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***In vivo* onset and duration of action varies for Botulinum neurotoxin A subtypes 1–5**

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Abstract

To date, over 40 subtypes of botulinum neurotoxins (BoNTs) have been identified. BoNTs are classified into 7 serotypes distinguished primarily by their antigenic properties, but also characterized by their unique SNARE targets and cleavage sites, host specificity, and duration of action. Sequencing efforts in the last decade have identified several subtypes within the serotypes. Subtypes are currently defined as distinct based solely on amino acid sequence comparison, with a similarity cut-off of 2.5 % difference. Ten subtypes have been identified for BoNT/A, which is the serotype associated with the most severe human botulism and also the most commonly used serotype for clinical purposes. Analyses of several of these subtypes have revealed distinct characteristics, ranging from differences in cell entry and enzyme kinetics to differences in potency in mice and cell-model specific potency. A long-term activity study in cultured primary neurons has indicated that BoNT/A1, 2, 4, and 5 have a similar duration of action, whereas BoNT/A3 has a significantly shorter duration of action. This report describes an *in vivo* mouse study, showing that after local injection BoNT/A2 resulted in faster onset of local paralysis than BoNT/A1, 3, 4, and 5, whereas BoNT/A3 resulted in significantly faster recovery of motor-neuron deficiency.

Introduction

Botulinum neurotoxins (BoNTs) are a family of toxins produced by a diverse group of gram positive, spore-forming, anaerobic bacteria termed *Clostridium botulinum* and a few related species. BoNTs are the causative agents of human and animal botulism, which is characterized by prolonged, descending flaccid paralysis due to an inactivation of the peripheral nervous system by the toxins (1). BoNTs are 150 kDa di-chain proteins consisting

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Ethical Statement

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

of a 100 kDa heavy chain (HC) and a 50 kDa light chain (LC) linked by a disulfide bond (2). The toxins enter neurons after specific binding of the HC to dual protein and ganglioside receptors, leading to endocytosis (3). The HC then mediates translocation of the LC into the cell's cytosol, where the disulfide bond between the HC and LC is cleaved, releasing the enzymatically active LC (4–7). The LC cleaves a member of the SNARE protein family, an essential component of the vesicle release machinery, thereby blocking neurotransmitter release resulting in flaccid paralysis (8, 9).

Early studies of BoNTs revealed that several immunologically distinct variants exist, which were classified as serotypes A–G. Each of the seven identified serotypes can be neutralized by antibodies raised against its toxoid, but not by antibodies to other serotype toxoids (10). Analyses of the seven serotypes further revealed other clinically important serotype specific characteristics, including distinct SNARE targets and cleavage sites, different duration of action, differential binding to distinct neuronal receptors, and host specificity (1). While BoNT/C and D cause animal botulism but are not usually associated with human botulism, BoNT/A, B, E, and F have all been isolated from human botulism cases, with BoNT/A and B causing the majority of the cases. BoNT/A results in the longest lasting and most severe botulism, which can last from several months to over a year. Due to its long duration of action and high potency, BoNT/A is also the most commonly used BoNT serotype for medical purposes. Thanks to advances of genetic and molecular methods in the past two decades, the existence of many more BoNT variants has been demonstrated in recent years. To date, over 40 different BoNT variants have been identified, and the list continues to grow (11, 12). These new variants have been categorized as sub-types of the current serotypes (denoted by numbers after the letter), based on amino acid sequence similarity and neutralization studies. As an arbitrary guideline, a 2.6 % amino acid cut-off requirement was set to define a new subtype within one serotype (13, 14). Subtypes therefore currently are only defined by their amino acid sequence absent any consideration of potential functional or structural differences or similarities.

In recent years, a few in depth functional analyses of BoNT/A subtypes 1–5 have revealed that each of these subtypes has unique functional characteristics (15–26). Most notable, BoNT/A4 has about 1,000-fold reduced activity compared to other subtypes, most likely due to less efficient cell entry and trafficking since the LC has only a slightly decreased catalytic activity compared to the BoNT/A1 LC (16). BoNT/A2 has been studied *in vitro* and *in vivo* in several labs, all of which found that this subtype appears more potent in cells and in causing local paralysis in rodents compared to BoNT/A1 (15, 16, 21–25). Cell entry kinetics and receptor studies indicate that this apparent greater potency is due to more efficient neuronal cell entry (15). BoNT/A3, on the other hand, appears to have slightly decreased potency *in vivo* and in cells (16, 18), which appears to be at least in part due to less efficient SNAP-25 hydrolysis or LC stability, as indicated by *in vitro* endopeptidase assays (16, 26). BoNT/A5 had similar potency to BoNT/A1 in mice and primary rodent cells, but cell-based studies indicated a lower potency in human derived neuronal cell models (16). This, however, could be due to various cell-model specific characteristics, and does not necessarily indicate species specificity. In addition, a thorough investigation of the catalytic activities of BoNT/A5 and A1 LCs revealed that despite having only 4 amino acid

differences, the BoNT/A5 LC has a greater SNAP-25 hydrolysis rate than A1 LC, most likely due to more favorable binding to the substrate (27).

In addition, both *in vivo* and cell-based assays indicate that BoNT/A2 and A3 may have distinct pharmacological properties compared to BoNT/A1 and A5. While injection of mice with high doses of BoNT/A1 and A5 results in classical symptoms of botulism including ruffled fur, progressive paralysis, labored breathing, and spasticity just prior to death, injection of equal amounts of BoNT/A2 and A3 resulted in distinct symptoms that were characterized by overall progressive paralysis until death with no associated spasticity (16, 18). In addition, local injection of rats in the hind limb with BoNT/A2 resulted in less toxin spread to the ipsilateral limb than similar injection with BoNT/A1 (22, 25).

Finally, a long-term study in primary rat spinal cord cells showed that while the LCs of BoNT/A1, 2, 4, and 5 all persisted in cells for at least 10 months and continued to hydrolyze SNAP-25, with only a very slow and steady reduction in activity, the LC of BoNT/A3 was only active for up to 3 months with a reduction in activity apparent at 2 weeks post intoxication (17). Here we describe the onset and duration of action of BoNT/A1–5 *in vivo* after local injection into mice using the digit abduction score (DAS) to measure local paralysis and rotarod analysis to determine motor-neuron deficiency. The data confirm an earlier onset of local paralysis by BoNT/A2, and a shorter duration of action of BoNT/A3 in mice.

Materials and Methods

Ethics statement

All animal experiments were approved by and conducted according to guidelines of the University of Wisconsin Animal Care and Use Committee.

Botulinum neurotoxin

BoNT/A1, /A2, /A3, and /A5 were purified from *C. botulinum* strains Hall A-*hyper*, Kyoto-F, CDC A3 (kindly provided by Susan Maslanka and Brian Raphael, Centers for Disease Control and Prevention) and A661222 as previously described (18, 28–30). Recombinant BoNT/A4 was expressed in a nontoxigenic strain of *C. botulinum* (Hall A-*hyper/tox*[−]) and purified as described (31). Purity of the toxins was confirmed by spectroscopy and SDS-PAGE analyses (16). The purified toxins were stored in phosphate buffered saline with 40 % glycerol at −20°C until use. Activities of the five subtype preparations were determined using a standard intraperitoneal mouse bioassay (MBA) as previously described (32, 33). The half-lethal dose of each toxin was defined as 1 mouse LD50 Unit (U). Specific activities of the toxins were 8 pg/U (A1), 7.9 pg/U (A2), 17 pg/U (A3), 12 ng/U (A4), and 7.3 pg/U (A5).

In vivo analyses of onset and duration of action of BoNT/A1–5

Groups of 5 female ICR mice (Harlan) were injected into the right gastrocnemius muscle with the indicated sub-lethal amounts of BoNT/A1, /A2, /A3, /A4, or /A5 in 10 µl of GelPhos buffer (30 mM sodium phosphate [pH 6.3] and 0.2% gelatin) using an insulin

syringe. The amount of toxin injected per dilution IM was confirmed by mouse bioassay(32, 33). Control mice (no toxin) were injected in the same manner with 10 μ l GelPhos buffer. The effect of the toxins was assessed using two different assays. To determine local paralysis, a digit abduction score (DAS) was determined for the injected limb. Mice were suspended briefly by their tail and the toe spread and limb position recorded as shown in Figure 1. The DAS was determined at 4 h intervals for the first 24 h after toxin injection, and every 24 h thereafter as shown. The average DAS and standard deviation of each mouse group (n=5) was calculated for each time point. Motor-neuron deficiency was further evaluated by Rotarod analysis (MED-Associates) using an accelerating cycle of 4–40 rpm over 5 min. Each group of mice underwent 3 rounds of running per time point on the Rotarod, and the time that mice were able to remain on the Rotarod was recorded. Mice were analyzed every 4 h after toxin injection for the first 24 h, and on a daily basis thereafter until able to complete the full 5 minute cycle.

Results

Previous studies in cultured neurons have indicated that BoNT/A2 enters neuronal cells faster than BoNT/A1, and that BoNT/A3 has a significantly shorter duration of action inside cultured neurons. In order to determine the onset and duration of action of BoNT/A1–5 *in vivo*, mice were locally injected with sub-lethal amounts of BoNT/A and were analyzed by DAS assay and Rotarod analysis. A DAS assay has been previously utilized to assess local paralysis in mice or rats after injection of BoNT/A1, and requires laboratory staff to visually determine the DAS score in each mouse by holding the mouse up by the tail and looking at the toe spread (Figure 1). Potential observer variation was reduced by repeated measurement of 5 mice in each treatment group by the same two observers throughout the study. In addition, Rotarod analysis was employed as a second, observer independent assay. This assay was used to measure the ability of mice to remain on a rotating rod with increasing rotation speed after local injection of BoNT/A subtypes. Correlation of the two assays was investigated in mice injected with 0.25, 0.5 and 0.75 U of BoNT/A1 into one hindleg. Both assays resulted in a dose-dependent response and recovery. However, the DAS assay resulted in a measurable response significantly sooner than the Rotarod, and recovery based on the DAS took about 3 days longer than recovery as determined by Rotarod analysis (Figure 2). Visual observation of the mice running on the Rotarod further showed that the mice were able to remain on the Rotarod using the three non-injected limbs, even when the injected limb was significantly paralyzed. However, mice showing signs of mild systemic symptoms such as ruffled fur and overall paralysis remained on the Rotarod for shorter periods of time. These data indicate that while the DAS assay records local paralysis, the Rotarod records overall motor-neuron deficiency of the entire animal.

Based on these data, the comparative onset of local paralysis after injection of mice with BoNT/A1–5 was determined by DAS assay, whereas the duration of action was determined by Rotarod (Figure 3). Injection of mice with BoNT/A2 resulted in a significantly more rapid increase in the DAS score compared to injection with the other BoNT/A subtypes tested in this study (Figure 3). The Rotarod analysis, on the other hand, was similar for BoNT/A1 and A2, with a similar onset and recovery. The time of recovery from maximal response at the highest dose to full recovery was about 11–12 days for both toxins. Injection

of BoNT/A3 resulted in a delayed increase in the DAS score as compared to BoNT/A1, as well as a delayed decrease in latency time on the Rotarod. While a similar maximal reduction in time mice remained on the Rotarod was reached when injected with similar doses of BoNT/A3 and A1, the mice injected with BoNT/A3 recovered much more rapidly than mice injected with BoNT/A1 at all doses, and required only 5 days for full recovery after maximum response at the highest dose (0.6 U). While the DAS response in mice injected with BoNT/A4 was similar to that of BoNT/A3 (slightly slower than BoNT/A1), the reduction in latency time on the Rotarod was significantly less pronounced at all doses tested, and mice recovered completely within 6 days after maximum paralysis was reached at the highest doses (0.6 U). However, since the maximum response was less pronounced than with the other BoNT/A subtypes and was more similar to the 0.3 U dose of BoNT/A1, this recovery time should be compared to the 0.3 U dose of BoNT/A1 (7 days from maximum response to full recovery). Mice injected with BoNT/A5 resulted in similar data as mice injected with BoNT/A1 in both the DAS assay and the Rotarod analysis. These data indicate a faster and more potent onset of local paralysis by BoNT/A2 compared to the other subtypes tested, a slightly delayed onset and more rapid recovery by BoNT/A3, and a similar onset and recovery by BoNT/A5 and BoNT/A1.

Discussion

The number of BoNT variants identified has dramatically increased over the last few years, and is continuing to increase further as more *Clostridium* strains are being analyzed by genetic and molecular methods. The large variety of these toxins indicates active toxin transfer and/or remodeling between *Clostridium* species, and it is likely that many more BoNT variants will be identified in the future. While the evolutionary reasons for this phenomenon and the significance of the BoNT gene cluster for these bacteria are currently unknown and presents an interesting question by itself (12), functional and structural analyses of the large variety of BoNTs is essential in understanding the properties of these toxins. Not only will functional analyses of BoNT variants add to our knowledge of the variants that are being assessed, but combining structural analyses with functional studies will reveal structure-function relationships that may be applicable to all BoNTs. Data from such studies are crucial to increase our understanding of both the pathogenicity and potential pharmaceutical benefits of these toxins.

BoNT/A1 is the most commonly used BoNT serotype for pharmaceutical purposes, because at the time of pharmaceutical development this variant was the most potent and longest lasting BoNT known. To date, 33 protein sequences of BoNT/A have been deposited in Genbank. While some of these sequences are 100% identical to each other due to re-naming of strains or isolation of several strains containing an identical toxin gene, a blast pairwise alignment grouped the sequences into 10 distinct groups, as previously described (11). However, only BoNT/A1–8 (14, 18, 29, 34, 35) have been officially classified as subtypes to date. Of those, only BoNT/A1–5 have been purified and comparatively analyzed for functional differences (16, 17, 27). These studies revealed distinctions in neuronal cell entry, pharmacologic behavior *in vivo*, as well as some minor differences in catalytic activity. Most notable, BoNT/A4 is about 1000-fold less potent than BoNT/A1; BoNT/A2 enters neuronal cells faster; and BoNT/A3 has a significantly shorter duration of action inside neuronal cells

(3 months vs over 10 months). In addition, pharmacological properties and *in vivo* potency of BoNT/A2 compared to BoNT/A1 have been extensively studied and indicate greater potency of BoNT/A2 in causing local paralysis and less toxin spread from the injection site (19–25). Recombinant or immuno-precipitated BoNT/A8 has recently been characterized with regards to antibody binding, receptor binding, and catalytic activity, and results indicated reduced ganglioside binding, reduced catalytic activity, and reduce activity in a mouse hemidiaphragm assay (34). However, further *in vitro*, cell-based and *in vivo* studies on this toxin after isolation from its native host are required to understand the pathogenicity and pharmacologic behavior of this toxin.

In this report, an *in vivo* study with humane endpoints was used to assess onset and duration of action of BoNT/A1–5 in the mouse model. The data comparing the DAS assay with a Rotarod analysis for BoNT/A1 indicated that the earlier response recorded by the DAS assay correlates with local paralysis whereas the response measured by Rotarod analysis appeared to correlate more with overall motor-neuron deficiency (Figure 2), which may be at least in part due to systemic spread of the toxin. Nevertheless, this assay is useful in assessing duration of action of BoNTs after injection of sub-lethal amounts, whereas the DAS assay appeared more useful for determining onset of action. Comparative analysis of the onset of local paralysis as measured by the DAS assay indicated an earlier onset of action by BoNT/A2 (Figure 3), which is in agreement with previous data suggesting greater *in vivo* potency after local injection (21, 24), as well as faster neuronal cell entry and greater potency in cultured neurons (15, 16). The duration of action as measured by Rotarod was similar for BoNT/A1 and A2, which also is in agreement with previous observations *in vivo* (24) and in cultured neurons (17). Onset of local paralysis by BoNT/A3 was slightly delayed, and duration of action was significantly shorter, confirming previous observations of lowered potency and shorter duration of activity in cultured neurons (16, 17). As expected based on previous cell-based studies (16, 17), BoNT/A5 resulted in similar onset and duration as BoNT/A1 (Figure 3). However, equal doses of BoNT/A4 resulted in a delayed and less pronounced onset of local paralysis. While the duration of action may appear shorter for BoNT/A4 (Figure 3), the maximum level of motor-neuron deficiency at all tested doses was also lower than that for the other subtypes and direct comparison to a dose of BoNT/A1 that resulted in a similar maximum level of paralysis (0.3 U) indicates a similar recovery rate. This is also supported by previous data that indicated a similar persistence of BoNT/A4 and /A1 LC activity in cultured neurons (17). Since BoNT/A4 is 1,000 times less potent than BoNT/A1, 1000-fold more protein had to be injected to assess this subtype, which may have led to more efficient and faster clearance of this toxin. Currently, the molecular mechanisms underlying the duration of action of BoNTs and recovery are not fully understood. It is important to further delineate these mechanisms to understand whether duration of action and recovery can be correlated between mice and humans.

The clinical applications and uses of BoNTs have been increasing steadily over the past years, now encompassing treatments for a variety of disorders including aesthetic applications, treatment of spasticity, reduction of hyperhidrosis, management of migraine headaches, pain disorders, and more (36–43). While expansion of the pharmaceutical uses of the two currently marketed BoNT variants, BoNT/A1 and B1, is continuously being

investigated, only a few studies focus on assessments of the potential benefits and unique pharmacologic behavior of other BoNT subtypes. With the continually growing number of BoNT variants, a potential treasure trove for discovery of new information on these toxins is presented to the botulinum neurotoxin community. The presented and described comparative studies of BoNT/A1–5 already have identified BoNT/A2 as a variant that has potential pharmacological benefits over the currently used BoNT/A1 (16, 17, 22, 23, 25). Further in depth functional studies of these and additional BoNT variants will increase our molecular understanding of these toxins, particularly when combined with structural studies. This holds great promise for improving BoNT based pharmaceuticals as well as aiding in the development of countermeasures to botulism.

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DAS Score

Figure 1.

DAS Scoring system. The digital abduction score measures the toe-spread after injection of sub-lethal amounts of BoNT into the gastrocnemius muscle. Mice are temporarily suspended by their tail and the paw and leg on the injected side are observed. A numbering system of 1–5 is assigned based as shows in the figure.

Figure 2.

Comparison of DAS assay and Rotarod assay. Groups of 5 mice were injected into the gastrocnemius muscle with 0.75, 0.5, 0.25, or 0 U of BoNT/A1 and assessed by DAS assay and Rotarod for up to 20 days. The average and standard deviation for the 5 mice at each time point are shown. The data for mice injected with 0 U did not change throughout the study period and are not shown in the graphs.

Figure 3.

Comparative analysis of onset and duration of action of BoNT/A1–5. Groups of 5 mice were injected with the indicated doses of BoNT/A1–5 (0 U controls are not shown, as there was no change in any parameters / groups). Onset of local paralysis was assessed by DAS assay for the first 72 h, and duration of action of assessed by Rotarod analysis until mice were able to remain on the Rotarod for the entire 5 min cycle.