Botulinum neurotoxin A activity is dependent upon the presence of specific gangliosides in neuroblastoma cells expressing synaptotagmin I

Brian C. Yowler, Richard D. Kensinger, and Cara-Lynne Schengrund*

Dept. of Biochemistry and Molecular Biology, The Pennsylvania State University College of Medicine, 500 University Drive, Hershey, PA 17033

*Corresponding Author: Cara-Lynne Schengrund, Dept. of Biochemistry and Molecular Biology, H171, The Pennsylvania State University College of Medicine, 500 University Drive, Hershey, PA 17033
Telephone: (717) 531-8048; Fax: (717) 531-7072
Email: cschengrund@psu.edu
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SUMMARY

Botulinum neurotoxin A (BoNT/A) is the deadliest of all known biological substances. While its toxicity makes BoNT/A a biological warfare threat, its biologic activity makes it an increasingly useful therapeutic agent for the treatment of muscular disorders. However, almost 200 years after its discovery, the neuronal cell components required for the activity of this deadly toxin have not been unequivocally identified. In this work, neuroblastoma cells expressing synaptotagmin I, a protein shown to be bound by BoNT/A, were used to determine whether specific gangliosides were necessary for BoNT/A activity as measured by SNAP-25 cleavage. Ganglioside GT1b was found to support BoNT/A activity significantly more effectively than GD1a, which was much more effective than GM1 when added to ganglioside deficient murine cholinergic Neuro 2a or to human adrenergic SK-N-SH neuroblastoma cells. While both cell lines expressed synaptotagmin I, SNAP-25 cleavage was not observed in the absence of complex gangliosides. These results indicate that 1) gangliosides are required for BoNT/A activity, 2) synaptotagmin I, in the absence of gangliosides, does not support BoNT/A activity, and 3) Neuro 2a cells are an efficient model system for studying the biological activity of BoNT/A.
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INTRODUCTION

Botulinum neurotoxin A (BoNT/A), one of seven serotypes of BoNT, is the deadliest of all known biological substances, being ~1 million times more poisonous than cobra toxin (1). While its toxicity makes BoNT/A a biological warfare threat, its biologic activity makes it an increasingly useful therapeutic agent (2,3). Produced as a single polypeptide chain by the bacterium Clostridium botulinum, BoNT/A (150kD) is subsequently cleaved into two functional subunits, held together by a disulfide bond. The 50kDa “light chain” is a zinc endoprotease. It catalyzes the cleavage of synaptosomal-associated protein of 25kD (SNAP-25) at the neuromuscular junction, thereby preventing neurotransmitter release, and inducing a flaccid paralysis (4). The 100kD “heavy chain” is made up of an N-terminal half, believed to mediate cytoplasmic entry of the light chain, and a C-terminal half (BoNT/A-HC), which mediates cellular adherence (5). While the enzymatic activity of the neurotoxin has been well defined, questions still exist regarding the molecular interactions needed for its cellular binding, internalization, and transport to its protein substrate. Identification of these interactions will provide guidance for developing potential inhibitors of its action.

It was 30 years ago that gangliosides were first mentioned as potential receptors for BoNT/A. Gangliosides, sialic acid containing glycosphingolipids, are found on the outer leaflet of cell membranes. Specifically, polysialylated gangliosides like GT1b [nomenclature according to Svennerholm (6)], were shown to be involved in BoNT/A neurotoxicity (7,8). Since then, GT1b has been shown to inhibit BoNT/A binding to synaptosomes (9), to quench BoNT/A fluorescence (10), and to be bound by BoNT/A when it was immobilized on either a thin layer chromatogram (11) or on plastic wells.
Specific gangliosides are required for BoNT/A activity (12). The dissociation constant for the adherence of BoNT/A-HC to GT1b-containing liposomes was recently reported to be \(~10^{-8}\) M (13). At the cellular level, cleavage of sialic acid residues by sialidase treatment of cultured cells isolated from spinal cord (14) and adrenergic chromaffin cells (15) was shown to reduce BoNT/A potency, while addition of a mixture of gangliosides to the sialidase treated chromaffin cells was found to restore it (16). In addition, a monoclonal antibody to GT1b was shown to antagonize the action of BoNT/A on rat superior cervical ganglion neurons (17). Most recently, GM2/GD2-synthase knockout mice were reported to show an increased time of survival when injected with a lethal dose of BoNT/A (18). Tetanus toxin, the other member of the clostridial toxin family, which has an affinity comparable to that of BoNT/A for gangliosides (13,19), was recently shown to use gangliosides as its cellular receptor (20). The crystal structure of tetanus toxin C-fragment complexed with a synthetic GT1b analogue indicated the presence of two binding sites in the \(\beta\)-trefoil domain. One of these two sites was also found in the \(\beta\)-trefoil domain of BoNT/A and is strongly believed to be a ganglioside binding site (21,22). All of these studies indicated that gangliosides might be receptors for BoNT/A.

In addition to gangliosides, a cell surface protein(s) may also be needed for BoNT/A adherence to cells. In contrast to more recent studies on chromaffin cells (16), early studies showed that BoNT/A binding to rat brain synaptosomes was trypsin sensitive (23,24). Also, a 140 kD protein in cholinergic synaptosomes prepared from Torpedo electric organs was identified as a BoNT/A binding protein. The interaction of BoNT/A with this 140 kD protein was both sialidase and trypsin sensitive (25). These studies, among others, led to the hypothesis that botulinum neurotoxins require a “double
Specific gangliosides are required for BoNT/A activity receptor”, comprised of protein and gangliosides (26). BoNT/B appears to follow this model. Nishiki et al. found that synaptotagmins I and II, in the presence of polysialylated gangliosides, serve as the receptors for BoNT/B (27-30). Synaptotagmins, proteins that serve as calcium sensors for synaptic vesicle fusion, have also been proposed as possible receptors for BoNT/A. Studies indicated that BoNT/A adhered to synaptotagmin I solubilized from rat brain synaptosomes, as well as to purified synaptotagmin I immobilized on plastic microtiter wells (31). In contrast to the observations made with BoNT/B, this binding was not enhanced by the presence of gangliosides (31).

In this work, the question of whether specific gangliosides were needed for BoNT/A to act on SNAP-25 in synaptotagmin I expressing murine cholinergic Neuro 2a and human adrenergic SK-N-SH neuroblastoma cells was addressed. Both cell types were found to be susceptible to BoNT/A only when they contained specific gangliosides. Interestingly, in their absence, BoNT/A activity was not observed, even though synaptotagmin I was present.
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EXPERIMENTAL PROCEDURES

Materials

BoNT/A was kindly provided by Dr B.R. DasGupta (University of Wisconsin, Madison, WI). Bovine brain gangliosides were purified by the method of Folch et al (32) or purchased from Matreya, Inc. (Pleasant Gap, PA). PPMP was also purchased from Matreya, Inc. SNAP-25 mouse monoclonal antibody (SMI 81) was purchased from Sternberger Monoclonals Inc. (Lutherville, MD), and mouse monoclonal antibodies to the luminal part of human synaptotagmin I were obtained from Synaptic Systems (Göttingen, Germany) and StressGen Biotechnologies Corp. (Victoria, BC, Canada). Neuro 2a (cholinergic murine neuroblastoma) and SK-N-SH (human adrenergic neuroblastoma) cells were purchased from the ATCC (Manassas, VA) and grown at 37°C in Dulbecco’s modified Eagle’s medium (DMEM) containing either 10% newborn bovine serum (Neuro 2a) or 10% fetal calf serum (SK-N-SH) in an atmosphere of 5% CO₂/95% air and 90% humidity. Human brain protein extracts were purchased from Clontech (Palo Alto, CA) and mouse brain extracts were kindly provided by the laboratory of Dr. Judith Bond (this university).

HPTLC analysis of lipid extracts of cultured cells

Neuro 2a (N2a) and SK-N-SH cells were seeded at a density of 1X10⁷ cells per 150cm² tissue culture flask. When ganglioside-depleted N2a cells were needed, they were grown in the presence of medium containing 7.5 µM PPMP, an inhibitor of glucosyl ceramide synthase. Forty-eight hours after seeding, the medium was replaced with serum-free medium or serum-free medium containing either GM1, GD3, GD1a, or GT1b
Specific gangliosides are required for BoNT/A activity (each at 50µg/ml). Twenty-four hours later, the cells were harvested. After washing with 5 ml of PBS, cells were dislodged by rapping the flask (N2a) or by adding 1 ml 0.25% Trypsin-EDTA and incubating at 37°C for 5 min (SK-N-SH). Trypsin was inactivated by the addition of 10ml of culture medium containing 10% serum. Cells were recovered by centrifugation at 250xg for 5 min. and the cell pellet washed with 5 ml PBS. After recovery by centrifugation, the cells were resuspended in 1ml H2O. Cellular lipids were then extracted as described by Kates (33). Briefly, 3.75 volumes of methanol:chloroform 2:1 (v/v) was added to the cell suspension and the mixture stirred at room temperature overnight. Cell debris was removed by centrifugation at ~250xg for 10 min. and the supernatant set-aside for future use. The pellet was resuspended in a volume of methanol:chloroform:H2O (2:1:0.8 v/v/v) equivalent to the total volume of the supernatant that was set aside, stirred for 2 hours at room temperature, centrifuged (~250xg, 10 min), and the supernatant removed. Combined supernatants were dried under N2 prior to exposure to 0.1 N NaOH in methanol for 1 hr at 37°C. Solvent was removed by evaporation under N2. Lipids were resuspended in H2O and desalted by size-exclusion chromatography on a PD-10 column (Pharmacia Biotech, Uppsala, Sweden), using H2O as the eluent. The void volume was collected and freeze-dried in a tared test tube. Lipid films were dissolved in chloroform: methanol (2:1 v/v) to give a final concentration of 10 µg/µl and an aliquot (200-400µg) applied to a silica gel 60 HPTLC plate. Chloroform:2-propanol:50mM KCl (2:13.4:4.6, v/v/v) was used to develop the plate and sialic acid-containing components were visualized using resorcinol (34). Densitometric analyses were performed using a Hewlett Packard ScanJet 6300C scanner and Scion Image software (Windows version of NIH Image). All bands used for
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densitometric analysis were within the linear range of approximately 2-10 nmol of lipid-bound sialic acid as determined by scanning and calibrating a range of ganglioside standards. Incorporated exogenous ganglioside was calculated by subtracting the value obtained for endogenous ganglioside from the total.

Western blot analysis to determine synaptotagmin expression by cultured cells

Cells were seeded at 5X10^5 cells (N2a) or 1X10^6 cells (SK-N-SH) per 25cm² flask and allowed to grow for 96 hrs with a change to fresh culture medium after 48 hrs. Cells were washed with PBS and harvested by scraping into PBS. Cells were recovered by centrifugation at 250xg for 5 min. and resuspended in 200-500 µl radioimmunoprecipitation (RIPA) buffer (50mM Tris-HCl, 150mM NaCl, 1% NP-40, 0.25% Na-deoxycholate, 1mM EDTA, pH 7.4) containing protease inhibitors (Halt Protease Inhibitor™, Pierce, Rockford, IL). An aliquot was taken to determine protein concentration (Bio-Rad protein assay, Hercules, CA), and the remaining sample combined with an equal volume of 2X sample buffer (125mM Tris-HCl pH 6.8, containing 2.5% SDS, 0.2% bromphenol blue, 25% glycerol, and 2.5% β-mercaptoethanol) and boiled for 5 min. Proteins in 20-80 µg of whole cell extract were separated by SDS-PAGE (5% stacking/ 7.5% running gel) (35) prior to transfer to a PVDF membrane (Immobilon-P, Millipore, Bedford, MA). After blocking with 5% non-fat dry milk in TBST [10 mM Tris, 150 mM NaCl, 0.1% (v/v) Tween-20] for 1 hr at room temperature, blots were probed with mouse monoclonal anti-synaptotagmin I antibody (1:1000) in 2% non-fat dry milk in TBST for 1 hr at room temperature. Following 3 washes with TBST, secondary antibody, HRP (horseradish peroxidase)-
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conjugated goat anti-mouse IgG (1:20,000, Sigma, St. Louis, MO) in 2% non-fat dry milk in TBST, was added. After 1 hr at room temperature, blots were washed 3 times with TBST and developed using SuperSignal West Femto Maximum Sensitivity Substrate™ (Pierce, Rockford, IL).

**Western blot analysis of BoNT/A activity in cultured cells**

N2a cells were seeded as described for analysis of synaptotagmin I content and grown in the presence of medium containing PPMP (concentrations of PPMP tested ranged from 1 – 10 µM with 7.5 µM used in most experiments). SK-N-SH cells were also seeded and grown as described for analysis of synaptotagmin I. After 48 hours, the medium on both cell types was removed and replaced with serum-free medium with or without ganglioside (GM1, GD3, GD1a, or GT1b, 50µg/ml) and in the case of N2a cells, with PPMP. Control cells were grown in the absence of added ganglioside and PPMP. Twenty-four hours later, the medium was changed to DMEM containing BoNT/A (6 nM), 10% serum, no added ganglioside, and for N2a cells, PPMP. Forty-eight hours later, the cells were harvested, as described above. Western blot analyses were used to determine the amount of intact and cleaved SNAP-25 present in 10-20 µg of whole cell extract (36). Densitometric analyses were performed as described above. To insure linearity, multiple exposures were taken of each blot, and all bands used for analysis were exposed at less than saturated levels.
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RESULTS

Ganglioside content of neuronal cell lines

HPTLC analyses indicated that N2a cells expressed detectable levels of gangliosides with mobilities comparable to those of the monosialoganglioside standards GM1, GM2, and GM3, and a relatively high level of a ganglioside that migrated with the disialoganglioside standard, GD1a (Figure 1). These gangliosides were previously shown to be the major ones expressed by N2a cells (37). On a molar basis, the gangliosides identified as GM1, GM2, and GM3 accounted for 62%, and that as GD1a for 38% of the gangliosides in N2a cells. PPMP treatment reduced the level of GD1a by 85 ± 3% (n=4). In contrast, resorcinol-positive lipids that migrated with the gangliosides GM2 and GM3 accounted for 95% and with GD1a for 5% of the gangliosides in SK-N-SH cells (Figure 2).

Added gangliosides were taken up by both cell lines (Figures 1 and 2). Densitometric analysis of resorcinol positive bands indicated that both cell lines took up equivalent molar amounts of added ganglioside. In N2a cells, grown in the presence of 7.5 µM PPMP, GD1a concentration was restored to only 40 ± 4% (n=4) of that of untreated cells. However, the added GD1a increased cellular levels of GD1a to 2.7 ± 0.4 (n=4) times that of PPMP treated cells grown in the absence of added GD1a. In the SK-N-SH cells, added GD1a increased cellular levels of GD1a to 2.3 ± 0.3 (n=4) times that of control cells.
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**Synaptotagmin I content of neuronal cell lines**

Synaptotagmin I was detected by Western blot analysis of N2a and SK-N-SH cell proteins (Figure 3). The identification of synaptotagmin I in PPMP-treated N2a cells (data not shown) verified that its expression was not altered by inhibition of ganglioside synthesis. In each analysis, the anti-synaptotagmin I antibody adhered to a protein in the appropriate control (mouse or human) brain extract that had the apparent mass of synaptotagmin I (65kD).

**Effect of ganglioside depletion on BoNT/A activity in Neuro 2a cells**

While cleavage of SNAP-25 was observed after exposure of N2a cells to 0.67nM BoNT/A (data not shown), essentially all of the SNAP-25 was cleaved when the concentration of BoNT/A was increased to 6 nM (Figure 4). In order to determine the effect of ganglioside depletion on BoNT/A activity, N2a cells were treated with increasing concentrations of PPMP, an inhibitor of glucosyl ceramide synthase, prior to a 24 hour exposure to BoNT/A. PPMP was used because concentrations of 5-10 µM had been shown to inhibit ganglioside synthesis in neurons (38). Western analysis of SNAP-25 cleavage indicated that as the PPMP concentration was increased, BoNT/A activity decreased (Figure 5). Exposure of the cells to either 5 µM or 10 µM PPMP resulted in complete inhibition of BoNT/A activity. Concentrations of PPMP above 10 µM were toxic to the cells. Therefore, PPMP concentrations in the range of 5-10 µM (usually 7.5 µM) were used. A 48 hr exposure to 7.5 µM PPMP reduced the GD1a concentration in N2a cells by 85 ± 3% (n=4) (Figure 1, lanes 3 and 4).
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The effect of PPMP on BoNT/A activity was reversible. Growth of PPMP-treated N2a cells in PPMP-minus medium for 48 hr prior to exposure to BoNT/A, restored the susceptibility of SNAP-25 to the protease activity of BoNT/A (data not shown).

Effect of the addition of specific gangliosides on BoNT/A activity in PPMP-treated N2a cells

Following 48 hrs of growth in the presence of medium with 10% serum containing 10 µM PPMP, N2a cells were grown in the presence of serum-free medium containing increasing concentrations of GT1b plus 10 µM PPMP. Twenty-four hrs later, cells were fed fresh medium with 10% serum containing BoNT/A (6 nM) and 10 µM PPMP and incubated at 37°C for another 24 hrs. Western analysis of SNAP-25 cleavage indicated that as the concentration of GT1b increased, BoNT/A activity increased (Figure 6). In subsequent experiments, cells were exposed to BoNT/A for 48 hrs. This resulted in almost complete cleavage of the SNAP-25 in untreated cells, and allowed measurement of the lower activities obtained when gangliosides other than GT1b were added to the cells.

Addition of exogenous GT1b (50µg/ml) to cells grown in the presence of PPMP, 24 hours prior to exposure to BoNT/A for 48 hrs, restored BoNT/A activity to 52 ± 6% (n = 3) of control levels, a value significantly (p<0.005) greater than the 21 ± 8% (n = 5) obtained when cells were treated with the same concentration of GD1a (Figure 4). Addition of GM1 had little effect: a small amount of cleavage was observed only upon overexposure of the band for intact SNAP-25. GD3 had no effect on BoNT/A activity (data for GD3 not shown).
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**BoNT/A activity in SK-N-SH cells**

Western blot analysis indicated that exposure of SK-N-SH cells to 6 nM BoNT/A for 48 hrs did not result in cleavage of SNAP-25 (Figure 7). However, if cells were maintained for 24 hr in serum-free medium containing either GD1a or GT1b (50 µg/ml) and then exposed to BoNT/A for 48 hrs, cleavage of SNAP-25 was observed. Exposure of the cells to GD1a resulted in cleavage of 25 ± 10% (n = 3) of the SNAP-25, while addition of GT1b resulted in cleavage of 67 ± 10% (n = 4) of the SNAP-25, a significant increase (p<0.005) relative to that induced by exposure to GD1a. Addition of GM1 was less effective (a small amount of cleavage was observed only upon overexposure of blots) than GD1a or GT1b at permitting BoNT/A activity.
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**DISCUSSION**

Botulinum neurotoxin was first described in the early 1800’s (39). Now, almost 200 years later, the molecules necessary for the neurotoxin to enter neurons and find its substrate have yet to be unequivocally identified. This lack of knowledge hampers development of treatments for botulism or improvement of its use as a therapeutic agent. If the components needed for the interaction of BoNT with neurons could be identified, inhibitors could be designed to prevent its uptake and thus limit its deadly activity. Inhibitors that could be easily administered, perhaps taken orally, would be of particular interest to victims of bioterrorism or military personnel who may be exposed to BoNT, especially in areas far from modern medical facilities.

Many of the early studies of BoNT/A implicated gangliosides as possible receptors. Gangliosides were shown to reduce the toxicity of BoNT/A in vivo (7-9), as well as to adhere to BoNT/A in a variety of in vitro experiments (9-13). Tetanus toxin, the other member of the clostridial toxin family, which has an affinity comparable to that of BoNT/A for gangliosides (13,19), was recently shown to recognize gangliosides as its cell surface receptor (20). In addition, the crystal structure of the tetanus toxin C-fragment complexed with a synthetic GT1b analogue indicated the presence of two binding sites in the β-trefoil domain. One of these two sites is also found in the β-trefoil domain of BoNT/A and is strongly believed to bind a ganglioside (21,22). However, the affinity of the interaction of BoNT/A with gangliosides has been traditionally believed to be weak in relation to other receptor/ligand interactions and therefore, unable account for the high potency of the toxin. For example, cholera toxin binds to its cell surface receptor, ganglioside GM1, with a $K_D$ in the range of $10^{-9}$ to $10^{-12}$ M (13,19,40,41).
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However, the recent report that the apparent dissociation constant for the adherence of BoNT/A to GT1b was $\sim 10^{-8}$M (13) supports the hypothesis that GT1b could function as its receptor.

In this work, BoNT/A activity was observed in N2a cells when it was added to the medium at concentrations similar to those reported for its dissociation constant. The observations that PPMP treatment of N2a cells resulted in an 85% decrease in GD1a synthesis and eliminated observable BoNT/A activity, while growing the PPMP-treated cells in the presence of GD1a or GT1b restored activity, indicate that specific gangliosides are needed for BoNT/A activity. These observations plus the fact that SK-N-SH cells, expressing primarily GM3 and GM2, were not susceptible to BoNT/A, unless they were grown in medium containing a gangliotetraose ganglioside, (GT1b > GD1a > GM1), indicate that specific gangliosides must be present in the cells in order for added BoNT/A to catalyze the cleavage of SNAP-25. The need for polysialylation was corroborated by the fact that GD1a or GT1b were more effective at restoring BoNT/A activity than GM1. This observation is in agreement with studies indicating that BoNT/A is neutralized by and has a greater affinity for GD1a and GT1b than GM1 (9,11,12,16).

The need for polysialylated gangliosides may explain the observation that in order to see levels of SNAP-25 cleavage in SH-SY5Y human neuroblastoma cells that were comparable to those observed in this study with control murine N2a cells, a 50-fold greater concentration of BoNT/A and a 24-hr longer exposure time were used (42). SH-SY5Y cells may be less susceptible to BoNT/A than N2a cells due to their lower expression of polysialylated gangliosides. The monosialylated gangliosides, GM1, GM2, and GM3 were shown to account for 71.5% of the total ganglioside sialic acid content of...
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the SH-SY5Y cells, while GD1a accounted for the rest (43). In the N2a cells used in this study, GD1a comprised 56 ± 3% (n=4) of the total ganglioside sialic acid. The higher content of polysialylated gangliosides in N2a cells may account for why activity was observed at a BoNT/A concentration below 1 nM. Combined, these observations indicate that N2a cells provide an effective model for studying the biological activity of BoNT/A.

Although addition of GD1a to N2a cells resulted in a cell-associated level of GD1a that was ~40% that of controls (Fig. 1), it only restored BoNT/A activity to ~20% of that seen in control cells. This observation coupled with the results obtained for the SK-N-SH cells indicated that there might be a relationship between cell-associated polysialylated gangliotetraose ganglioside concentration and BoNT/A activity. Further support for such a relationship was provided by the observation that when PPMP-treated N2a cells were grown in the presence of higher concentrations of GT1b they became more susceptible to BoNT/A-catalyzed cleavage of SNAP-25.

Due to the potency of BoNT/A, some believe the receptor must be a cell surface protein exhibiting a very high affinity for the toxin. Since the leading candidate for a protein receptor is currently synaptotagmin (31), its expression and relation to BoNT/A activity were studied. The reported K_D for BoNT/B adherence to synaptotagmin I reconstituted with gangliosides is 2.3 nM (29), which is the same order of magnitude as the concentration of BoNT/A used in these studies. Western analyses indicated that both cell lines used in this work express synaptotagmin I. The observation that BoNT/A was unable to catalyze the cleavage of SNAP-25 in either the PPMP-treated N2a cells or SK-N-SH cells indicates that in the absence of gangliosides, synaptotagmin I is unable to efficiently mediate BoNT/A activity. However, these observations do not negate the
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hypothesis that when gangliosides are present, synaptotagmin I might serve as a receptor for BoNT/A. Combined, these observations indicate that in the absence of specific gangliosides synaptotagmin I cannot support BoNT/A activity.

The results reported here do not define the role gangliosides have in the action of BoNT/A, only that they are essential. However, based on the studies discussed previously, it is probable that the neurotoxin binds to polysialylated gangliotetraose gangliosides during its interaction with the cell. The results of this work indicate that these gangliosides are required for the biological activity of botulinum neurotoxin A. The requirement for specific gangliosides supports the hypothesis that a possible way to prevent BoNT/A from acting on neurons would be to inhibit its interaction with cell surface gangliosides. Studies are in progress to design ganglioside-based inhibitors of BoNT/A activity.

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Specific gangliosides are required for BoNT/A activity

FOOTNOTES

The abbreviations used are: GM1, Gal(β1-3)GalNAc(β1-4)[NeuAc(α2-3)]Gal(β1-4)Glcβ1-cer; GM2, GalNAc(β1-4)[NeuAc(α2-3)]Gal(β1-4)Glcβ1-cer; GM3, NeuAc(α2-3)Gal(β1-4)Glcβ1-cer; GD3, NeuAc(α2-8)NeuAc(α2-3)Gal(β1-4)Glcβ1-cer; GD1a, NeuAc(α2-3)Gal(β1-3)GalNAc(β1-4)[NeuAc(α2-3)]Gal(β1-4)Glcβ1-cer; GT1b, NeuAc(α2-3)Gal(β1-3)GalNAc(β1-4)[NeuAc(α2-8)NeuAc(α2-3)]Gal(β1-4)Glcβ1-cer; ganglioside nomenclature according to Svennerholm (6); PPMP, d,l-threo-l-phenyl-2-hexadecanoylamino-3-morpholino-propanol HCl; BoNT/A, botulinum neurotoxin serotype A; HPTLC, high performance thin layer chromatography; PVDF, polyvinylidene fluoride; PBS, phosphate buffered saline; N2a, Neuro 2a; HRP, horseradish peroxidase; TBST, tris buffered saline with Tween-20 (0.1%), pH 7.4.
Specific gangliosides are required for BoNT/A activity

FIGURE LEGENDS

Figure 1. Ganglioside composition of N2a cells. 20µg of ganglioside standards were spotted in lanes 1 and 2 of an HPTLC plate. All cells, except control (lane 3), were grown in the presence of PPMP (7.5 µM) for 48 hr. Culture medium was changed to serum-free DMEM with the addition of 7.5µM PPMP (lanes 4 – 7) and either no lipid (lane 4), or 50 µg/ml of either GM1 (lane 5), GD1a (lane 6), or GT1b (lane 7). Following a 24 hr incubation, cells were harvested, the cell pellets taken up in H2O, and their lipids extracted with chloroform: methanol (1:2). 200 µg of lipid extract were applied to the HPTLC plate and the plate was developed in chloroform:2-propanol:50mM KCl (2:13.4:4.6 v/v/v). Gangliosides were visualized with resorcinol spray.

Figure 2. Ganglioside composition of SK-N-SH cells. 20µg of ganglioside standards were spotted in lanes 1 and 2 of an HPTLC plate. Cells were grown for 48 hr prior to the addition of no lipid (lane 3), or 50 µg/ml of either GM1 (lane 4), GD1a (lane 5), or GT1b (lane 6) in serum-free medium. Following a 24 hr incubation, cells were harvested, the cell pellets taken up in H2O, and their lipids extracted with chloroform: methanol (1:2). 200 µg of lipid extract were applied to the HPTLC plate and the plate was developed in chloroform:2-propanol:50mM KCl (2:13.4:4.6 v/v/v). Gangliosides were visualized with resorcinol spray.

Figure 3. Synaptotagmin expression by SK-N-SH and Neuro 2a cells. Extracts of 20-80 µg of SK-N-SH cells (lane 1), Neuro2a cells (lane 2), and mouse brain (lane 3) protein
Specific gangliosides are required for BoNT/A activity were separated by SDS-PAGE under reducing conditions on a 5% stacking/7.5% running gel. Following transfer to PVDF, the immunoblot was probed with anti-synaptotagmin I and then with a HRP-conjugated goat anti-mouse antibodies. Bands were visualized using SuperSignal West Femto Maximum Sensitivity Substrate™.

**Figure 4. BoNT/A activity in Neuro2a cells.** Cells were seeded and allowed to grow in the absence (lane 1 and 2) or presence (lanes 3-6) of PPMP (7.5 µM) for a total of 120 hrs. Forty-eight hours after seeding, medium was replaced with serum-free DMEM containing no lipid (lanes 1-3), or 50 µg/ml of either GM1 (lane 4), GD1a (lane 5), or GT1b (lane 6). Following a 24 hr incubation, medium was replaced with fresh DMEM containing BoNT/A (6nM, except on cells in lane 1). After a 48 hr exposure, cells were harvested in RIPA buffer. Twenty micrograms of protein in each whole cell extract were separated by SDS-PAGE under reducing conditions on a 5% stacking/13% running gel. Following transfer to PVDF, the immunoblot was probed with an anti-SNAP-25 monoclonal antibody followed by a HRP-conjugated goat anti-mouse antibody. Bands were visualized using SuperSignal West Pico Chemiluminescent Substrate™ and exposure to film.

**Figure 5. Effect of PPMP treatment of N2a cells on BoNT/A activity.** Cells were seeded and allowed to grow for a total of 96 hours in the presence of 0 (lane 1), 1 (lane 2), 2.5 (lane 3), 5 (lane 4), or 10 (lane 5) µg/ml of PPMP. Seventy-two hours after seeding, cells were exposed to 6nM BoNT/A for 24 hours, and then harvested in RIPA buffer. Twenty micrograms of protein in each whole cell extract were separated by SDS-
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PAGE under reducing conditions on a 5% stacking/13% running gel. Following transfer to PVDF, the immunoblot was probed with an anti-SNAP-25 monoclonal antibody followed by a HRP-conjugated goat anti-mouse antibody. Bands were visualized using SuperSignal West Pico Chemiluminescent Substrate™ and exposure to film.

**Figure 6. Effect of the addition of GT1b to PPMP-treated N2a cells on BoNT/A activity.** Cells were seeded and allowed to grow in the presence of PPMP (10 µM) for a total of 96 hrs. Forty-eight hours after seeding, the medium was replaced with serum-free DMEM containing 0 (lane 1), 1 (lane 2), 5 (lane 3), 10 (lane 4), 25 (lane 5), or 50 (lane 6) µg/ml of GT1b. Following a 24 hr incubation, the medium was replaced with medium containing BoNT/A (6nM). After a 24 hr exposure, the cells were harvested in RIPA buffer. Twenty micrograms of protein in each whole cell extract were separated by SDS-PAGE under reducing conditions on a 5% stacking/13% running gel. Following transfer to PVDF, the immunoblot was probed with an anti-SNAP-25 monoclonal antibody followed by a HRP-conjugated goat anti-mouse antibody. Bands were visualized using SuperSignal West Pico Chemiluminescent Substrate™ and exposure to film.

**Figure 7. BoNT/A activity in SK-N-SH cells.** Cells were seeded and allowed to grow for 48 hr prior to the addition of no lipid (lane 2), or 50 µg/ml of either GM1 (lane 3), GD1a (lane 4), or GT1b (lane 5) in serum-free DMEM. Following a 24 hr incubation, the medium was replaced with medium only (lane 1) or with medium containing 6 nM
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BoNT/A (lanes 2-5). After a 48 hr exposure, cells were harvested in RIPA buffer. Twenty micrograms of protein in each whole cell extract were separated by SDS-PAGE under reducing conditions on a 5% stacking/13% running gel. Following transfer to PVDF, the immunoblot was probed with an anti-SNAP-25 monoclonal antibody followed by a HRP-conjugated goat anti-mouse antibody. Bands were visualized using SuperSignal West Pico Chemiluminescent Substrate™ and exposure to film.
Figure 4

SNAP-25
c-SNAP-25

1  2  3  4  5  6
Botulinum neurotoxin A activity is dependent upon the presence of specific gangliosides in neuroblastoma cells expressing synaptotagmin I
Brian C. Yowler, Richard D. Kensinger and Cara-Lynne Schengrund

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