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Characterization of Botulinum Neurotoxin A Subtypes 1 Through 5 by Investigation of Activities in Mice, in Neuronal Cell Cultures, and *In Vitro*

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Botulinum neurotoxins (BoNTs) are synthesized by *Clostridium botulinum* and exist as seven immunologically distinct serotypes designated A through G. For most serotypes, several subtypes have now been described based on nominal differences in the amino acid sequences. BoNT/A1 is the most well-characterized subtype of the BoNT/A serotype, and many of its properties, including its potency, its prevalence as a food poison, and its utility as a pharmaceutical, have been thoroughly studied. In contrast, much remains unknown of the other BoNT/A subtypes. In this study, BoNT/A subtype 1 (BoNT/A1) to BoNT/A5 were characterized utilizing a mouse bioassay, an *in vitro* cleavage assay, and several neuronal cell-based assays. The data indicate that BoNT/A1 to -5 have distinct *in vitro* and *in vivo* toxicological properties and that, unlike those for BoNT/A1, the neuronal and mouse results for BoNT/A2 to -5 do not correlate with their enzymatic activity. These results indicate that BoNT/A1 to -5 have distinct characteristics, which are of importance for a greater understanding of botulism and for pharmaceutical applications.

Botulinum neurotoxins (BoNTs) are synthesized by *Clostridium botulinum* and by rare strains of *Clostridium baratii* and *Clostridium butyricum* (1, 2). BoNTs have traditionally been classified into seven immunologically distinct serotypes designated A through G (3). The amino acid sequences of BoNTs within one serotype can differ by from 0.9% to 36%, thus representing different subtypes/genetic variants of the same serotype (2). All BoNTs are synthesized as 150-kDa polypeptides and processed by posttranslational proteolytic cleavage to yield distinct heavy chains (HCs) and light chains (LCs) linked by a disulfide bond. The HC can be further functionally separated into the HC-carboxy terminus (H_C) and HC-amino terminus (H_N). The H_C is responsible for neuronal cell (NC) receptor recognition and binding leading to endocytosis, while the H_N facilitates translocation of the LC into the neuronal cell cytosol (reviewed in reference 4). The LC confers the enzymatic activity of the toxin acting as a zinc-dependent endopeptidase, which specifically cleaves intracellular soluble N-ethylmaleimide-sensitive factor (NSF) attachment protein receptors (SNAREs) on the presynaptic vesicle and neuronal membrane, thus inhibiting neurotransmitter release (reviewed in reference 4). Each BoNT serotype acts at distinct SNARE targets and sites (5), with BoNT/A cleaving SNAP-25 between amino acids Gln^{197} and Arg^{198} (6), which is also presumed to be true for all known BoNT/A subtypes. The fully functional HC and LC regions are necessary for entry, trafficking, and intoxication of cells.

BoNT is recognized by the Centers for Disease Control (CDC) as a category A tier 1 select agent because of its potency and potential to be misused as a bioterrorism weapon (7). Currently, the Food and Drug Administration (FDA) has approved two serotypes, A1 (abo-, ona-, and incobotulinumtoxin A) and B1 (rimaotulinumtoxin B), for treatment of various indications in humans. BoNT/A1 is the predominant serotype of botulinum neurotoxin used for pharmaceutical applications (8, 9) and is the best-studied BoNT. Its extraordinary potency (10), its long duration of action, and its localized activity in the inhibition of neuromuscular activity upon intramuscular injection have contributed to the use of BoNT/A as a valuable pharmaceutical for treatment of a variety of clinical as well as aesthetic applications (reviewed in reference 11). Since the first therapeutic use of BoNT/A1 for the treatment of strabismus in 1977 (12), the understanding of these toxins has significantly increased as more clinical and aesthetic treatments have been approved and basic research has progressed. However, much remains unknown of BoNT/A, particularly of BoNT/A subtypes other than A1.

Seven subtypes of BoNT/A termed A1 to A7 based on sequencing have been previously described (13–18). Having been described only recently, little is known about the A6 and A7 subtypes, and they have yet to be purified as 150-kDa holotoxins. Amino acid similarity between BoNT/A subtypes ranges from 98% (A1 versus A5) to 84% (A1 versus A3) (19). It remains unclear how these amino acid differences affect the subtypes' biologic activity, and only a few differences in characteristics have been elucidated. BoNT/A2 has been shown to enter cells faster than BoNT/A1 (20), to more potently inhibit the grip strength in rats after local administration, and to be more potent in the hemidiaphragm assay (21, 22). In addition, grip strength analysis after local injection in rats indicated that BoNT/A2 diffusion to the contralateral leg was reduced nearly 3-fold compared to A1 diffusion and no axonal transport was observed (21). For BoNT/A3, intoxication symptoms significantly different from those seen

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with A1 were observed when it was injected intravenously (i.v.) in mice at high concentrations (10^3 U) and manifested first in paralysis of the front legs, hind legs, and whole body followed by death (23). In contrast, the typical symptoms of BoNT/A1 intoxication include ruffled fur, labored breathing, descending flaccid paralysis, and spasticity just prior to death (24). Similar experiments investigating symptoms of intoxication have not been conducted for other subtypes. Additionally, various investigators have shown differences in the ability of monoclonal and polyclonal antibodies to bind to or neutralize various subtypes within the same serotype (23, 25–27).

The central goal of this study was to further characterize purified BoNT/A1 to -5 150-kDa neurotoxins and to better understand the unique properties of each subtype *in vivo*, in neuronal cell models, and *in vitro*. The results indicate that, unlike those of BoNT/A1, the activities of BoNT/A2 to -5 subtypes in the *in vivo* mouse model do not consistently correlate with their enzymatic activity and their behavior in neuronal cells of different species and origin. Additionally, the findings reported herein suggest means to improve clinical treatments with BoNT-based pharmaceuticals, including reduced immunogenicity and potentially different modes of action.

MATERIALS AND METHODS

All the work described in this article was approved by the University of Wisconsin—Madison Institutional Biosafety Committee. All animal experiments were approved by and conducted according to guidelines of the University of Wisconsin Animal Care and Use Committee.

Neuronal cells. Primary rat spinal cord (RSC) cells and human induced pluripotent stem cell (hiPSC)-derived neurons (iCell Neurons; Cellular Dynamics International, Madison, WI) were prepared and maintained in serum-free medium as previously described (28–30). Primary mouse spinal cord (MSC) cells were prepared essentially as previously described (31). Briefly, pregnant C57BL/6NHsd mice were purchased from Harlan (Harlan Laboratories, Madison, WI), and spinal cords were removed from fetal mice at gestation day 14, dissociated with TrypLE (Invitrogen), and plated on 0.01% poly-L-ornithine (Sigma)- and 8.3 μg/cm^2 matrigel (BD Biosciences)-coated 96-well Techno Plastic Products (TPP) plates at a density of 50,000 cells/well. The cells were allowed to mature for at least 18 days before use. The primary cells were maintained in serum-free culture medium (Neurobasal medium supplemented with 2% B27, 2 mM GlutaMAX, and 100 units/ml penicillin and streptomycin [all from Invitrogen]). The hiPSC-derived neurons were maintained in the medium supplied by Cellular Dynamics. HIP Neurons and accompanying media were supplied from Global Stem (Rockville, MD) and grown according to the manufacturer's instructions with the exception that they were plated onto poly-L-ornithine (PLO)-laminin-coated plates before differentiation, and differentiation medium was supplemented with 2 μM all-trans-retinoic acid (Stemgent, Cambridge, MA) and 5 μM purmorphamine (CalBiochem). After 5 days of differentiation, the cells were kept in the same plate and differentiation media were replaced with maintenance media (supplied by GlobalStem). The neurons were matured for 18 days prior to use in the toxin assays. All cell types were incubated at 37°C in a humidified 5% CO_2 atmosphere.

Neuronal cell receptor analysis. Protein expression of SV2 isoforms was analyzed in the MSC cells and the HIP Neurons by the following method. Cells were harvested in M-PER protein extraction reagent (Thermo Scientific) supplemented with Halt protease inhibitor cocktail (Thermo Scientific)–1% EDTA–1% Triton X-100 and centrifuged at ~14,000 × *g* for 10 min to pellet the cell lysates. Lithium dodecyl sulfate (LDS) sample buffer (Invitrogen) was added to the supernatant to 1×, and samples were heated at 70°C for 10 min. Western blots were probed using primary antibodies against SV2-A (mouse), SV2-B (mouse), and SV2-C (rabbit) isoforms (Synaptic Systems, Göttingen, Germany) and secondary alkaline phosphatase (AP)-conjugated anti-rabbit and anti-mouse antibody (KPL, Rockville, MD). These antibodies recognize human, rat, and mouse proteins with no cross-reactivity among isoforms. Proteins were detected using chemiluminescent CDP-Star reagent (Invitrogen) and X-ray film (MidSci, St. Louis, MO) as previously described (29).

Botulinum neurotoxin. The 150-kDa holotoxins of BoNT/A1, -2, -3, and -5 were purified from *C. botulinum* strains Hall A *hyper*, Kyoto-F, CDC A3, and A661222 as previously described (23, 32–34). The purified toxins were stored in 40% glycerol–phosphate-buffered saline at −20°C until use. Purity was assessed by separating 1 μg of nonreduced and reduced toxins (100 mM dithiothreitol [DTT]) on a 4 to 12% NuPAGE Bis-Tris gradient gel (Invitrogen) and visualizing the proteins by Coomassie blue staining.

BoNT/A4 was recombinantly expressed in a nontoxigenic strain of Hall A *hyper* constructed as previously described (35) and using the nucleotide sequence from strain 657Ba (15) with a pMTL80000 vector system (36).

Activities of the five subtype preparations were determined using a mouse bioassay (MBA) as described previously (24, 37) and periodically confirmed throughout the study. Briefly, groups of four female ICR mice (Harlan) (18 to 20 g body weight) were injected intraperitoneally (i.p.) with 500 μl of gelatin phosphate buffer (30 mM sodium phosphate [pH 6.3] and 0.2% gelatin) containing serial dilutions of the BoNT/A subtypes (32). Mice were observed for 4 days from the time of injection for botulism symptoms and death. Surviving and dead mice were counted and specific activities calculated using the method of Reed and Muench (38). Specific activities in mouse (50% lethal doses [LD_{50}] are indicated in U/mg) were 1.25×10^8 (A1), 1.27×10^8 (A2), 5.8×10^7 (A3), 1.01×10^5 and 1.25×10^5 (A4), and 1.4×10^8 (A5).

Intravenous assays of BoNT/A1, -2, and -5 in mice. Female ICR mice (18 to 20 g; Harlan) were injected intravenously with 10^3 U of BoNT/A1, -2, or -5 in 100 μl of gelatin phosphate buffer (32), as has previously been described for BoNT/A3 (23). Three mice per subtype were injected and injections repeated as necessary. Mice were observed for symptoms of botulism from the time of injection until death.

BoTest. A BoTest botulinum neurotoxin detection kit (BioSentinel Pharmaceuticals, Madison, WI) was used following the manufacturer's instructions. Ten-fold serial dilutions of the BoNT/A subtypes were prepared in triplicate, and a negative control without BoNT added was included. All toxin samples were reduced prior to sensor addition by adding 100 mM DTT into the reaction buffer. The 96-well plate (Nunc; black, flat bottom) was incubated for up to 18 h at 30°C. Fluorescence emissions were read on a BioTek Synergy H1 Hybrid reader. The absorbance ratio of 470 nm/526 nm was determined, and the EC_{50} (the concentration that produces 50% of the maximal possible effect) was estimated by nonlinear regression using GraphPad PRISM 6 (GraphPad Software Inc.). At least three independent assays using triplicate samples per subtype were performed during the study, and the averages and standard deviations of the EC_{50}s of 3 to 5 independent assays were determined for each subtype.

NCB BoNT assays. For all neuronal cell-based (NCB) assays, the neuronal cells were exposed to the indicated concentrations of BoNT/A subtypes in 50 μl of the respective culture medium for 48 h. All samples were tested in a minimum of triplicate experiments, and a negative control without BoNT was included. After the specified exposure time, the extracellular solution containing BoNT was removed and cells were lysed in 1× lithium dodecyl sulfate (LDS) sample buffer (Invitrogen) and analyzed by Western immunoblotting for SNAP-25 cleavage as previously described (28–30). Cleaved and uncleaved SNAP-25 bands were quantified by densitometry using a Foto/Analyst FX system and TotalLab Quant software (Fotodyne). Data plots and EC_{50}s (four parameters—variable slope) were generated using PRISM 6 software.

Statistical analyses. Statistics were performed in PRISM 6 using one-way analysis of variance (ANOVA) and in Excel using Student's *t* test as

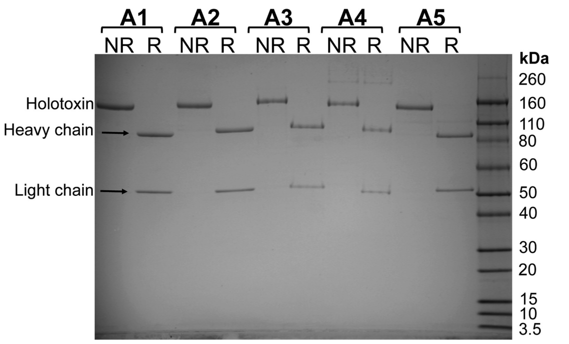

FIG 1 Purified 150-kDa BoNT/A1 to -5. BoNT/A1 to -5 were isolated from *C. botulinum*, and purity was assessed by SDS-PAGE gel analysis and Coomassie staining. The 150-kDa protein toxins were either not reduced (N) or reduced (R) with dithiothreitol to the 100-kDa heavy chain and 50-kDa light chain.

indicated. Differences were considered statistically significant with *P* values less than 0.05.

RESULTS

The *in vivo* intoxication symptoms for BoNT/A5 resemble those for BoNT/A1, while the BoNT/A2 symptoms closely resemble those for BoNT/A3. The 150-kDa BoNT/A1 to -5 subtypes were isolated from *Clostridium botulinum*, and purity was confirmed by SDS-PAGE gel analysis. Reduction of the 150-kDa BoNTs with DTT resulted in distinct 100-kDa and 50-kDa bands, and no bands corresponding to complex or other proteins were observed (Fig. 1).

A previous report from our laboratory showed that mice treated intravenously (i.v.) with BoNT/A3 at 10^{3-4} U/ml exhibited botulism intoxication symptoms that were uniquely different from those of mice injected with A1 at equal concentrations (23). To determine if the other BoNT/A subtypes provoked unique intoxication symptoms, mice were injected i.v. with 10^4 U/ml of A2 or A5 and observed until the time of death. BoNT/A5-treated mice showed ruffled fur, labored breathing, descending flaccid paralysis, and spasticity just prior to death that were analogous to the symptoms seen with BoNT/A1-treated mice (data not shown). Conversely, mice injected with BoNT/A2 exhibited botulism symptoms similar to those seen with BoNT/A3, presenting with paralysis of the front legs, hind legs, and the whole body followed by death (data not shown). Additionally, BoNT/A2-injected mice died about 20 min faster (at around 60 min postinjection) than A1- or A5-treated mice (~80 to 90 min postinjection). Taken together, these *in vivo* mouse data indicate that there are distinct somatic responses to intoxication of BoNT/A subtypes at high concentrations.

BoNT/A4 has 1,000-fold lower activity in mice than BoNT/A1. The specific toxicities of BoNT/A2, -3, and -5 in mice have previously been reported and differ only marginally from that observed for BoNT/A1 (23, 32–34). In order to determine the specific activity of BoNT/A4, mice were injected intraperitoneally (i.p.) with serial dilutions of BoNT/A4 (24, 37). The specific-activity determination of two independent purifications of 150-kDa holotoxin preparations resulted in similar activities (1.01×10^5 and 1.25×10^5 U/mg), both about 1,000-fold less than that typically observed for BoNT/A1. This indicates that BoNT/A4 has ~1,000-fold-lower specific activity than A1 (1.25×10^8 U/mg) in mice.

The BoNT/A subtypes have distinct *in vitro* enzymatic activities. Differences in the enzymatic activity of BoNT/A1 to -4 have previously been reported for recombinantly expressed LCs (39). However, in those earlier studies, truncated (amino acids [aa] 1 to 425), recombinantly produced LCs in *Escherichia coli* were used, and A5 had not yet been identified. To determine whether there are differences in the *in vitro* SNAP-25 cleavage activity of the native, reduced BoNT/A1 and -5 holotoxins, the enzymatic activities were assayed using the BoTest, which uses a truncated SNAP-25 reporter construct of amino acids 141 to 206 flanked by cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP), thus forming donor-acceptor Förster fluorescence resonance energy transfer (FRET) upon cleavage of the reporter construct (40). Changes in fluorescence emissions reflect real-time SNAP-25 cleavage BoNT/A events (40). The FRET values of all five subtypes were determined at 30 min, 1 h, 2 h, 15 h, 18 h, and 24 h. The activity of all subtypes increased markedly over the first 2 h, while after 15 h the reactions were complete for all subtypes. The 2-h and 18-h data points are shown (Fig. 2A), and average EC_{50}s (concentrations that produce 50% of the maximal possible effect) from at least three independent assays are presented in Table 1. We observed some variability in this assay in repetitive

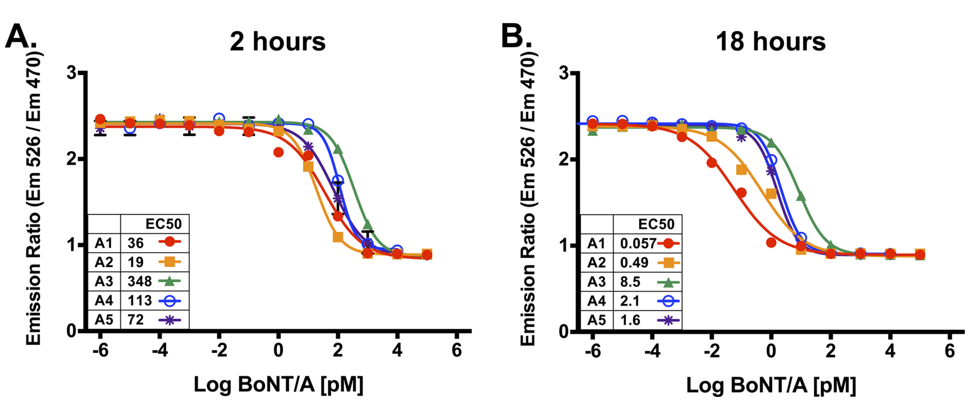

FIG 2 Comparison of *in vitro* SNAP-25 cleavage activities of BoNT/A subtypes using the endopeptidase BoTest (BioSentinel Pharmaceuticals). Changes in fluorescence were measured after 2 h (A) and 18 h (B). The emission ratio was plotted as a function of toxin concentration where the emission ratio is the value in relative fluorescence units (RFU) of the FRET peak (526 nm) divided by the RFU value of the donor/CFP peak (470 nm). Results from one representative BoTest experiment are shown.

TABLE 1 BoTest data at 2 and 18 h

	EC_{50} [pM ± SD]	
BoNT	2 h	18 h
A1	19.4 ± 13.6	0.063 ± 0.018
A2	44.9 ± 21.5	0.32 ± 0.15[a]
A3	235 ± 105[a]	5.2 ± 2.86[a]
A4	129 ± 14.8[a]	1.18 ± 0.79[a]
A5	68.9 ± 5.04[a]	1.34 ± 0.76[a]

[a] EC_{50} value significantly statistically different from that determined for BoNT/A1 (P = 0.05).

experiments for unknown reasons, and the *in vitro* assays were performed at least 3 times with different reagent kits to obtain a compilation for summarizing the data.

Within the first 2 h, there were no apparent differences in the enzymatic activities of A1 and A2 and only minor differences between A1 and A5, with EC_{50}s of 19.4 ± 13.6 pM for A1, 44 ± 21.5 pM for A2, and 68.9 ± 5.14 pM for A5 (Table 1). The curves for A3 and A4 lagged behind those of the other subtypes at as early as 30 min, and the lag became more pronounced over time (with ~10 times and ~7 times greater EC_{50}s at 2 h (A3, 235 ± 105 pM; A4, 129 ± 14.8 pM; A1, 19.4 ± 13.6 pM) and were approximately ~90 and ~20 times greater at 18 h (A3, EC_{50} = 5.2 ± 2.86 pM; A4, 1.2 ± 0.76 pM; A1, 0.06 ± 0.018 pM) (Table 1). This indicates that A3 and A4 are significantly less active *in vitro* in this system than the other BoNT/A subtypes (Fig. 2B). The curves for A2 and A5 consistently diverged at 18 h, resulting in EC_{50}s significantly different from those of A1 (A1, 0.06 ± 0.018 pM; A2, 0.32 ± 0.15; A5, 1.34 ± 0.76 (Fig. 2B). This indicates that A2 and A5 have ~5- and ~2-times-lower activity than A1 at 18 h (Table 1), which could be due to lower catalytic activity, lower affinity for the substrate, or other factors such as lower stability of the reduced toxins. Although BoNT/A4 is 1,000-fold less active in mice than A1 (1.25×10^5 U/mg versus 1.25×10^8 U/mg), in the *in vitro* assay the EC_{50}s were decreased only slightly at equal molar concentrations at 2 h (~7-fold) and ~20-fold at 18 h. Taken together, these data suggest that in the *in vitro* BoTest, A1 and A2 had greater enzymatic activity than BoNT/A3, -4, and BoNT/A5 and that BoNT/A3 had the lowest enzymatic *in vitro* activity.

BoNT/A subtypes have distinct activities in different neuronal cell models. BoNT/A1 to -5 activity was determined in various types of neuronal cells to determine species-specific and neuronal class differences. Primary mouse spinal cord (MSC) cells (31, 41), primary rat spinal cord (RSC) cells, and human induced pluripotent stem cell (hiPSC)-derived neurons from two different sources (iCell Neurons and HIP neural stem cell-derived neurons) were treated with serial dilutions of the five BoNT/A subtypes for 48 h. The iCell Neurons comprise a mixture of primarily glutamatergic neurons and GABAergic neurons similar to that seen with forebrain cells, and the differentiated HIP neural stem cells primarily comprise a mixture of cholinergic, GABAergic, and glutamatergic neurons and were directed to enrich the motor neuron population by addition of retinoic acid and purmorphamine. EC_{50}s for BoNT/A1 in RSC cells and iCell Neurons have previously been documented to be between 0.2 and 0.3 U or around 0.25 pM (28–30).

The EC_{50}s for A1 were about 0.14 pM in mouse spinal cord (MSC) cells and about 0.25 pM in RSC cells and iCell Neurons ($P > 0.05$) (Fig. 3A, B, and C and Table 2). The HIP Neurons were less sensitive to BoNT/A1, with an EC_{50} of about ~2.2 pM ($P < 0.0001$). In MSC cells, there were only minor differences of from 0.14 to 0.46 pM ($P > 0.05$) in the EC_{50}s for A1, A3, and A5 and an about 1,000-fold decrease in the activity of A4 that was similar to the i.p. BoNT activity in mice. In contrast, the EC_{50}s of A2 in all cell models, including mouse cells, were consistently ~10-fold lower than those of A1 (~0.015 to 0.29 pM; $P < 0.01$), indicating increased activity in neuronal cells compared to the other subtypes and to the mouse assay (Fig. 3 and Table 2). BoNT/A3 and -5 had EC_{50}s in rat spinal cord cells that were ~5-fold-greater than those

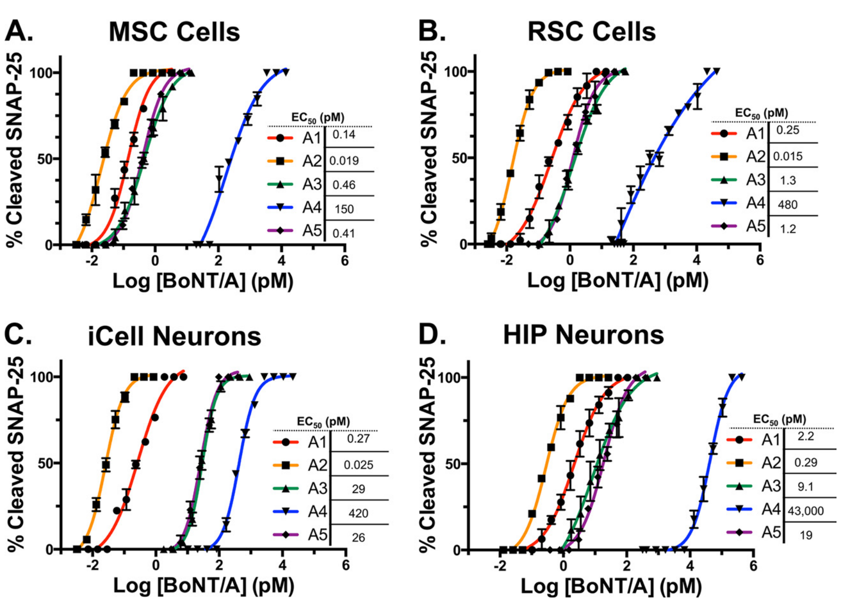

FIG 3 Sensitivity comparison of primary mouse spinal cord (MSC) cells (A), rat spinal cord (RSC) cells (B), iCell Neurons (C), and HIP Neurons (D) after exposure to BoNT/A subtypes for 48 h. Cell types were exposed to the indicated concentrations of BoNT/A subtypes, and cell lysates were analyzed for SNAP-25 cleavage by Western blotting. Data from three Western blots were quantified by densitometry, and data plots and EC_{50}s were generated.

TABLE 2 Summary of EC_{50}s of BoNT/A subtypes in MSC cells, RSC cells, iCell Neurons, and HIP Neurons

	EC_{50} [pM][a]			
BoNT (concn)	MSC cells	RSC cells	iCell Neurons	HIP Neurons
A1 (1.25×10^8 U/mg)	0.14	0.25	0.27	2.2
A2 (1.27×10^8 U/mg)	0.019**	0.015****	0.025****	0.29****
A3 (5.8×10^7 U/mg)	0.46	1.3***	29****	9.1****
A4 (1.25×10^5 U/mg)	150****	480****	420****	43,000****
A5 (1.37×10^8 U/mg)	0.41	1.2***	26****	19****

[a] For statistical analyses, * represents $P < 0.05$, ** represents $P < 0.01$, *** represents $P < 0.001$, and **** represents $P < 0.0001$.

of A1 (EC_{50} = ~1.3 and 1.2 pM versus 0.25 pM; $P < 0.001$) and showed marked changes in EC_{50}s in the human neuron models. While BoNT/A3 was ~107-fold less active than BoNT/A1 in iCell Neurons (EC_{50} = 29 pM versus 0.27 pM; $P < 0.0001$), it was only about 4-fold less active in the HIP Neurons (EC_{50} = 9.1 and 2.2 pM; $P < 0.001$) (Fig. 3D). Similarly, BoNT/A5 was about 100-fold less active than BoNT/A1 in iCell Neurons (EC_{50} = 26 versus 0.27 pM; $P < 0.0001$) and about 9-fold less active in the HIP Neurons (EC_{50} = ~19 and 2.2 pM; $P < 0.0001$). Strikingly, while BoNT/A4 had relatively similar levels of activity in MSC cells, RSC cells, and iCell Neurons (EC_{50} = ~150 to 480 pM), there was a significant difference in activity levels in the two human cell models. In the motor neuron-enriched population of HIP Neurons the activity was ~20,000-fold lower than that of A1 (EC_{50}s = ~43,000 and 2.2 pM; $P < 0.0001$), and in the forebrain-like iCell Neurons it was only about 1,500-fold lower than that of A1 (EC_{50} = ~420 pM versus 0.27 pM; $P < 0.0001$). Note that the specific activity in mice was 2-fold lower for A3 and ~1,000-fold lower for A4 than for the other subtypes and that, unlike an *in vitro* enzymatic assay, a cell-based assay requires fully functional toxin to enable specific cell binding and translocation into the cell's cytosol as well as enzymatic cleavage of the SNARE protein. A correlation of the activity in different cell models with activity in mice is shown in Table S1 in the supplemental material.

To ensure that the differences in the EC_{50}s of A3 and A5 in human neurons were not due to a delayed cell entry or slowed SNAP-25 cleavage, hiPSC-derived neurons were exposed to the BoNTs for 72 h. This exposure resulted in the same EC_{50}s as a 48-h exposure (data not shown), indicating that SNAP-25 cleavage by BoNT/A3 and -5 was complete after 48 h. Additionally, to determine if the differences in EC_{50}s for MSC cells and HIP Neurons were a result of differences in subtypes interacting with cell surface receptors, the expression of receptor SV2 isoforms (42) was assayed via Western immunoblot analysis. Expression of SV2-C was detected at about equal levels in MSC cells and HIP Neurons. A strong SV2-A band was detected in HIP Neurons, but only a very weak band was observed in the MSC cells (Fig. 4).

For all the cell types tested, 100% cleavage of SNAP-25 was reached with all subtypes. Consistently across the subtypes, the iCell neuronal assay resulted in the steepest dose-response curves. The mouse and rat primary spinal cord models and the HIP Neuron assays resulted in sigmoidal curves with a flatter slope.

In summary, these data indicate that, compared to BoNT/A1, BoNT/A3 and -5 are significantly less active in the human iPSC-derived forebrain neurons (iCell Neurons) and slightly less active in human iPSC-derived motor-neuron enriched populations (HIP Neurons). While the decrease in A4 activity compared to A1 activity in iCell Neurons correlates to the decreased activity in the *in vivo* mouse model, it was further decreased in the HIP neuronal model. Activity in rodent primary spinal cord cells was decreased only slightly for BoNT/A3 and -5 compared to BoNT/A1. BoNT/A2, on the other hand, appears to have been at least 10-fold more active than BoNT/A1 in all cells tested. All EC_{50}s and specific activities are summarized in Table 2 and in Table S1 in the supplemental material.

DISCUSSION

The focus of this study was to elucidate the differences in toxicities among BoNT/A1 to -5 using *in vivo* assays and an *in vitro* assay and several neuronal cell models. Our results revealed substantial differences among the BoNT/A subtypes in various experimental systems, including *in vitro*, *in vivo*, and neuronal cell-based assays.

The results of the *in vitro* BoTest endopeptidase assay revealed that within smaller time frames (≤2 h), BoNT/A subtypes have similar EC_{50}s, with the exception of A3 and A4, which were ~10- and 7-fold less active, respectively. This decrease in activity was even more pronounced with longer assay times (Table 1 and Fig. 2). After longer incubations (≥15 h), the EC_{50}s of A2 and A5 also increased, indicating significant differences (~ 5- and 20-fold) in enzymatic activity compared to A1 (Table 1). This could be due to lower catalytic activity or reduced SNAP-25 binding affinity, and other factors such as lower stability of the reduced toxins in the reaction buffer cannot be excluded. Further enzymology and mutational studies are necessary to understand the differences in the observed activities. An amino acid alignment of the five subtypes reveals that the 8 aa residues (His223, Glu224, His227, Glu261, Glu262, Glu351, Arg363, and Try366) known to be required for catalytic activity in A1 are 100% conserved across all five subtypes analyzed here (43) (Fig. 5), suggesting that the observed differences are due to other factors such as structural modifications, decreased stability, or differences in substrate affinity. Of the 10 aa residues (Met106, Ser110, Leu322, Lys337, Lys340, Met344, Ile348, Asn353, Lys356, and Phe357) that comprise a hydrophobic patch believed to be necessary for recognition of and binding to SNAP-25 in BoNT/A1 (19), A3 is the only subtype with differences (19, 44), which suggests that the significantly lower activity observed with BoNT/A3 in this assay may be due to lower substrate binding. Previous data using recombinant truncated LCs, however, reported no differences in the K_m and k_{cat} values of A1 and A3 LCs in enzyme kinetics assays using a similar substrate (GST-SNAP-25; aa 141 to 206) (39). This aa-141-to-206 SNAP-25 fragment is conserved among rat, mouse, and human and contains all SNAP-25 domains that have been shown to be involved in BoNT/A1 LC binding and catalytic activity (reviewed in reference 4).

Activity of the BoNT/A subtypes was further studied in neuronal cells. Four different cell models from different species were

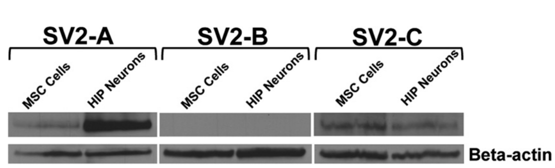

FIG 4 Expression of SV2 isoforms A, B, and C in mouse spinal cord (MSC) cells and HIP Neurons.

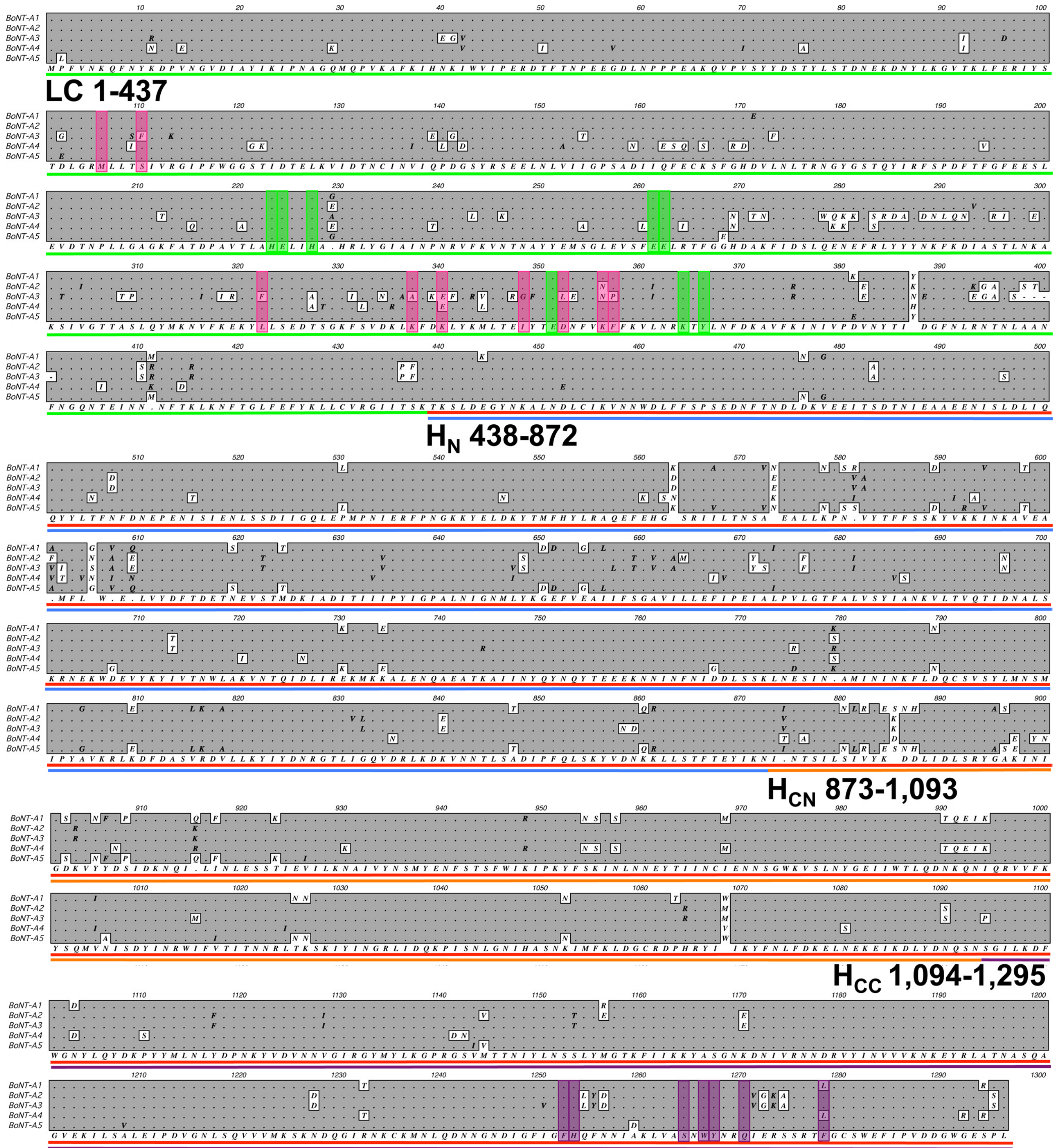

FIG 5 Sequence alignment of BoNT/A subtypes 1 to 5. The alignment indicates where amino acids differ within (bolded letters) and between (boxed letters) classes, the light chain is underlined in green, the heavy chain is underlined in red, the HC-amine end is underlined in orange, and the HC-carboxyl end is underlined in purple. Residues boxed in pink are involved in substrate binding. Residues boxed in green are necessary for LC catalytic activity. Residues highlighted in purple are involved in ganglioside binding and recognition.

employed. The MSC cells, which have been used previously by our group and other groups as a BoNT model (31), were chosen to directly compare the activity of BoNT/A subtypes in a cell model with activity in the mouse bioassay, as the neurons are derived from the same species. The RSC cells have previously been established as a sensitive and reproducible cell model for BoNT detection (28, 30) and represent another rodent cell model of the same origin as MSC cells (spinal cord) but from a different rodent species. Furthermore, the hiPSC-derived neurons are human neuronal cell models, one of which (the iCell Neuron model) has recently been developed as a human-specific and sensitive model (29) and contains primarily glutamatergic and GABAergic neurons most representative of human forebrain cells (29). The second iPS model used (HIP Neurons) is differentiated under conditions that enrich cholinergic neurons.

BoNT/A1 had approximately equal EC_{50}s ($\sim$0.14 to 0.27 pM) when exposed to the spinal cord cells and human iCell Neurons (Fig. 3A, B, and C), which is consistent with previous reports (29). Alternatively, BoNT/A2 had EC_{50}s 10-fold lower than those of A1 in all cell types. These results indicate A2 has comparatively greater activity in neuronal cell models than in the *in vivo* mouse bioassay. However, we indicated in a previous report an EC_{50} of $\sim$0.1 U for A2 in RSC cells, which was only about 2-fold lower than that for A1 (20). While we presently cannot explain this difference, it is interesting that the specific activity in mice of the previous A2 stock was over 3-fold lower than that tested here (4.3×10^8 U/mg versus 1.27×10^8 U/mg) and that the molar activities of A2 in cells in the two studies were therefore similar. Further research is required to determine if the MBA and NCB assays can correlate for BoNT/A2 as they do for BoNT/A1 and to determine which assay would more accurately depict the activity after local injection in humans.

BoNT/A4 had about 1,000-to 2,000-fold-reduced activity compared to A1 in the mouse and rat spinal cord cell models and in the human iCell Neurons similar to the 1,000-fold reduction in activity in the *in vivo* mouse model. When tested in the HIP Neurons, however, BoNT/A4 was about 20,000 times less active than A1, indicating that the HIP Neurons used here are less susceptible to BoNT/A4. Interestingly, BoNT/A4 was $\sim$1,000-fold less active in mice than A1 and had relatively similar EC_{50}s in the different cell types using mouse LD_{50} units as a reference, except in the HIP Neurons (see Table S1 in the supplemental material). These results suggest that the activity of BoNT/A4 in neuronal cell models correlates with that in mice except for HIP Neurons, which are less sensitive for an unknown reason. On the other hand, the *in vitro* enzymatic activity was reduced only about 7-fold (2 h) and 19-fold (18 h) compared to that of BoNT/A1 (Table 1). Thus, BoNT/A4 has only slightly reduced catalytic activity compared to A1 but is at least 1,000-fold less active in mice and in cells. This suggests that BoNT/A4 is significantly less efficient at entering cells than A1. Since the amino acid residues thought to be required for interaction with cell surface gangliosides are 100% conserved among all BoNT/A subtypes (19), A4 may interact with cell surface receptors differently than A1.

Interestingly, both BoNT/A3 and -5 had significantly decreased activity in the human neurons compared to the other subtypes, with a much greater decrease in the forebrain-representative iCell Neurons (29 pM and 26 pM compared to 0.27 pM for A1) than in the motor-neuron enriched HIP Neurons (9.1 pM and 19 pM versus 2.2 pM for A1) (Table 2). The EC_{50}s of BoNT/A3 and -5 in both the RSC and MSC cells, on the other hand, were relatively similar to that of BoNT/A1. This indicates potential species-specific differences in the activity of A3 and A5 and potential neuronal subpopulation differences in human cells. SNAP-25 is 100% conserved between rat and human, while mouse SNAP-25 is 95.6% similar. The nine amino acid differences are confined to a 31-amino-acid region between residues 58 and 89. These differences are upstream of the α- and β-exosite binding sites and the A-specific cleavage site known to be essential for toxin activity (19). Thus, the seemingly species-specific differences in A3 and A5 activity are probably not due to differential SNAP-25 cleavage or exosite binding.

Another possible explanation for the observed differences may be the differential binding to the neuronal cell surface receptors. Previous Western blot analyses detected expression of all SV2 isoforms in RSC cells but expression of only SV2-A in iCell Neurons. Quantitative PCR of iCell Neurons confirmed the SV2-A is the predominantly expressed isoform, with 100-fold and 1,000-fold less SV2-B and SV2-C, respectively (29). Our data indicate that SV2-C expression is detectable by Western blotting in MSC cells and HIP Neurons at nearly equivalent levels but that SV2-A is detected at greater levels in HIP Neurons than in MSC cells (Fig. 4). These data indicate the possibility that differences in the expression of the SV2 isoforms in different cell models may impact the activity of the BoNT/A subtypes.

While it has previously been reported and is widely accepted that SV2 is the primary protein receptor for BoNT/A1, with SV2C having the highest affinity (42), FGFR3 has recently been reported to be a higher-affinity receptor for BoNT/A1 than SV2 (45). Thus, future studies are required to determine whether the differences in A3 and A5 activity in human cells are due to differential SV2 receptor expression in the different NCB models or to other factors such as other protein receptors, ganglioside binding, glycosylation, or neuronal activity.

It had previously been shown that BoNT/A3 had unusual botulism intoxication symptoms in mice compared to BoNT/A1 when injected at 10^4 U/ml (23). To determine if A2 and A5 had effects more in line with the canonical A1 symptoms, mice were injected intravenously with 10^4 U/ml and observed until death. BoNT/A2-injected mice exhibited symptoms of botulism most closely in line with those seen with A3-injected mice, showing front-leg, hind-leg, and whole-body paralysis followed by death. Moreover, A2-injected mice died faster than those injected with A1, indicating that A2 has a faster onset of intoxication than A1. The different symptoms seen in mice with BoNT/A1 and -5 compared to -A2 intriguingly suggest that the different subtypes may target different classes of neurons.

Although the MBA has been used for many decades for analyzing and determining titers of BoNTs (24), several new methods for detecting BoNTs have been developed, including both *in vitro* and *in vivo* simulation assays (reviewed in reference 46). In some of these assays, the sensitivity is greater than that of the MBA. For the purpose of detecting fully active toxin, the only acceptable assays are those that take into account all sequential steps of cellular intoxication: HC binding to neuronal cell receptors, uptake of holotoxin, translocation of LC into the cell cytosol, and, ultimately, enzymatic activity of the LC on neuronal substrates. Only an assay that considers these four sequential steps can allow an accurate determination of BoNT potency. As supported here, neuronal cell-based (NCB) assays, which meet all of these requirements, are excellent models for determination of BoNT potency (28, 29, 47–53). However, correlation to the MBA still has to be confirmed for most of these assays, and the presented data suggest that this correlation should be determined for each BoNT subtype and formulation.

The data shown here indicate that for BoNT/A1, the *in vivo* mouse data and results from several NCB models appear to correlate with the MBA. However, for A2, there was a discrepancy between the MBA and the NCB assays tested in this study compared to the A1 results, and yet the A2 results had good correlation within different NCB models. For A3 and A5, there was a divergence of human cell-based assays and rodent cell-based assays, suggesting potential species-specific differences. The differences in activity of A3, A4, and A5 in the two human neuronal cell models additionally indicate distinct behaviors of these toxins in different neuronal populations. Taken together, the results suggest that for BoNT/A subtypes other than BoNT/A1, differences in specific activity determined by the *in vivo* MBA are not necessarily indicative of *in vitro* activity or activity in various cell models. It currently is not clear which model will best predict activity in humans after local injection.

Future developments of pharmaceutical BoNTs may include different sero- or subtypes and/or recombinant toxins or targeted toxins. It is important to consider the human subject and inherent differences in characteristics of various cell-based models and animal models when designing potency assays for current and future pharmaceutical BoNT preparations.

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