incubated in PBT with AlexaFluor 555-conjugated donkey-anti-mouse antibody (2 μg/mL, Invitrogen) and AlexaFluor 488-conjugated α-bungarotoxin (1 ng/mL, Invitrogen) overnight at 4°C. Tissue was then rinsed with PBT, washed three times for 1 h with PBT, once for 5 min with PBS, and fixed in 1% formaldehyde/PBS. Muscle fragments were cut near the pin insertion sites and mounted on a microscope slide under a coverslip using Vectashield hard set mounting medium (Vector Lab). Slides were kept at 4°C overnight, and examined by confocal microscopy using a NIKON LSM-510 microscope equipped with argon and HeNe lasers.

Accumulation of BoNT/A *ad* at the NMJ *in vivo*

Six week old female ICR mice (18 to 22 g, n=5) were injected *ip* with 1 μg of BoNT/A *ad* in 100 μL of 0.5% BSA/PBS. This amount constitutes approximately 1 mouse LD_{50}. The mice were observed for up to 12 hours after injection, at which time they were euthanized with CO_2. Diaphragm nerve-muscle preparations were prepared as described above.

Binding/ SNAP25 cleavage assay in primary spinal cord cells

Matured primary rat spinal cord (RSC) cells [11] were exposed to 0.0027 nM *wt* BoNT/A (20 pg, ~3 mouse LD_{50} Units) or various amounts of BoNT/A *ad* (0 – ~300 nM) in 50 μl of culture medium (Neurobasal with B27, Glutamax, and penicillin/streptomycin, Invitrogen). After incubation for 48 h, cells were harvested by lysis in 1 × LDS sample buffer, and analyzed by Western blot as described [11].

Competition assay

A 6-well plate with RSC cells (seeded at 1×10^6 cells/well) was cooled on ice for 10 min followed by one wash in cell stimulation medium (ice-cold culture medium supplemented with 56 mM KCl and 0.5 mM $CaCl_2$). The cells were then exposed to 0 – 500 nM BoNT/A *ad* in 1.25 mL of ice-cold cell stimulation medium for 10 min. Another 1.25 mL of the same ice-cold medium containing the same concentration of BoNT/A *ad* as well as 0.5 nM *wt* BoNT/A was added and incubation was continued on ice for an additional 20 min. After toxin removal, the cells were washed twice with ice-cold culture medium, fresh culture medium was added, and the plates were moved to a 37°C, 5% CO_2 incubator and incubated for 4 h prior to analysis as above.

Analysis of BoNT/A *ad* internalization into primary rat spinal cord cells

Primary rat spinal cords were purchased from BrainBits; rat spinal cord (RSC) cells were extracted according to manufacturer's recommendation, seeded into collagen-coated 6-well plates (BD Biosciences) at a density of 200,000 cells/well and cultured for 19 days prior to treatment and fractionation. The cells were either: 1) untreated; 2) treated with 30 nM BoNT/A *ad*; 3) treated with cross-linked/inactivated 30 nM BoNT/A *ad* (performed by incubating BoNT/A *ad* dichain in PBS at room temperature with 200 mM formaldehyde/50

mM sodium borohydride followed by dialysis [16]). Following 8h treatments, cells were washed twice with PBS and harvested with trypsin/EDTA (5 min, 37°C). Cell pellets were collected by centrifugation (4°C, 500 g, 3 min). Pellets were washed twice in PBS; washes were collected as a control lacking BoNT/A *ad*. The cytosolic fraction was extracted from the cell pellet with digitonin as described [17]. Crude cell extract (supernatant) was collected by centrifugation (500 g, 5 min) and cleared by centrifugation at 100,000 g for 10 min. The total protein concentration in all extracts was normalized after measurement with micro BCA protein assay kit (Pierce). Fractions were incubated with anti-SNAP25 (N19) goat polyclonal antibody (5 μl, Santa Cruz) 2 hours, 4°C. A mixture of protein A and Protein G magnetic beads (Invitrogen) was added, followed by 1 hour incubation at 4°C with gentle shaking. Beads were separated from the supernatant, briefly washed with PBS, resuspended in 1 × SDS sample buffer (Bio-Rad), and solution, containing antibodies and proteins released from the beads was separated on a 10–14% PAGE gel (Bio-Rad) and blotted as described [10]. SNAP25 and BoNT/A *ad* light chain were visualized using rabbit polyclonal antibodies against SNAP25 (Abcam) and mouse monoclonal antibody, F1-40, against BoNT/A LC [15], respectively.

Results

BoNT/A *ad* lacks metalloprotease activity in neuronal cells and competes with *wt* BoNT/A

To confirm absence of residual metalloprotease activity of BoNT/A *ad*, the toxin derivative was directly incubated with primary rat spinal cord (RSC) cultures at concentrations up to ~300 nM BoNT/A *ad* for 48 hours. Although this assay can detect femtomolar amounts of *wt* BoNT/A [11], no SNAP25 substrate cleavage was detected with BoNT/A *ad*, indicating no intracellular metalloprotease activity of BoNT/A *ad* (Fig, 1, **Panels A** and **B**). The ability of BoNT/A *ad* to specifically bind to neuronal BoNT/A surface receptors was further examined by a competition assay. The cells were stimulated by addition of KCl and $CaCl_2$ to the medium to expose the BoNT/A protein receptor SV2 on the neuronal cell surface. Pre-incubation of the RSC cells with 500 nM BoNT/A *ad* blocked intracellular SNAP25 cleavage induced by 0.5 nM *wt* BoNT/A in a dose dependent manner (Fig. 1, **Panel C**). These data demonstrate that BoNT/A *ad* specifically competes with *wt* toxin, probably by preventing its neuronal cell entry.

BoNT/A *ad* has very low *in vivo* toxicity in mice

Mouse toxicity of the non-catalytic BoNT/A *ad* was tested by intraperitoneal injection of doses ranging from 0.3125 to 10 μg per animal with 2× incremental increases. Mice were observed for up to 96 h for symptoms of botulism (Table 1). The estimated LD_{50} [18] was 1.12 μg BoNT/A *ad* dichain, or approximately 50 μg/kg, which is about 100,000-fold higher than the LD_{50} of *wt* BoNT/A. The LD_{50} for BoNT/A *ad* single chain (non-processed) was also determined and was 8.9 μg, which is ~10 fold higher, consistent with previous observations made for *wt* BoNT/A [5]. Based on these experiments, CDC excluded BoNT/A *ad* from the Select Agent list in 2009.

BoNT/A *ad* accumulates at the neuromuscular junction *in vitro* and *in vivo*

Next, the specificity of BoNT/A *ad* binding to presynaptic sites of the neuromuscular junction (NMJ) was analyzed *in vitro* by exposure of murine diaphragm preparations to BoNT/A *ad* followed by immunohistochemical analysis using a LC-specific monoclonal antibody F1-40 [15] to localize BoNT/A *ad* and a fluorescently labeled α-bungarotoxin (α-BTX) as a marker for post-synaptic sites. Fig. 2, **Panel A** demonstrates that BoNT/A *ad* binds specifically to NMJs *in vitro* and appears to colocalize with α-bungarotoxin. On Fig. 2, **Panel B**, with the specimen mounted with partial side view of the section (merged image), it is possible to see that red LC *ad* signal on the top of the panel (white arrows) is at

least partially separated from the green BTX-labeled AChR (post-synaptic marker) signal, a staining pattern which is consistent with a pre-synaptic clostridial toxin binding site as described [19].

BoNT/A *ad* accumulation at the NMJ was analyzed *in vivo* after *ip* injection into mice followed by immunohistochemical analysis of the diaphragm (Fig. 2, **Panel C**). LC *ad* accumulation at NMJs was observed in a similar pattern to the *in vitro* data, although the BoNT/A *ad* signal was weaker. The weaker signal may be due to the significantly lower total protein load to which the NMJ was exposed *in vivo*, and/or may indicate active ongoing internalization of the LC *ad* by motor neurons at the NMJ, as was described for internalization of a related clostridial toxin (tetanus toxin) derivative [19].

BoNT/A LC *ad* associates with SNAP25 in the neuronal cytosol

To determine if the residual *in vivo* toxicity of BoNT/A *ad* at high doses was related to the ability of LC *ad* to bind SNAP25 in the neuronal cell cytosol without mediating cleavage, co-immunoprecipitation analysis was performed after exposure of RSC culture to BoNT/A *ad*. As shown in Fig. 3, the ~52 kDa band corresponding to LC *ad* (**Panel B**, lane 2) was co-precipitated with an anti-SNAP25 antibody. Neither SNAP25 nor LC *ad* were detected in a control experiment using BHK fibroblasts treated with BoNT/A *ad* under the same conditions (not shown). This indicates that the LC *ad* enters the neuronal cell cytosol and associates with SNAP25.

Discussion

The modular structure [3] and trafficking mechanism of BoNT/A creates the potential for its utilization as a delivery vehicle for carrying therapeutic agents to the neuronal cytoplasm. Clostridial toxin-based approaches to targeted delivery are being evaluated in diverse therapeutic fields, including enzyme replacement therapy for lysosomal storage disorders [20], treatment of oxidative injury [21], modulation of apoptosis [22], inhibition of neurotransmitter secretion [23], and replacement/supplement of defective/absent SMN protein in spinal muscular atrophies [24].

Attempts to develop BoNTs as delivery vehicles have often relied on trafficking studies with chemically labeled fluorescent derivatives of *wt* BoNT. These studies are difficult to interpret because it is impossible to distinguish between LC and HC entities of the holotoxin after uptake. Moreover the heterogeneity produced by chemical labeling makes it difficult to confidently attribute the behavior of the fluorescent label as representative of native BoNT trafficking and biological activity (because the most intensely fluorescent-labeled molecules are likely to be the most intensely structurally perturbed). Consequently, the most intense fluorescent signal can result from molecules poorly related to native trafficking, while a small population of unmodified molecules could be responsible for residual biological activity [25].

The majority of studies exploring targeted delivery have focused on utilization of the receptor-targeting portion of clostridial toxins to provide specificity of neuronal uptake, and few studies have addressed the translocation step required for cytosolic delivery of the intended cargo [26]. To our knowledge, there are no reports of BoNT-based delivery vehicles able to mediate translocation of the LC, or cargo with therapeutic value from the endosomal compartment to the cytoplasm. By maintaining the integrity of BoNT structural domains required for native trafficking and translocation pathways, our constructs and expression systems for BoNT derivatives have been specifically designed to direct LC or LC-associated cargo delivery into the cytosol [10].

For *wt* BoNT/A, evidence of LC delivery to the neuronal cytosol is primarily based on demonstrating intracellular cleavage of its substrate, SNAP25. Internalization to the neuronal cytoplasm has not been directly visualized for the LC of *wt* BoNT/A by fluorescence or immunodetection due to technical difficulties. The available polyclonal antibodies do not discriminate between trafficking of HC and LC toxin subunits, and are less immunoreactive with LC than HC [27]. Although high specificity and sensitivity anti-LC monoclonal antibodies recently became available [15], none have thus far been reported to detect *wt* LC in the cytoplasm after neuronal uptake. The BoNT/A-binding domain of SV2 is exposed on the luminal side of synaptic vesicles and is only present at the cell surface when regulated exocytosis is active. Because *wt* BoNT/A disables the mechanism of it's own uptake, thereby preventing transport of SV2 to the cell surface, very few BoNT/A molecules are internalized per neuron [6]. In contrast, because BoNT/A *ad* does not cleave SNAP25, the mechanism for its continued uptake and internalization has not been disabled. BoNT/A *ad* should, therefore, have an increased capacity to accumulate in the neuronal cytosol; and indeed, we found detectable levels of LC *ad* complexed to SNAP25 in the cytosolic fraction of neuronal cultures (Fig. 3). Retaining the ability to accumulate to high levels in neuronal cytoplasm is an extremely important feature required for any BoNT-based delivery vehicle.

The co-immunoprecipitation of the BoNT/A *ad* LC, containing two amino acid mutations (E_{224}>A; Y_{366}>A) [10], with SNAP25 indicates that aside from the active core, there are multiple amino acids in the LC scaffold that form exosites interacting with SNAP25, similar to the interactions in co-crystallization complexes of a similar enzymatically inactive (E_{224}>Q; Y_{366}>F) LC of BoNT/A with SNAP25 described earlier [28]. The ability of the BoNT/A *ad* LC to accumulate in neurons and bind SNAP25 is likely related to the toxicity observed at very high doses in the mouse LD_{50} experiments.

The large size, multi-domain structure, critical disulfide bonding, and mechanical sensitivity of BoNTs make it challenging to express recombinant full-length BoNT proteins that retain native configuration and trafficking. Several laboratories have described the expression of full-length BoNTs, likewise rendered atoxic by multiple point mutations to the active core of the LC metalloprotease. Although these recombinant BoNT derivatives are immunogenic and provide protection against challenge with active toxin, very limited information has been provided on the physiological properties of these proteins [29–31]. If these BoNT derivatives retained the native trafficking properties of the *wt* toxin, they should likewise accumulate atoxic LC when tested in neuronal cultures. Yet the majority of these BoNT derivatives designed to be vaccines have been reported to be non-toxic at doses even higher than those we report here for BoNT/A *ad*. This may reflect either less efficient translocation of LC of these derivatives to the cytosol or failure to bind SNAP25.

The data presented here are consistent with BoNT/A *ad* being structurally and functionally similar to *wt* BoNT/A, but lacking the toxin metalloprotease function. This opens a new potential therapeutic window for BoNT/A *ad* utilization as a new pharmaceutical as well as a molecular vehicle for targeting agents to the neuronal cytoplasm.

Acknowledgments

We thank Peter Hallock from Dr. Steven Burden laboratory (Skirball Institute of Biomolecular Medicine at NYU School of Medicine) for technical assistance related to dissection and processing of mouse diaphragm. Support for these studies was provided by USDA CRIS Project 5325-42000-043-00D to LHS, as well as by the National Institute Of Allergy And Infectious Diseases Award Number R21AI082826 to EAJ and R21 AI072466 to EAJ and KI. Authors thank Dr. Eric Milner for reading and careful editing of the manuscript.

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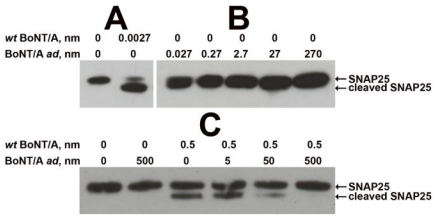

Figure 1. BoNT/A *ad* lacks intracellular metalloprotease activity and competes with *wt* BoNT/A for binding to neuronal receptors

Panels A and B: Binding/cleavage assay. *wt* BoNT/A (**Panel A**) or BoNT/A *ad* (**Panel B**) were incubated with primary rat spinal cord (RSC) cells at the indicated concentrations for 48 h, and cell lysates were analyzed for BoNT/A-specific SNAP25 cleavage by immunoblot.

Panel C: Competition assay. Primary rat spinal cord cells were pre-incubated with BoNT/A *ad* in stimulation medium at the indicated concentrations for 10 min., followed by incubation with *wt* BoNT/A for an additional 20 min. as indicated. After 4 h, cell lysates were analyzed for BoNT/A-specific SNAP25 cleavage by immunoblot.

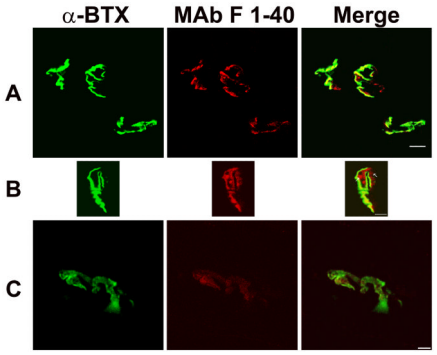

Figure 2. BoNT/A *ad* is targeted to presynaptic sites at neuromuscular junction *in vitro* (Panels A and B) and *in vivo* (Panel C)

Samples were incubated with BoNT/A *ad* as described in Methods and probed with a specific anti-BoNT/A LC antibody (red channel) and bungarotoxin (green channel) as a marker for post-synaptic sites. **Panels A – C:** Whole mouse diaphragm nerve-muscle preparations. **Panel A** (scale bar 10 μm) frontal view of the NMJ, and **Panel B** (scale bar 5 μm) mixed, frontal and side views. **Panel C** (scale bar 5 μm) shows the results of *in vivo* NMJ labeling after *ip* injection with BoNT/A *ad* as described in Methods. Image scanning was performed on Nikon LSM 510 confocal microscope equipped with argon and HeNe lasers, producing excitation lines of 488 and 568 nms.

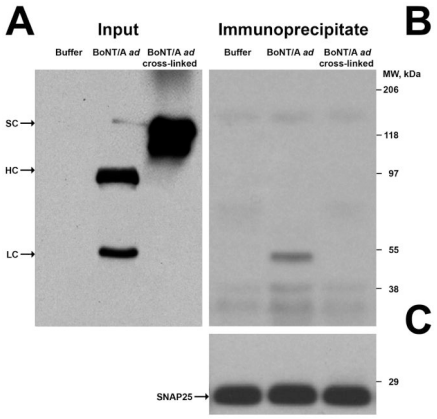

Figure 3. BoNT/A *ad* LC binds intracellular SNAP25 after exposure of primary rat spinal cord cells to BoNT/A *ad*

Rat spinal cord cells were exposed to buffer (lane 1), to 30 nM BoNT/A *ad* (lane 2), or to 30 nM crosslinked/inactivated BoNT/A *ad* (lane 3) as described in Methods. Cells were fractionated, and cytosolic extracts were incubated with anti-SNAP25 (N19) goat polyclonal antibodies, followed by pulling down immunoprecipitates with Protein A and Protein G magnetic beads as described in Methods. **Panel A:** Immunoblot of input proteins probed with anti-BoNT/A polyclonal antibody; **Panel B:** Immunoblot of immunoprecipitates probed with anti-BoNT/A LC monoclonal antibody, F1-40. **Panel C:** Immunoblot of immunoprecipitates probed with rabbit polyclonal antibodies against SNAP25, showing relative amounts of recovered SNAP25 from each sample. Arrows indicate BoNT/A *ad* single (SC), heavy (HC), or light (LC) chains and SNAP25. MW markers are shown on the right.

Table 1

[a] Mouse bioassay results.

µg/mice	BoNT/A ad dichain, Total mice:		BoNT/A ad single chain, Total mice:	
	# alive	# dead	# alive	# dead
0.3125	5	0	5	0
0.625	5	0	5	0
1.25	2	3	5	0
2.5	0	5	5	0
5	0	5	5	0
10	0	5	2	3

[a] All mice in a positive control group injected with 40 pg of *wt* BoNT/A died.