Entry of Botulinum Neurotoxin Subtypes A1 and A2 into Neurons

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ABSTRACT Botulinum neurotoxins (BoNTs) are the most toxic proteins for humans but also are common therapies for neurological diseases. BoNTs are dichain toxins, comprising an N-terminal catalytic domain (LC) disulfide bond linked to a C-terminal heavy chain (HC) which includes a translocation domain (H_N) and a receptor binding domain (H_C). Recently, the BoNT serotype A (BoNT/A) subtypes A1 and A2 were reported to possess similar potencies but different rates of cellular intoxication and pathology in a mouse model of botulism. The current study measured H_C A1 and H_C A2 entry into rat primary neurons and cultured Neuro2A cells. We found that there were two sequential steps during the association of BoNT/A with neurons. The initial step was ganglioside dependent, while the subsequent step involved association with synaptic vesicles. H_C A1 and H_C A2 entered the same population of synaptic vesicles and entered cells at similar rates. The primary difference was that H_C A2 had a higher degree of receptor occupancy for cells and neurons than H_C A1. Thus, H_C A2 and H_C A1 share receptors and entry pathway but differ in their affinity for receptor. The initial interaction of H_C A1 and H_C A2 with neurons may contribute to the unique pathologies of BoNT/A1 and BoNT/A2 in mouse models.

KEYWORDS botulinum toxin, gangliosides, synaptic vesicles, synaptic vesicle protein 2, TIRF microscopy, clostridial neurotoxins, *Clostridium botulinum*, toxins

Botulinum neurotoxins (BoNTs) are AB exotoxins secreted by several species of the genus *Clostridium*. BoNTs are single-chain 150-kDa proteins cleaved by either bacterial or host proteases to a dichain comprising a 50-kDa light chain (LC) and a 100-kDa heavy chain (HC) linked by an interchain disulfide bond. The HC includes a translocation domain (H_N) and receptor binding domain (H_C) (1). The H_C includes an N-terminal subdomain (H_{CN}) of limited known function and a C-terminal subdomain (H_{CC}) that confers neuron specificity by binding dual neuron-specific receptors. There are seven BoNT serotypes (A to G) (2). BoNT serotype A (BoNT/A) binds a ganglioside and synaptic vesicle glycoprotein 2 (SV2) (3–6), which allows rapid toxin entry via synaptic vesicles. Acidification of the synaptic vesicle lumen triggers H_N to form a channel to facilitate LC translocation into the cytosol. Intracellular LC cleaves a SNARE (soluble N-ethylmaleimide-sensitive factor [NSF] attachment protein receptor) protein (7–12). SNARE protein cleavage inhibits exocytosis of cholinergic synaptic vesicles at neuromuscular junctions. BoNT LCs are long-lived proteases, sustaining paralysis in humans for several months depending on the serotype (2), which led to the licensing of BoNT/A and BoNT/B for human therapies (13, 14).

BoNT serotypes include subtypes neutralized by serotype-specific antisera (15–17). Informatics has classified eight BoNT/A subtypes (A1 to A8), which vary by ~10 to 15% in amino acid identity (15, 16, 18–21). While BoNT/A1 and BoNT/A2 cleave SNAP25 with similar kinetics (22–24) and possess similar potencies in the mouse model of botulism (BoNT/A1 and BoNT/A2 had specific activities of 1.25×10^8 and 1.27×10^8 50% lethal doses [LD_{50}] [in U/mg], respectively, in mice) (25, 26), BoNT/A1 and BoNT/A2 elicit

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FIG 1 H_cA2 and H_cA1 binding to neurons. Rat primary cortical neurons were cooled to 4°C. Neurons were incubated with 40 nM FLAG-H_cA1, FLAG-H_cA2, or FLAG-H_cT for 30 min. Cells were washed, fixed, and stained for FLAG and synaptophysin 1 (Syp1). (A) Representative images of neurons stained for FLAG (red) and Syp1 (green), with an inset of the merged images below. (B) FLAG intensity was normalized to Syp1 intensity, and data from eight independent fields were averaged. (C) Colocalization of FLAG (H_c) with Syp1 was measured, using Pearson's correlation coefficients (ImageJ). Scale bar, 20 μm. The data sets shown are representative of replicate experiments and were evaluated using one-way ANOVA with GraphPad Prism version 5. For all figures, significance is expressed as follows: ns, not significant and $P > 0.05$; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$.

different paralytic symptoms in mice (24). For example, BoNT/A1 appears to be more potent than BoNT/A2 for grip strength on the contralateral side (27), and BoNT/A1 shows more contralateral spread than BoNT/A2, which is prevented by colchicine treatment in a rat model (26, 27). In addition, BoNT/A2 is more potent than BoNT/A1 in cultured primary neurons (24, 28). Retrograde movement of BoNT/A1 has been observed in mice (29–31) and cultured neurons (32), but BoNT/A2 trafficking has not been examined. Thus, to identify potential differences between BoNT/A1 and BoNT/A2, entry of H_cA1 and H_cA2 into neurons was studied. This study identifies receptor occupancy as a primary difference in H_cA1 and H_cA2 binding to neurons, which may contribute to the unique pathologies of BoNT/A1 and BoNT/A2 in mouse models.

RESULTS

H_cA1 and H_cA2 binding to neurons. To analyze the initial membrane binding step, rat cortical neurons were cooled to 4°C and incubated with 40 nM receptor binding domain (H_c) of BoNT/A1 (H_cA1), BoNT/A2 (H_cA2), or tetanus neurotoxin (H_cT) (Fig. 1).

FIG 2 H_cA2 and H_cA1 show unique entry into synaptic vesicles. Rat primary cortical neurons were incubated with 40 nM FLAG-H_cA1 or FLAG-H_cA2 for 5 min in buffer containing 5.6 mM potassium (low K⁺) or 56 mM potassium (high K⁺). Cells were washed, fixed, and stained for FLAG (red) and Syp1 (green). Upper panels, representative images are shown. Bottom left panel, FLAG intensity was normalized to Syp1 intensity. Bottom right panel, Pearson's correlation coefficients between FLAG and Syp1 were measured using ImageJ. Quantification was averaged from eight independent fields. Scale bar, 20 μ m.

H_cA2 bound neurons at $\sim$ 10-fold-higher efficiency than H_cA1 (Fig. 1B). H_c colocalization with synaptophysin 1 (Syp1) was measured using Pearson's correlation coefficients. In this experimental system, Pearson's coefficients of >0.6 indicate colocalization, those of 0.3 to 0.6 indicate partial colocalization, and those of <0.3 indicate segregation, as previously determined (33). Syp1 is a marker for synaptic vesicles (34–36). At 4°C, Pearson's coefficients were $\sim$ 0.2 for H_cA1 and Syp1 and $\sim$ 0.3 for H_cA2 and Syp1 (Fig. 1C), which indicated that both H_cA1 and H_cA2 were segregated from Syp1. In similar experiments at 4°C, bound H_cA1 and H_cA2 also segregated from SV2a (Pearson's coefficients of <0.3), the protein receptor for BoNT/A (data not shown) (4). The observed differences in Pearson's coefficient between H_cA1 and Syp1 and H_cA2 and Syp1 were statistically significant but were not considered biologically significant. H_cT was a positive control for proteins that bind neurons at 4°C (33). Together, the data show that the initial binding of H_cA1 and H_cA2 to neurons occurs independent of synaptic vesicle proteins and that H_cA2 had a higher initial binding to neurons than H_cA1.

H_cA1 and H_cA2 entry into neurons. Upon establishing low-temperature binding properties and ganglioside binding specificity, entry of H_cA1 and H_cA2 into rat cortical neurons was measured at 37°C. In low-K⁺ buffer, which emulates resting neurons, H_cA1 and H_cA2 showed partial colocalization with Syp1, where more H_cA2 than H_cA1 was cell associated with Syp1. This indicated that H_cA2 had a higher probability of being associated with synaptic vesicle proteins on the cell membrane than H_cA1. Upon membrane depolarization, both H_cA1 and H_cA2 colocalized with Syp1 (Fig. 2). H_cA2 also entered rat primary hippocampal neurons more efficiently than H_cA1 (Fig. 3). A dose-response assessment (Fig. 4A and B) showed that bound H_cA1 increased with increasing H_c and that H_cA1 colocalization with Syp1 increased with increasing H_c and approached the level of H_cA2 association with Syp1 (Fig. 4C). Together, these data

FIG 3 H_cA2 and H_cA1 entry into rat primary hippocampal neurons. Rat primary hippocampal neurons were incubated with 40 nM FLAG-H_cA1 or FLAG-H_cA2 for 5 min in buffer containing 5.6 mM potassium (low K⁺) or 56 mM potassium (high K⁺). Cells were washed, fixed, and stained for FLAG-H_cs and synaptophysin 1. Upper panels, representative images are shown. Bottom left panel, FLAG intensity was normalized to Syp1 intensity. Bottom right panel, Pearson's correlation coefficients between FLAG and synaptophysin 1 were measured using ImageJ. Quantification was averaged from eight independent fields. Scale bar, 20 μ m.

show that H_cA2 has higher affinity for synaptic vesicle proteins than H_cA1 on resting neurons and that both H_cA1 and H_cA2 localize with synaptic vesicle proteins upon membrane depolarization.

H_cA1 and H_cA2 compete for entry into neurons. A competition experiment determined if H_cA1 and H_cA2 used a similar pathway to enter neurons. Neurons were incubated with 40 nM hemagglutinin (HA)-tagged H_cA1 or H_cA2, alone or with 400 nM untagged H_cA1 or H_cA2 (Fig. 5). H_cA1 and H_cA2 effectively competed for H_cA1 association with neurons, while H_cA2 was more effective than H_cA1 as a competitor for H_cA2 association with neurons. H_cT of tetanus neurotoxin was a negative control for competing with H_cA1 or H_cA2 for cell association with synaptic vesicles, as we previously observed that H_cT does not bind SV2 (33). H_cT was less effective in competing for H_cA1 than either H_cA1 or H_cA2 (Fig. 5). The ability of H_cA1 and H_cA2 to compete with the homologous and heterologous H_cA subtype supports the entry of both H_cs into similar synaptic vesicles. H_cA2 also appears a more efficient competitor for entry, possibly due to a higher affinity for gangliosides than H_cA1.

Ganglioside interactions of H_c with neurons and Neuro2A cells. H_cA1 binds gangliosides GT1b and GD1a via the terminal 4-galactose and the 5-sia sugars (5, 37). We previously showed that W1266, a component of the ganglioside binding pocket of H_cA, was required for ganglioside binding, as H_cA1(W1266A) failed to bind gangliosides and did not have a detectable effect on H_cA1 structure (PDB 4IQP) (38). Since H_cA1 and H_cA2 are ~90% identical, we predicted that W1266 in H_cA2 would also contribute to ganglioside binding. H_cA2 binding and entry into neurons were W1266

FIG 4 Concentration-dependent entry of H_cA1 into synaptic vesicles. Rat cortical neurons were incubated with HA-H_cA1 (20 to 80 nM) or HA-H_cA2 (20 nM) for 5 min in buffer containing 5.6 mM potassium (low K⁺) or 56 mM potassium (high K⁺). Cells were fixed and processed for immunofluorescence. (A) Representative images are shown. (B) The average intensity of HA (H_c)/Syp1 was calculated from eight fields. (C) Pearson's coefficients were calculated to determine colocalization between HA-tagged protein and Syp1. The experiment was performed in triplicate, and the values were averaged. Scale bar, 20 μ m.

dependent (Fig. 6). The similar binding of H_cA2(W1266A) and H_cA2 to resting neurons (low K⁺) at 37°C may be due to ganglioside-independent association with SV2 on the cell membrane, consistent with two independent receptor binding interactions, via gangliosides and SV2. Note that the inability of H_cA2(W1266A) to enter synaptic vesicles upon membrane depolarization is consistent with the need for dual receptor interaction to facilitate intracellular entry, as previously observed with tetanus toxin (39).

The ganglioside binding specificities of H_cA1 and H_cA2 were tested in Neuro2A cells, a mouse neuroblastoma cell line. Neuro2A cells express synaptic vesicle proteins, including SV2c, the BoNT/A receptor, but do not express complex gangliosides at levels comparable to those for primary neurons (40). Exogenous ganglioside-enriched Neuro2A cells were incubated with H_cA1 and H_cA2 and analyzed for bound H_c (Fig. 7). In ganglioside-enriched Neuro2A cells, both H_cA2 and H_cA1 localized to SV2c-positive perinuclear vesicles, with ~4-fold more H_cA2 cell associated than H_cA1. H_cA2 also had a higher baseline entry into Neuro2A cells without addition of exogenous gangliosides, which may reflect H_cA2's greater affinity for gangliosides than H_cA1. GT1b and GD1a contain sialic acid at the position 5 of the ganglioside, which appeared to target H_cA1 and H_cA2 into the cells. Association with each ganglioside was sufficient to deliver H_cA1 and H_cA2 into SV2-containing vesicles. GD1b-mediated H_c entry was low for both H_cA1 and H_cA2, but the difference did not reach statistical significance for H_cA1. This

FIG 5 H_cA2 and H_cA1 compete for entry into synaptic vesicles. Rat primary cortical neurons were incubated with 40 nM HA-tagged H_cA1 (right) or HA-H_cA2 (left) for 5 min in buffer containing 56 mM potassium (high K⁺) and 400 nM untagged competitor HC (H_cA1, H_cA2, or H_cT). Cells were washed, fixed and, stained for HA (H_c) and synaptophysin 1 (Syp1). Upper panels, representative images of neurons. Lower panel, HA intensity (H_cA1 and H_cA2) was normalized to Syp1 intensity, and data were averaged from eight independent fields.

could reflect the limitation of the resolution of the assay, or binding to b-series gangliosides may have a different H_c entry rate than a-series ganglioside-mediated H_c entry. The higher association of H_cA2 with the plasma membrane without addition of gangliosides may be due to association with endogenous a-series gangliosides or SV2c on the plasma membrane. Thus, H_cA2 has a higher affinity for gangliosides than H_cA1, but H_cA2 and H_cA1 have same ganglioside binding specificity.

Entry of H_cA in Neuro2A cells. A time course evaluated the entry of H_cA1 and H_cA2 into Neuro2A cells. At each time point, individual cells were scored for perinuclear H_cA (SV2-associated vesicles), using a rubric assessing random fields at each time point (Fig. 8A). The intensity of GT1b-dependent, internalized H_cA1 was lower at all time points than that of H_cA2. After 20 min, H_cA1 perinuclear localization had not reached the intensity of that of H_cA2 (Fig. 8B). The initial rate of uptake for the H_cs was measured by quantifying the percentage of cells that contained at least 2 H_cA perinuclear localized puncta (Fig. 8C). At 0.5 min, H_cA1 and H_cA2 had similar percentages of cells with 2 or more puncta, while at later times, H_cA2 entry into cells approached a 2-fold-higher rate than that of H_cA1. Note that H_cAs were rapidly internalized, with

FIG 6 W1266 mediates H_cA2 binding to neurons. Rat primary cortical neurons were cooled to 4°C and incubated with 40 nM FLAG-tagged H_cA2(W1266A) or H_cA2 for 20 min. Cells were washed, fixed, stained for FLAG epitope and synaptophysin 1 (Syp1), processed, and analyzed. (A and B) Representative images of neurons stained for FLAG. (C) FLAG intensity was normalized with Syp1 intensity. (D) Neurons were maintained at 37°C and incubated with 40 nM H_cA2(W1266A) or H_cA2 for 5 min in buffer containing 5.6 mM potassium (low K⁺) or 56 mM potassium (high K⁺).

~50% of cells containing internalized H_cA at 0.5 min. Thus, the initial internalization rates of H_cA1 and H_cA2 are similar, and H_cA2 internalization is more efficient than that of H_cA1 due to a greater receptor occupancy.

Entry of site-mutated H_cA1 into neurons. An informatics assessment of the amino acids surrounding the ganglioside showed that residues 1271 to 1274 were unique between H_cA1 and H_cA2 (37). Gain-of-function mutations were used to assess a role for these residues in H_c entry. Mutations were engineered into H_cA1 at a loop adjacent to the ganglioside binding site which included four amino acid substitutions between H_cA1 and H_cA2, H_cA1(IERS1271-1274VGKA). Resting and upon membrane depolarization, H_cA1(IERS1271-1274VGKA) possessed entry properties intermediate between those of H_cA1 and H_cA2 (Fig. 9). This supports a role for the loop from residue 1271 to 1274 in H_c entry.

Entry of an H_c chimera into neurons. The ganglioside binding site resides in the C-terminal subdomain of the H_c (residues 1090 to 1296), while the SV2 binding site resides within the C-terminal subdomain on an adjacent face of the H_c and close to the N-terminal jellyroll subdomain (residues 870 to 1090) (41–43). Contributions of subdomains in neuron entry were examined with H_cA1 and H_cA2 chimeras, fusing the N-terminal subdomain with the C-terminal subdomain of reciprocal H_c. H_cA1A2 contained the N-terminal subdomain of H_cA1 and the C-terminal subdomain of H_cA2, while H_cA2A1 contained the N-terminal subdomain of H_cA2 and the C-terminal sub-

FIG 7 H_cA2 and H_cA1 share ganglioside binding specificity. Neuro2A cells were loaded with 20 μ g/ml purified gangliosides (GT1b, GD1b, GD1a, or GM1a) for 4 h. Cells were incubated with 40 nM HA-H_cA1 or HA-H_cA2 for 20 min at 37°C. Cells were fixed and stained for HA and SV2c. Upper panel, representative images. Bottom left panel, HA intensity (H_c) was normalized to SV2c intensity. Bottom right panel, schematic of the four gangliosides used in this experiment, with the 5-sialic acid-galactose bound by H_cA highlighted in gangliosides GT1b and GD1a. Scale bar, 50 μ m.

domain of H_cA1. H_cA2A1 could not be purified to sufficient homology and was not utilized further in this study (Fig. 10A). In resting neurons, H_cA1A2 binding to neurons was similar to that of H_cA2 (Fig. 10B and C), and upon membrane depolarization, H_cA1A2 showed increased association, which approached that of H_cA2 (Fig. 10D). This indicated that the C-terminal subdomain of H_cA appears to be sufficient for synaptic vesicle entry.

DISCUSSION

BoNT/A1 and BoNT/A2 possess similar potencies but have unique rates of entry into neurons and unique pathologies in mouse models of botulism (28). The current study resolved the sequential entry of H_cA1 and H_cA2 entry into neurons, identifying an initial ganglioside-dependent step and a subsequent step involving association with synaptic vesicles. Competition experiments in neurons supported H_cA1 and H_cA2 entry into the same population of synaptic vesicles. The rates of cell entry were similar for the two subtypes, while H_cA2 had a higher degree of receptor occupancy than H_cA1. Thus, H_cA2 and H_cA1 share receptors and entry pathways but differ in their affinity for their receptor.

The current studies resolved the sequential entry of H_cA1 and H_cA2 into neurons with an initial association which was dependent upon ganglioside binding. H_cA2 bound primary neurons with greater affinity than H_cA1, independent of the association with synaptic vesicle marker proteins. At 37°C, in resting neurons H_cA2 had a greater association with synaptic vesicle proteins than H_cA1, but upon membrane depolarization, both H_cS colocalized with synaptic vesicles. H_cA2 and H_cA1 entered into similar populations of synaptic vesicles, with H_cA2 competing more efficiently for entry than H_cA1. Assessment of H_cA1A2 implicated the C-terminal subdomain of the H_c (H_cC) as the primary determinant for neuron binding and synaptic vesicle association. Thus, the unique entry of H_cA1 and H_cA2 into synaptic vesicles may contribute to the unique rates of neuron intoxication and pathologies elicited by BoNT/A1 and BoNT/A2.

While early studies implicated gangliosides as host receptors for the clostridial

FIG 8 H_CA1 and H_CA2 entry into Neuro2A cells. Neuro2A cells were loaded with 20 μ g/ml purified GT1b or left unloaded (none) for 4 h. Cells were washed and incubated with 40 nM FLAG-H_CA1 or FLAG-H_CA2 for 1.25, 2.5, 5, 10, or 20 min at 37°C. Cells were fixed and stained for FLAG and SV2c. (A) Representative images of FLAG-reactive cell-associated material at 1.25- and 20-min incubations. (B) Individual cells within random fields were scored for internalized perinuclear FLAG intensity and assigned an arbitrary number (0, 1, or 2). Representative cells are shown by score, with cells scored as 0 containing no intensity, 1 containing intermediate intensity, and 2 containing the most intensity. The cumulative score was divided by the total number of cells per field and plotted. At least 10 fields, or >100 cells per condition, in three replicates were counted. (C) The rate of endocytosis for H_CA1 and H_CA2 was assessed by incubation of a 40 nM concentration of either protein with Neuro2A cells for 0.5, 1, or 2 min. Individual cells were scored as positive for internalized signal if at least 2 FLAG-reactive puncta were present within the perinuclear region (SV2c marker) of a cell. Data are presented as percentage of cells containing internalized signal. Linear regression was performed and slopes plotted for H_CA1 and H_CA2. An unpaired Student *t* test between H_CA1 and H_CA2 in GT1b-loaded Neuro2A cells was performed at each time point. Scale bar, 50 μ m.

neurotoxins (44, 45), Montecucco and Schiavo proposed that clostridial neurotoxins utilized dual host receptors, “a protein and a lipid,” to generate high-affinity binding to neurons (46). Subsequently, synaptotagmin was identified as the BoNT/B protein receptor (47, 48), and SV2 was identified as the BoNT/A protein receptor (4, 6, 41), where stimulation of synaptic vesicle cycling enhanced the efficiency of H_C entry. The current study supports a sequential utilization of the dual BoNT host receptors. At low temperature, H_Cs bound neurons but segregated from synaptic vesicle marker proteins, while at 37°C, H_Cs associated with synaptic vesicle proteins, which increased upon membrane depolarization. The greater association of H_CA2 with synaptic vesicle proteins in resting neurons, relative to that of H_CA1, implicates a greater receptor occu-

FIG 9 H_cA1(IERS1271-1274VGKA) possesses a response to membrane depolarization of neurons intermediate between those of H_cA1 and H_cA2. Rat primary cortical neurons were incubated with 40 nM FLAG-H_cA1, H_cA1(IERS1271-1274VGKA), or H_cA2 in buffer containing 5.6 mM potassium (low K⁺) or 56 mM potassium (high K⁺) at 37°C for 5 min. Cells were washed, fixed, and stained for FLAG epitope (H_c) and synaptophysin 1 (Syp1). (A) Representative images for FLAG intensity. (B) FLAG intensity was normalized with Syp1 intensity and shown as mean values with standard error. (C) Colocalization of FLAG with Syp1 was measured, using Pearson's correlation coefficients (ImageJ).

pancy for H_cA2, which may explain why BoNT/A2 cleaves SNAP-25 more rapidly than BoNT/A1 in cultured neurons (28).

Rapid SNAP-25 cleavage in neuron cultures may be a result of unique interactions of H_cA2 with gangliosides. H_cA2 is more efficient at entering Neuro2A cells despite similar rates of uptake of H_cA1 and H_cA2 (Fig. 8). The greater association of H_cA2 than of H_cA1 for gangliosides observed in cooled and resting neurons (Fig. 1 and 2) may lie in H_c determinants outside amino acid interactions within the H_c ganglioside binding pocket, since H_cA1 and H_cA2 possess 7 of 8 identical amino acids that contact GT1b: Y1117, E1203, F1252, H1253, S1264, W1266, S1275, and R1276 (37). Y1117 is not conserved in H_cA2 and is instead a Phe (Fig. 11C). However, introduction of the Y1117F mutation into H_cA1 did not enhance the binding properties of H_cA1 for neurons (data not shown). This indicates that the loss of the -OH from the aromatic R group at residue 1117 did not enhance ganglioside binding. Examination of ganglioside-bound H_cA1 (PDB 2VU9) versus ganglioside-free H_cA1 (PDB 3BTA) provides a candidate mechanism for the observed differential ganglioside binding affinities of H_cA1 and H_cA2, where a loop comprising residues 1269 to 1275 that lies adjacent to the ganglioside binding pocket appears “flipped away” from the ganglioside binding site upon GT1b occupancy (Fig. 11). We hypothesize that regions adjacent to the ganglioside binding pocket may

FIG 10 The C-terminal subdomain contributes to binding and entry of H_c into synaptic vesicles. (A) Schematic of H_c domains of BoNT/A1 (white) and BoNT/A2 (black), with the constructed H_cA1A2 chimera. Rat primary cortical neurons were incubated with 20 nM HA-H_cA1, HA-H_cA2, or HA-H_cA1A2 for 5 min in buffer containing 5.6 mM potassium (open, low K⁺) or 56 mM potassium (solid, high K⁺). Cells were washed, fixed, and stained for HA epitope (H_c) and synaptophysin 1 Syp1. (B) Representative images. (C) HA intensity was normalized to Syp1 intensity and shown as mean values with standard error. (D) Colocalization between HA and Syp1 was measured, using Pearson's coefficients. Scale bar, 40 μm.

contribute to the greater affinity of H_cA2 for gangliosides than H_cA1, without influencing the ganglioside specificity of the H_cs. The observation that H_cA1(IERS1271-1274VGKA) possessed an intermediate association with neurons, relative to those of H_cA1 and H_cA2 (Fig. 10), supports this hypothesis. Additional distanced amino acids, not yet identified, may also contribute to the observed affinity of H_cA2 for gangliosides.

Recently, two reports identified residues of BoNT/A1 involved in SV2 binding. Benoit and colleagues (43) crystalized H_cA1 complexed with the fourth luminal loop (L4) of SV2c, which formed a right-handed quadrilateral β-helix and interacted with the H_cA1 β-sheets 40 and 41 (residues 1140 to 1153; secondary structure assignment as defined by Lacy and Stevens [49]) (43). This SV2 interaction site is located in the C-terminal subdomain of H_cA1 yet distant from the ganglioside binding pocket. Although several of the H_c-SV2 contacts were via main-chain interactions, four residues at the interface of the interaction site when mutated resulted in decreased binding between H_cA1 and SV2c (43). This suggests that structural packing contributes to the H_c-SV2 interaction, rather than the R-group composition, and is consistent with regions outside the SV2-L4 contributing to SV2 affinity (Fig. 11). Strotmeier and colleagues used site-directed mutagenesis to determine residues contributing to the interaction of BoNT/A1 with

FIG 11 Molecular organization of the dual receptors of BoNT/A. (A) Schematic of BoNT/A1 (PDB 3BTA) light chain (LC) and heavy chain (HC). (B) BoNT/A1-H_c-GT1b complex (PDB 2VU9) with the bound SV2-L4 loop (blue) (PDB 4JRA) binding site. Residues 1143 to 1149 of H_cA1 interacting with SV2-L4 are indicated (orange). The ganglioside binding pocket is shown (magenta). (C) H_cA1 ganglioside binding site (GT1b), including H1251, S1264, W1266, and Y1267 (magenta). Adjacent to the GT1b site is Y1117 (cyan). (D) The loop from residue 1271 to 1274 from the BoNT/A structure with side chains and surrounding main chain is overlaid in red. This loop opens upon GT1b occupancy (black) as opposed to unbound (red).

SV2c-L4 (42). SV2c-interacting residues were identified at the interface of the N and C subdomains of H_cA1, which partially overlapped the region crystalized with the SV2-L4 loop. While making substantial contributions toward deciphering H_c-SV2 interactions, neither study explains the subtype specificity of BoNT/A1 and BoNT/A2 with SV2. The current study showed H_cA1A2 responded to membrane depolarization, which supports the localization of the SV2 binding site within the C-terminal subdomain. Our hypothesis is that the primary interaction between the H_c and SV2 is mediated by the C-terminal subdomain of the H_c. The determination that main-chain interactions contribute to H_cA1-SV2-L4 association (43) is consistent with a region(s) outside the H_cA1-SV2-L4 also contributing to BoNT/A-synaptic vesicle interactions (Fig. 11). These interactions may reside within other regions of SV2, including N-linked glycans, or may involve other synaptic vesicle proteins, since synaptic vesicle proteins form higher-order complexes and in synaptic vesicle lysates (50), and earlier studies showed that H_cA1 bound synaptic vesicle protein complexes (51). Current studies are assessing the role of the N-terminal and C-terminal subdomain interfaces of H_cA1 and H_cA2 to test for potential sites of interaction with SV2.

Despite the dissimilarities in occupancy between H_cA1 and H_cA2 observed in neurons and Neuro2A cells, the potencies in the mouse model of BoNT/A1 and

BoNT/A2 are similar (26). Thus, the differential properties of the H_C reported in the current study may contribute to the rate of neuronal cell intoxication and unique BoNT pathologies associated with the various BoNT/A subtypes (25, 52). This implies that absolute neurotoxin potency can be uncoupled from the observed unique pathologies of the BoNT/A subtypes in mouse models of botulism. The symptoms associated with intoxication are different for BoNT/A1 and BoNT/A2 in mice, where intravenous injection of BoNT/A1 caused ruffled fur, labored breathing, descending paralysis, and spasticity before death (52), while intravenous injection of BoNT/A2 caused paralysis of the front legs, hind legs, and whole body, followed by death (24). Further, BoNT/A1 diffuses more readily than BoNT/A2 to the contralateral side due to retrograde movement (27, 29, 53); whether retrograde trafficking of BoNT plays a role in the symptoms elicited by the toxins remains unknown. The symptoms of other BoNT subtypes appear to be related to their similarity. BoNT/A3 shares 93% amino acid identity with BoNT/A2, and both cause similar symptoms in mice (52). BoNT/A5 shares 97% identity with BoNT/A1 and causes paralysis analogous to BoNT/A1 in mice (24). Further elucidation of the molecular mechanisms that differentiate BoNT/A1 and BoNT/A2 will contribute to our understanding of how clostridial neurotoxins elicit paralysis and how to modulate these neurotoxins to enhance therapeutic potency.

MATERIALS AND METHODS

Subcloning and production of epitope tagged-H_Cs. *Escherichia coli* codon-optimized DNA encoding H_CA1 (S877 to L1296) and H_CA2 (K871 to L1296), also referred to as H_C/A1 and H_C/A2, were synthesized by EZBiolab and GenScript, respectively. DNA encoding each H_CA was subcloned into a modified pET-28a (Novagen), which introduced 6-His and 3-FLAG epitopes N-terminal to the H_C. DNAs encoding H_CA1 and H_CA2 were also subcloned into a modified pET-28a vector to replace the 3-FLAG epitope with three repeats of the human influenza virus hemagglutinin epitope (HA) (YPYDVPDYA), which is recognized by a monoclonal rat antibody (clone 3F10; Roche). The pET-28a vector containing each H_C was transformed into *E. coli* BL21(DE3) (Stratagene) for protein expression. Bacterial cell culturing and protein purification by sequential column chromatography were performed as previously described (54). Proteins were resolved and normalized by SDS-PAGE. FLAG-H_C and HA-H_C showed similar properties with regard to binding and entry into neurons (A. R. Kroken and J. T. Barbieri, unpublished data). Mutated forms of H_CA1 were generated, using a QuikChange site-directed mutagenesis kit (Stratagene), and chimeras between H_CA1 and H_CA2 were generated by overlap PCR. Point mutations and chimeras were confirmed by DNA sequencing.

Cell culturing of primary neurons. E18 rat primary cortical, spinal, or hippocampal neurons (BrainBits, LLC) were cultured on 24-well glass-bottom dishes (MatTek) coated with poly-D-lysine (Sigma) and laminin (Life Technologies) in Neurobasal medium (Life Technologies) supplemented with B27, Glutamax (Life Technologies), and Primocin (InvivoGen) according to supplier directions. On day 7, half of the culture medium was changed, and cells were analyzed on days 10 to 14. Neuro2A cells (ATCC) were plated on glass coverslips coated with collagen I and cultured in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), 0.1% sodium bicarbonate, 1× nonessential amino acids, 1 mM sodium pyruvate, 50 units/ml penicillin, and 50 µg/ml streptomycin (Life Technologies).

Entry of H_Cs into neurons and Neuro2A cells. Cells were washed twice with Dulbecco's phosphate-buffered saline (DPBS), incubated with H_Cs in buffer for 5 min at 37°C or chilled at 4°C for 10 min, and incubated with H_Cs. Buffers were either high potassium (high K⁺) to stimulate membrane depolarization and Ca²⁺-dependent synaptic vesicle cycling (56 mM KCl, 2.2 mM CaCl₂, 15 mM HEPES, 145 mM NaCl, 0.5 mM MgCl₂, pH 7.4) or low potassium (low K⁺) to emulate resting membranes (5.6 mM KCl, 2.2 mM CaCl₂, 15 mM HEPES, 145 mM NaCl, 0.5 mM MgCl₂, pH 7.4). Cells were washed twice and fixed in 4% (wt/vol) paraformaldehyde in DPBS for 15 min. Neuro2A cells were loaded with 10 µg gangliosides (GT1b, GD1b, GD1a, or GM1a; Matreya) in 0.5 ml (20 µg/ml) of complete MEM containing 0.5% FBS for 4 h prior to incubation with H_Cs in low-potassium buffer. Cells were washed twice and fixed in 4% (wt/vol) paraformaldehyde in DPBS for 15 min.

Immunofluorescence of fixed neurons or Neuro2A cells. Cells were permeabilized with 0.1% Triton X-100–4% formaldehyde in DPBS for 15 min, washed twice, incubated with 150 mM glycine for 15 min, washed twice, and then incubated in 10% (vol/vol) FBS–2.5% (wt/vol) fish skin gelatin (Sigma)–0.1% Triton X-100–0.05% Tween 20 (blocking solution) for 1 h. Fixed cells were incubated with primary antibodies (mouse anti-FLAG, 1:10,000 [Sigma]; rat anti-HA, 1:2,000 [Roche]; guinea pig anti-synaptophysin 1, 1:2,000 [Synaptic Systems], or rabbit anti-SV2C, 1:2,000 [Synaptic Systems]) at 4°C overnight. Cells were then washed 3 times for 5 min, and Alexa-labeled secondary antibodies (1:1,000; Life Technologies) were incubated at room temperature for 1 h. Cells were washed 4 times for 5 min, with the final wash containing DAPI (4',6'-diamidino-2-phenylindole) stain, and fixed with 4% paraformaldehyde. Neurons were preserved in CitiFluor AF-3 (Electron Microscopy Sciences), and Neuro2A cells were mounted on slides containing ProLong Gold (Life Technologies) and sealed with nail polish.

Imaging and analysis of stained cells and neurons. Micrographs were acquired with a Nikon TE2000 inverted total internal reflection fluorescence (TIRF) microscope equipped with a Nikon Apo TIRF 100×/1.49 numerical aperture (NA) oil objective or a Plan Apo VC 60×/1.40 NA oil objective and CoolSnap HQ2 camera (Photometrics) with Nikon Elements or Metamorph acquisition software. At least 8 fields were imaged for each experimental replicate, and fields were chosen while observing synaptophysin 1 (Syp1) or SV2c staining to limit bias. Intensity and Pearson's correlation coefficients were measured using ImageJ (NIH) and the Intensity Correlation Analysis plugin (Wright Cell Imaging Facility). The numerical values for FLAG/Syp1 and other normalized intensity are expressed as arbitrary units (AU). Data sets shown are representative of replicate experiments and were evaluated by one-way analysis of variance (ANOVA) statistics, using GraphPad Prism version 5: ns, not significant ($P > 0.05$); *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; and ****, $P \leq 0.0001$. Data sets for H₂A1 and H₂A2 uptake in Neuro2A cells were evaluated by the unpaired Student *t* test, using GraphPad Prism version 5; significance cutoffs were as described above.

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