Molecular Basis for Tetanus Toxin Coreceptor Interactions[†]

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ABSTRACT: Tetanus toxin (TeNT) elicits spastic paralysis through the cleavage of vesicle-associated membrane protein-2 (VAMP-2) in neurons at the interneuronal junction of the central nervous system. While TeNT retrograde traffics from peripheral nerve endings to the interneuronal junction, there is limited understanding of the neuronal receptors utilized by tetanus toxin for the initial entry into nerve cells. Earlier studies implicated a coreceptor for tetanus toxin entry into neurons: a ganglioside binding pocket and a sialic acid binding pocket and that GT1b bound to each pocket. In this study, a solid phase assay characterized the ganglioside binding specificity and functional properties of both carbohydrate binding pockets of TeNT. The ganglioside binding pocket recognized the ganglioside sugar backbone, Gal-GalNAc, independent of sialic acid-(5) and sialic acid-(7) and GM1a was an optimal substrate for this pocket, while the sialic acid binding pocket recognized sialic acid-(5) and sialic acid-(7) with "b"series of gangliosides preferred relative to "a" series gangliosides. The high-affinity binding of gangliosides to TeNT HCR required functional ganglioside and sialic acid binding pockets, supporting synergistic binding to coreceptors. This analysis provides a model for how tetanus toxin utilizes coreceptors for high-affinity binding to neurons.

Clostridium botulinum neurotoxins, serotypes A−G (BoNTs),[1] and *Clostridium tetani* neurotoxin (TeNT) are zinc proteases that inhibit synaptic vesicle fusion with the plasma membrane through the cleavage of soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins (*1–4*). BoNTs act at the peripheral neuromuscular junction which results in flaccid paralysis, while TeNT initially binds to peripheral neurons and retrograde traffics to the central nervous system where glycine release is blocked in TeNT-intoxicated inhibitory interneurons to elicit spastic paralysis. TeNT and BoNT are structurally similar, sharing 30−40% sequence homology (*5*). TeNT and BoNTs are produced as ∼150 kDa dichain proteins, comprising a light chain (LC, 50 kDa) and a heavy chain (HC, 100 kDa) linked by a disulfide bond. The LC encodes the zinc protease domain; TeNT, BoNT/B, BoNT/D, BoNT/F, and BoNT/G cleave vesicle-associated membrane protein-2 (VAMP-2) (*6, 7*), while BoNT-A and BoNT-E cleave the 25 kDa synaptosome-associated protein (SNAP-25) (*6, 8*). BoNT/C cleaves both SNAP-25 and syntaxin (*9–11*). The HC is composed of an N-terminal translocation domain (HCT) and a C-terminal receptor binding domain (HCR). The HCT forms a pore in the endosome membrane that is responsible for a pH-dependent translocation of the LC into the cytosol (*12*).

Current models implicate a coreceptor mechanism in the binding of the clostridial neurotoxins to neurons, which comprises a ganglioside and a protein that sequentially cooperate to form a high-affinity toxin binding site (*13*). Gangliosides, sialic acid-containing glycosphingolipids, are located on the outer leaflet of cell membranes and contain a common "core" (asialo-GM1) consisting of Gal(β1−3)-GalNAc(β1−4)Gal(β1−4)Glc(β1−1)Cer to which one or more *N*-acetylneuraminic acids (sialic acids) are bound, yielding "a" and "b" series gangliosides (*14, 15*). Gangliosides of the "b" series, especially GT1b and GD1b, have the highest affinity for BoNT/A. The dissociation constant of BoNT/A-HCR for GT1b-containing liposomes was recently reported to be $\sim 10^{-8}$ M (*16, 17*). In contrast, BoNT/D appears unique and binds phosphatidylethanolamine as a receptor rather than gangliosides (*18*). Structures of the ganglioside binding pockets that include a tryptophan are conserved among TeNT and BoNTs except for BoNT/D, which suggests a common ganglioside binding pocket. In addition to a ganglioside binding pocket, a second carbohydrate binding site, termed the sialic acid binding site, has been described uniquely for the association of TeNT with host cell membranes (*19*). While gangliosides are components of the neurotoxin receptor, the protease sensitivity of TeNT binding to both synaptosomes and neuronal cells implicates a role for a protein(s) as a receptor component (*20*). Unlike BoNTs, TeNT binding to neurons is sensitive to phospholipase C, implicating a role for a glycosylphosphatidylinositol-anchored glycoprotein as a membrane recep-

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[1] Abbreviations: TeNT, tetanus toxin; VAMP-2, vesicle-associated membrane protein-2; BoNTs, *C. botulinum* neurotoxins, serotypes A−G; TeNT, *C. tetani* neurotoxin; SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor; LC, light chain; HC, heavy chain; SNAP-25, 25 kDa synaptosome-associated protein; HCT, N-terminal translocation domain; HCR, C-terminal receptor binding domain; DPBS, Dulbecco's phosphate-buffered saline; BSA, bovine serum albumin.

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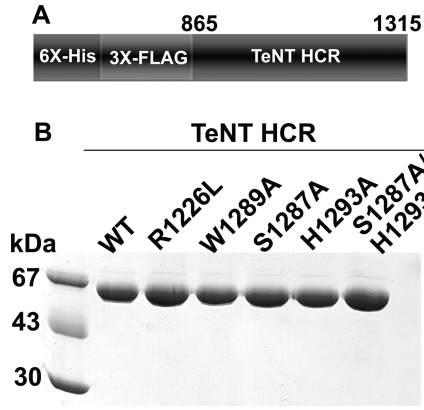

FIGURE 1: Wild-type and mutated TeNT HCRs. (A) Schematic diagram of TeNT HCR fusion proteins (residues 865−1315). (B). Recombinant His6-3×FLAG-TeNT HCRs were purified from *E. coli* using nickel affinity chromatography as described in Materials and Methods. Purified TeNT HCRs (5 μg) were separated by SDS−PAGE and visualized with Coomassie blue.

tor (*21*). This sialic acid binding site in TeNT may represent the interaction of TeNT with a sialic acid-containing glycoprotein receptor. Both the ganglioside and sialic acid binding pockets are required for the expression of neuronal toxicity of TeNT (*19, 22*). TeNT bound two molecules of GT1b, supporting a model of two independent carbohydrate binding pockets (*19*). The cocrystal of a GT1b analogue and the TeNT HCR showed that the ganglioside and sialic acid binding pockets of TeNT were located in different regions of TeNT (*23–25*). According to the cocrystal structure of the GT1b analogue and TeNT HCR, the hydrophobic faces of the Gal4-GalNAc3 sugar rings are stacked against the indole ring of W1289 and the polar faces are hydrogen bonded to the protein. However, the cocrystal structure cannot explain why TeNT has a higher affinity for GT1b and GD1b than for GM1a (*26–28*), as GT1b, GD1b, and GM1a share the same Gal4-GalNAc3 backbone.

While earlier studies assessed the binding of the TeNT HCR to gangliosides, in our study, the specificity of an individual carbohydrate binding pocket was tested using a quantitative solid binding assay. These studies resolve minimal functional gangliosides for each carbohydrate binding pocket. The data supported synergistic interactions between two carbohydrate binding pockets for high-affinity binding of TeNT to neurons.

MATERIALS AND METHODS

Production and Purification of Wild-Type (wt) and Mutated TeNT HCRs. pET-28a (Novagen) was modified to contain a 3×FLAG epitope directly downstream of the histidine tag as previously described (Figure 1A) (*29*). A synthetic TeNT HCR gene was synthesized that comprised a codon bias optimized for expression in *Escherichia coli*. Restriction sites *Kpn*I and *Pst*I were incorporated at the 5′ and 3′ ends, respectively, to allow subcloning into the modified pET28a expression vector. Site-directed mutations were introduced into the gene encoding the TeNT HCR by QuikChange site-directed mutagenesis (Stratagene). The mutations were confirmed by DNA sequence analysis. *E. coli* BL-21(DE3) cells were transformed with the pET28-TeNT HCR and grown overnight on an LB agar with 50 μg/mL kanamycin at 37 °C. Transformants were stored at −80 °C in 12% (v/v) glycerol. *E. coli* cells (pET-28a-TeNT HCR) were grown overnight on an LB agar plate with 50 μg/mL kanamycin and inoculated into LB medium (400 mL) containing the same antibiotic, at 30 °C for 2 h at 250 rpm to an OD_{600} of ∼0.6 when 0.1 mM IPTG was added, followed by an overnight culture at 250 rpm and 16 °C. Cells were harvested, lysed with a French press, and clarified by centrifugation and filtration (cellulose acetate). The HCR was purified from the filtered lysate using a Ni^{2+}-NTA spin column (Qiagen). Fractions containing purified HCRs were dialyzed overnight against Dulbecco's phosphate-buffered saline (DPBS) with calcium and magnesium. Purified proteins were then stored either at −20 °C in the presence of 40% (v/v) glycerol or undiluted at −80 °C (Figure 1B).

Alignment of the GT1b-TeNT HCR and the Syt-II-BoNT/B HCR. The GT1b-TeNT HCR (PDB entry 1FV2) was aligned with the Syt-II-BoNT/B HCR (PDB entry 2NM1) using PyMOL version 1.0.

TeNT HCR Binding to Gangliosides. Gangliosides GT1b, GD1b, GD1a, and GM1a were diluted to 2.5 μg/mL in methanol, added to 96-well plates (100 μL/well, 0.25 μg of total ganglioside), and allowed to dry for 16 h at room temperature (RT). Plates were then blocked with 50 mM Na_2CO_3 (pH 9.6) buffer containing 1% (w/v) bovine serum albumin (BSA) for 1 h at 4 °C before being washed three times with DPBS. Binding assay mixtures containing 800 nM TeNT HCRs in 2-fold serial dilutions of 50 μL/well in DPBS containing 1% BSA were incubated for 1 h at 4 °C. Plates were then washed three times with 300 μL of DPBS per well. Mouse HRP-conjugated anti-FLAG antibody (Pierce, 1:10000 final dilution) was added and the mixture incubated for 45 min at 4 °C. Plates were washed three times with 300 μL of DPBS per well and developed with 100 μL of Ultra-TMB (Pierce) per well for 30 min at RT. Absorbance (450 nm) was read after quenching with 100 μL of 1 M H_2SO_4 per well.

TeNT HCRs Binding to Rat Cortical Neurons. Rat E18-cortical neurons (BrainBits) were cultured on laminin-coated glass coverslips in neurobasal medium supplemented with 2 mM glutamine and B27 supplement for 10−14 days prior to use. Cells were treated with 10, 40, 200, or 800 nM TeNT HCRs for 30 min at 4 °C in fresh neurobasal medium. Cells were washed three times with DPBS, fixed with 4% (w/v) paraformaldehyde in DPBS for 30 min at 37 °C, and permeabilized with 0.1% Triton X-100 with 4% formaldehyde in DPBS for 15 min at RT. The bound HCR was detected by immunofluorescence, using mouse HRP-conjugated α-FLAG antibody (clone M2, 1:4000 dilution) and then goat α-mouse IgG Alexa488 (Molecular Probes; 1:500 dilution). Images were captured with a Nikon TE2000 microscope equipped with a CFI Plan Apo VC 60× oil, NA 1.4-type objective using a Photometrics CoolSnap EZ camera. Image analyses were performed using Metamorph version 6, and figures were compiled using Photoshop CS (Adobe).

Inhibition of TeNT Action by TeNT HCRs. Rat cortical neurons were incubated with 6.7 nM TeNT alone or with 800 nM wild-type and mutated TeNT HCRs for 30 min at 37 °C. Cells were then washed with DPBS and incubated for an additional 48 h at 37 °C in fresh neurobasal medium and conditioned neurobasal medium (1:1). Following treatment, cells were washed three times with DPBS, fixed with

4% (w/v) paraformaldehyde in DPBS for 15 min at RT, permeabilized with 0.1% Triton X-100 and 4% formaldehyde in DPBS for 10 min at RT, and stained with mouse α-VAMP-2 (clone 69.1, Synaptic Systems). Bound antibodies were visualized using α-mouse IgG Alexa488.

RESULTS

Resolution of Two Independent Binding Sites for Gangliosides and Sialic Acid in the TeNT HCR. The receptor binding pocket of the BoNT/B HCR has recently been cocrystallized with a peptide of Syt-II (*24, 25, 30*). Superposition of the GT1b-TeNT HCR (*23*) with the Syt-II-BoNT/B HCR (*24, 25*) showed that the TeNT HCR shared structural homology with the BoNT/B HCR (Figure 2A). The ganglioside binding sites of TeNT and BoNT/B aligned well, while the Syt-II binding pocket of BoNT/B overlapped with the sialic acid binding site of TeNT HCR (Figure 2A,C). TeNT has two functional carbohydrate binding sites (*19, 23*): a ganglioside binding site consisting of W1289, H1271, and S1287 and a sialic acid binding pocket which overlaps with Syt-II binding sites of BoNT/B (Figure 2C). When Syt-II was modeled on the structure of the TeNT HCR, R1226 was located at the bottom of the sialic acid binding pocket with the guanidinium group extending into the Syt-II peptide (Figure 2C). The sialic acid binding pocket and ganglioside binding pocket of the TeNT HCR were independent physical structures (Figure 2B). This was consistent with a cocrystal that was determined for a GT1b ganglioside analogue and TeNT HCR (*23*) and the determination that TeNT HCR could bind two molecules of GT1b (*19*). In this study, four gangliosides (GT1b, GD1b, GD1a, and GM1a) (Figure 3) were used to characterize the specificity of individual ganglioside and sialic acid binding pockets of point-mutated TeNT HCRs that were defective in either the ganglioside binding pocket (W1289A, H1293A, S1287A, or S1287A/H1293A), the sialic acid binding pocket (R1226L), both pockets (R1226L/W1289A), or the wild-type TeNT HCR.

TeNT HCRs Binding to Gangliosides GT1b, GD1b, GD1a, and GM1a. Earlier studies showed that the affinity of TeNT was greater for "b" series gangliosides (GD1b, GT1b, and GQ1b) than for GM1a (*26–28*). Using a solid phase binding assay, apparent affinities (termed B_{50}, the concentration of the HCR required to achieve 50% of maximal binding) and B_{max} (saturation binding of the HCR to immobilized gangliosides) were established for wt and mutated TeNT HCRs with respect to GT1b, GD1a, GD1b, and GM1a (Figure 3). Mutated TeNT HCRs were characterized on the basis of previous studies showing that R1226 within the sialic acid binding pocket and W1289 within the ganglioside binding pocket are key residues in each pocket where mutation to either residue weakened binding to GT1b and rat brain synaptosomes (*19*). Therefore, HCR-R1226L has a functional ganglioside binding pocket and HCR-W1289A a functional sialic acid binding pocket, while HCR-R1226L/W1289A has neither functional binding pocket. Experimental conditions in the solid phase binding assay were established in which TeNT HCR-wt exhibited a dose-dependent binding to GT1b.

(i) Ganglioside Binding to the Ganglioside Binding Pocket of TeNT (HCR-R1226L). HCR-R1226L bound to GD1a and GM1a with higher affinities ($B_{50} \sim 75$ nM) than either GT1b or GD1b ($B_{50} > 800$ nM) (Figure 4), indicating that "a" series

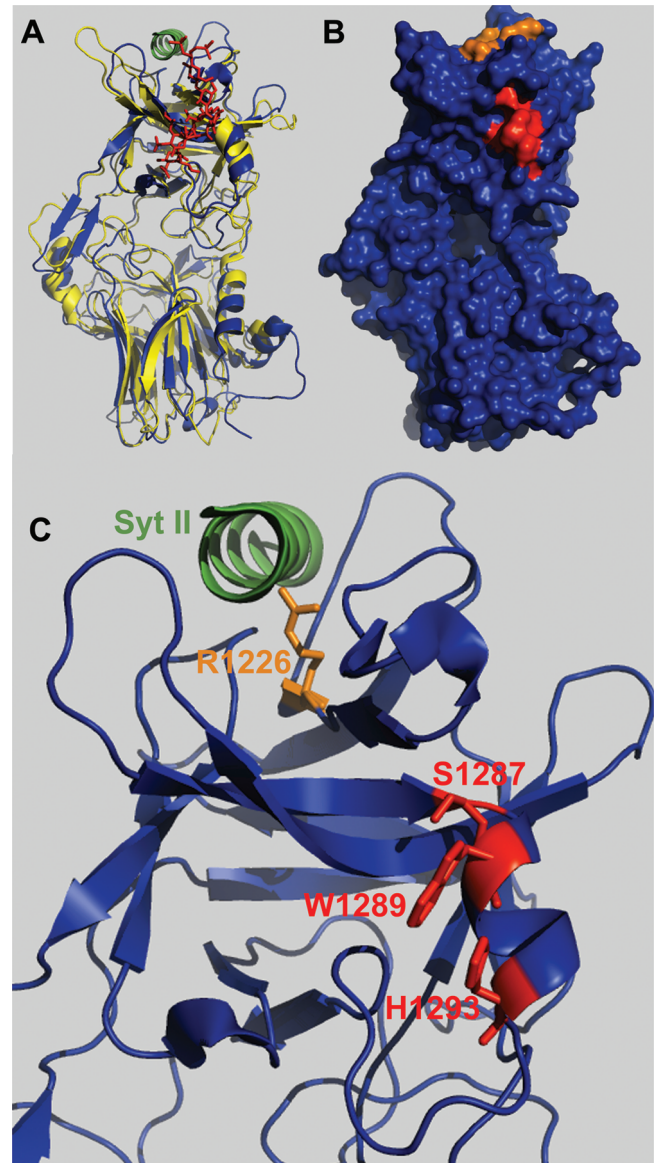

FIGURE 2: Physical relationship between the ganglioside and sialic acid binding pockets of the TeNT HCR. (A) The GT1b-TeNT HCR (PDB entry 1FV2) was aligned with the Syt-II-BoNT/B HCR (PDB entry 2NM1), using PyMOL version 1.0. Shown are the BoNT/B HCR (yellow), Syt-II (green), and the TeNT HCR (blue) with the GT1b analogue (red). (B) Surface organization of the TeNT HCR (blue; PDB entry 1FV2) with a ganglioside binding pocket (red) and a sialic acid binding pocket (orange). (C) Ganglioside and sialic acid binding pockets of the TeNT HCR. R1226 of the sialic acid binding pocket (orange stick) and S1287, W1289, and H1293 of the ganglioside binding pocket (red sticks) are shown.

gangliosides are preferred substrates for the ganglioside binding pocket. The similar binding profile of HCR-R1226L for GM1a and GD1a indicated that sialic acid-(5) did not contribute to binding in the ganglioside binding pocket, while the weaker binding of HCR-R1226L to "b" series gangliosides indicated that sialic acid-(7) interfered with ganglioside binding to this pocket. Other experiments observed that HCR-R1226L does not bind GA1 (GM1a without Sia-6) (data not shown), implicating a role for Sia-6 in the binding of ganglioside to the ganglioside pocket.

(ii) Ganglioside Binding to the Sialic Acid Binding Pocket of TeNT (HCR-W1289A). HCR-W1289A showed limited binding to each ganglioside tested (B_{max} did not achieve saturation at the highest concentration of HCR tested). Under

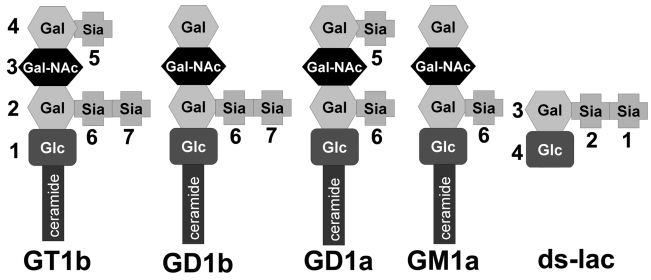

FIGURE 3: Schematic illustrations of GT1b, GD1a, GD1b, and GM1a and disialyllactose. Disialyllactose is α-Neu5Ac-(2→8)-α-Neu5Ac-(2→3)-β-D-Gal-(1→4)-D-Glc (Sia-1-Sia-2-Gal-3-Glu-4) indicated as ds-lac.

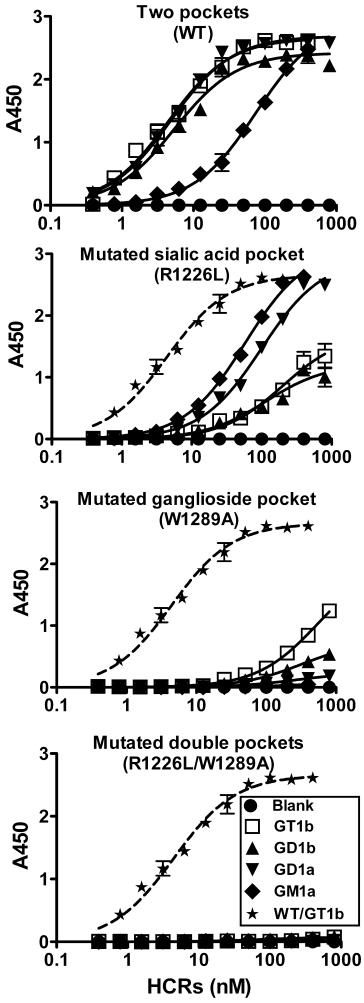

FIGURE 4: Binding of TeNT HCR-wt and mutated TeNT HCRs to immobilized gangliosides. Binding of the indicated TeNT HCRs (wt, R1226L, W1289A, and R1226L/W1289A) to GT1b, GD1a, GD1b, and GM1a immobilized on microtiter plates. Binding experiments were performed in DPBS at 4 °C for 1 h. The amount of bound HCRs was detected by HRP-conjugated anti-FLAG antibody and ultra-TMB. The plates were read at 450 nm after reactions had been quenched with H_2SO_4.

these binding conditions, "b" series gangliosides were better substrates for the sialic acid binding pocket than "a" series gangliosides (GT1b > GD1b > GD1a > GM1a). Increasing the amount of ganglioside did not yield detectable HCR-W1289A binding to GM1a (data not shown), indicating that GM1a was a poor substrate for the sialic acid binding pocket and that sialic acid-(6) was not recognized by the sialic acid binding pocket of the TeNT HCR.

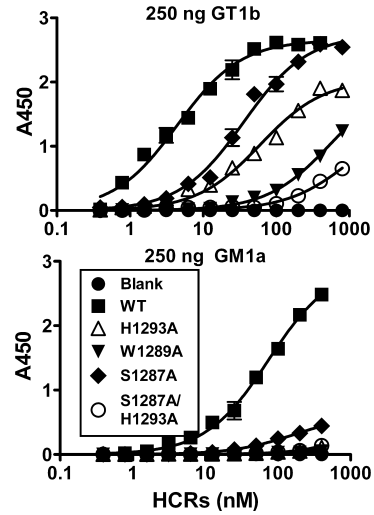

FIGURE 5: Binding of TeNT HCR-wt and mutated TeNT HCRs to immobilized gangliosides. Binding of the indicated TeNT HCRs (wt, S1287A, H1293A, and S1287A/H1293A) to GT1b and GM1a immobilized on microtiter plates. Binding experiments were performed in DPBS at 4 °C for 1 h. The amount of bound HCRs was detected by HRP-conjugated anti-FLAG antibody and ultra-TMB. The plates were read at 450 nm after reactions had been quenched with H_2SO_4.

(iii) Ganglioside Binding to Two Carbohydrate Binding Pockets of TeNT-wt. HCR-wt bound GT1b, GD1b, and GD1a with similar affinities ($B_{50} \sim 5$ nM) and GM1a with a lower affinity ($B_{50} \sim 75$ nM). The similar binding affinity of HCR-wt and HCR-R1226L for GM1a was consistent with GM1a binding only to the ganglioside pocket. The high-affinity binding of GT1b, GD1b, and GD1a to HCR-wt relative to the binding observed for either point-mutated HCR indicated that in the presence of two functional binding pockets ganglioside binding is synergistic.

(iv) Ganglioside Binding to Doubly Mutated Binding Pockets of TeNT. HCR-R1226L/W1289A with double mutated pockets did not bind any of the gangliosides that were tested.

To extend the functional characterization of TeNT HCR binding at the ganglioside binding pocket, three additional mutations within this pocket were analyzed, HCR-H1293A, HCR-S1287A, and HCR-S1287A/H1293A (*19*) (Figure 5); H1293 supports the alignment of W1289 in the ganglioside binding pocket. Relative to HCR-wt, HCR-S1287A and HCR-H1293A had lower affinities for GT1b, which was more pronounced in the binding profiles of GM1a. The doubly mutated protein HCR-S1287A/H1293A exhibited reduced binding affinities for GT1b, and almost baseline binding to GM1a. This indicated that multiple mutations within the ganglioside binding pocket of TeNT HCR have additive effects on the binding affinity for the ganglioside binding pocket.

Binding of TeNT HCRs to Rat Cortical Neurons. Singly and doubly mutated TeNT HCRs and HCR-wt were assayed for the ability to bind rat cortical neurons at 4 °C (Figure 6). TeNT HCR-wt exhibited a dose response for the binding to primary neurons. HCR-R1226L and HCR-W1289A exhibited detectable binding at 800 nM, the highest concentration tested, while binding of HCR-R1226L/W1289A to neurons was not detected. HCR-S1287A and HCR-H1293A at 200 nM exhibited detectable binding to neurons, but not doubly mutated HCR-S1287A/H1293A (Figure 6). Thus, as

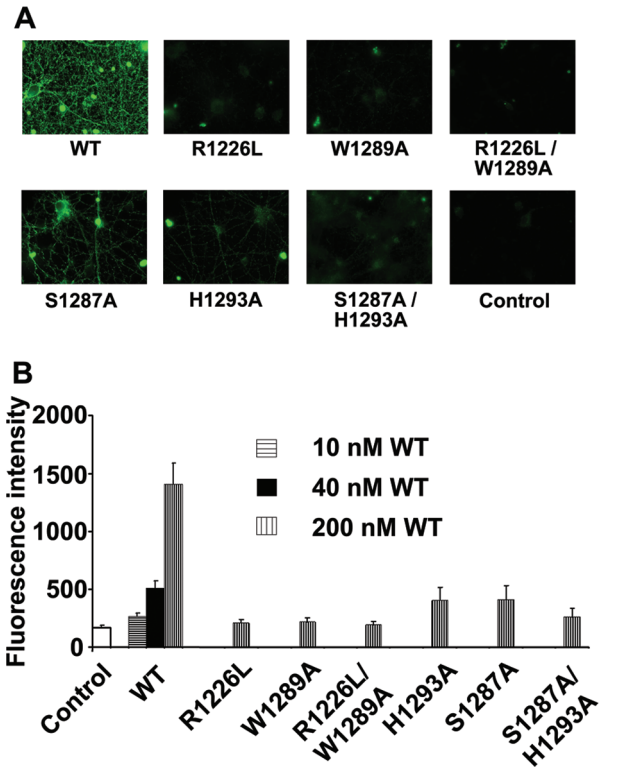

FIGURE 6: Binding of TeNT HCR-wt and mutated TeNT HCRs to rat cortical neurons. TeNT HCR-wt, HCR-R1226L, HCR-W1289A, HCR-S1287A, HCR-H1293A, HCR-S1287A/H1293A, and doubly mutated HCR-R1226L/W1289A were incubated with rat cortical neurons at 4 °C for 30 min. Cells were washed, and HCR bound to neurons was detected by immunofluorescence using mouse α-FLAG antibody followed by Alexa488-coupled secondary antibody. The amount of fluorescence was determined in 20 random fields and averaged and is presented with the standard deviation. (A) Indicated TeNT HCRs at 200 nM bound to rat cortical neurons. (B) Quantified binding of TeNT HCR-wt and mutated HCRs at the indicated concentrations.

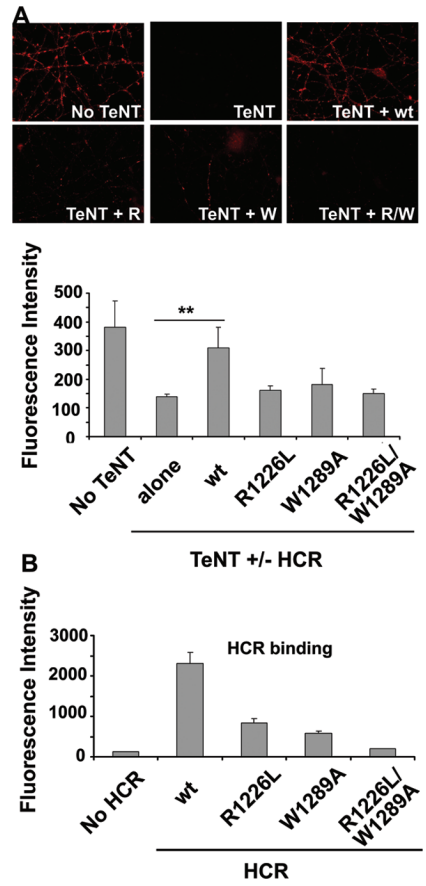

FIGURE 7: Inhibition of TeNT cleavage of VAMP-2 in rat cortical neurons by wild-type and mutated HCRs. (A) Rat cortical neurons were incubated for 30 min at 37 °C with 6.7 nM TeNT or wild-type or the indicated mutated TeNT HCRs (at 800 nM) or not treated (no TeNT). Cells were washed and incubated for an additional 48 h at 37 °C in fresh neurobasal medium and conditioned neurobasal medium (1:1). The cleavage of substrate VAMP-2 was visualized using mouse α-VAMP-2 (clone 69.1) which recognizes only full-length VAMP-2. The amount of fluorescence was determined in 20 random fields and averaged and is presented with the standard deviation. The extent of VAMP-2 cleavage by TeNT was reduced ~60% by TeNT HCR-wt (asterisks indicate *t* test, $P < 0.001$, $n = 20$). (B) Rat cortical neurons were incubated for 30 min at 4 °C alone or with the indicated HCR at 800 nM. HCR bound to neurons was detected by immunofluorescence using mouse α-FLAG antibody followed by Alexa568-coupled secondary antibody.

observed for purified gangliosides, both carbohydrate binding pockets of TeNT-HCR were needed to achieve high-affinity binding to primary neurons. In addition, multiple mutations within the ganglioside binding pocket had an additive effect on binding to primary neurons.

TeNT HCRs Inhibit TeNT Cleavage of VAMP-2 in Neurons. TeNT was incubated alone or with either wild-type or mutated TeNT HCRs on neurons for 30 min, washed, and incubated for 48 h when VAMP-2 cleavage was assayed. Under these experimental conditions, TeNT cleaved 75% of VAMP-2 relative to control cells. HCR-wt inhibited TeNT cleavage by 60% ($P < 0.001$; *t* test; $n = 20$) (Figure 7), while neither HCR-R1226L, -W1289A, nor -R1226L/W1289A inhibited TeNT activity. The inhibition of cleavage was consistent with the ability of TeNT HCR-wt to bind to neurons with a higher affinity than the point-mutated HCRs and correlated HCR binding with function.

DISCUSSION

A double-receptor model has been proposed for the entry of botulinum and tetanus neurotoxins into neurons (*31*). Crystal structures of BoNT/B bound to Syt-II and sialyllactose support the double-receptor model (*24, 25, 32*), and an earlier study showed that the TeNT HCR had two carbohydrate binding pockets (*19*). The study presented here utilized a solid phase binding assay to resolve the ganglioside binding properties to each carbohydrate binding pocket. The ganglioside binding pocket of TeNT exhibited preferred binding to "a" series gangliosides; the sialic acid binding pocket of TeNT preferred binding to "b" series gangliosides via sialic acid-(7), and "a" series gangliosides bind with low affinity via sialic acid-(5) (Figure 8). The relative affinities of TeNT HCR-wt and the mutated forms of TeNT HCRs at the ganglioside and sialic acid binding pockets in the solid phase assay and in cultured primary neurons supported a synergistic model of binding to the coreceptors. This study is the first quantification of the physical properties of the individual coreceptor binding pockets of tetanus toxin.

Rummel and co-workers used mass spectroscopy to show that TeNT HCR-wt bound two molecules of GT1b (*19*). The ganglioside binding pocket is formed by residues W1289, H1271, and S1287. In this binding site, the hydrophobic faces of Gal4 and GalNAc3 are stacked against the aromatic ring

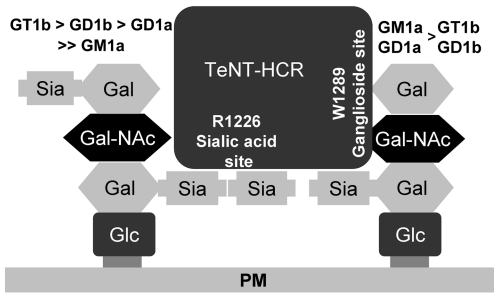

FIGURE 8: Two-pocket binding model of TeNT. TeNT HCR can bind to two different molecules at the plasma membrane using the ganglioside binding pocket and the sialic acid binding pocket. The ganglioside binding pocket can bind to Gal-GalNAc of the ganglioside (illustrated by GM1a). The sialic acid binding pocket can bind to the sialic acid of the ganglioside (illustrated by GT1b). Three types of molecules may bind to the sialic acid binding pocket of TeNT: sialic acid within either a ganglioside or a glycosylated protein or peptide sequence within a protein that mimics sialic acid.

of W1289 and form hydrogen bonds with H1271, S1287, and D1222. H1293 and Y1290 appear to align W1289 within the pocket (*23*). The sialic acid pocket is a shallow binding site formed by residues R1226, N1216, D1214, D1147, and Y1229. Sialic acid-(5) and sialic acid-(7) interact with the sialic acid binding pocket of TeNT. However, the cocrystal structure of GT1b and TeNT HCR did not explain why the TeNT HCR bound GT1b and GD1b with higher affinity than GD1a and GM1a (*26–28*). In this study, HCR-W1289A bound the four gangliosides in the following order: GT1b > GD1b > GD1a > GM1a (Figure 4). This suggests that sialic acid-(5) and sialic acid-(7) bind to the sialic acid binding pocket and that sialic acid-(7) binds better than sialic acid-(5). The fact that HCR-R1226L bound to GD1a ($B_{50} \sim$ 75 nM) with a lower affinity than HCR-wt ($B_{50} \sim$ 5 nM) supported a lower-affinity binding of sialic acid-(5) to the sialic acid binding pocket. The crystal structure of disialyllactose, α-Neu5Ac-(2→8)-α-Neu5Ac-(2→3)-β-D-Gal-(1→4)-D-Glc (Sia-1-Sia-2-Gal-3-Glu-4) (Figure 3, ds-lac), and the TeNT HCR (PDB entry 1YYN) (*33*) showed that the two sialic acid residues bind to the sialic acid binding pocket with the same set of residues: D1147, D1214, N1216, and R1226. Gal3 and Glu4 of disialyllactose do not interact with the protein and are solvent-exposed. R1226 and N1216 can form hydrogen bonds with both sialic acid-(1) and sialic acid-(2) of disialyllactose. Both D1147 and D1214 can form hydrogen bonds with either sialic acid-(1) or sialic acid-(2) of disialyllactose instead of water-mediated interactions in the crystal structure of monosialic acid and the TeNT HCR (PDB entry 1DFQ). Therefore, disialic acid is a better substrate than monosialic acid for the sialic acid binding pocket because of the stronger interactions between disialic acid and the TeNT HCR. This is consistent with the current observation that the "b" series of gangliosides are better substrates for the sialic acid binding pocket than the "a" series of gangliosides. In addition, HCR-W1289A did not bind GM1a, which indicated that sialic acid-(6) did not bind to the sialic acid binding pocket. Spatial constraints may be responsible for the inability of GM1a to bind to the sialic acid binding pocket, where sialic acid-(6) is too close to the ceramide and cannot extend into the sialic acid binding pocket.

Angstrom et al. (*27*) tested binding of the ^{125}I-labeled TeNT HCR to serial dilutions of GM1a but did not observe GM1a binding. However, the concentration of the ^{125}I-labeled TeNT HCR used in that assay may have been below the concentration needed to bind to the ganglioside binding pocket. On the other hand, Critchley et al. (*26*) and Holmgren et al. (*28*) observed TeNT binding to GM1a. In our system, 50% TeNT HCR binding to GM1a was observed at ∼75 nM and was specific, since under identical conditions the BoNT/A HCR did not bind GM1a (data not shown).

GT1b, GD1b, and GD1a bound to both carbohydrate binding pockets of TeNT, which indicated that both the sialic acid binding pocket and the ganglioside binding pocket are accessible within the HCR. The observation that HCR-wt bound GT1b, GD1b, and GD1a with an affinity higher than that observed for the binding to individual binding pockets suggests that binding to the TeNT HCR was synergistic. Two other observations supported a synergistic binding model for TeNT in which the binding of HCR-wt to cortical neurons was stronger that expected from the binding properties of the individual binding pocket-mutated HCRs (Figure 6) and only HCR-wt, but not individual binding pocket-mutated HCRs, inhibited TeNT cleavage of VAMP-2 (Figure 7). In addition, the inhibition profile of the TeNT HCRs was similar to the binding affinities of the TeNT HCRs for cortical neurons, correlating TeNT HCR binding with function.

The identity of the TeNT protein receptor has yet to be resolved. Earlier studies showed that TeNT binding to rat brain membranes was sensitive to heating and protease treatment in physiological buffer (*20*), suggesting that TeNT binds to a protein receptor on neurons. Herreros et al. identified a candidate protein receptor for TeNT as a 15 kDa N-glycosylated protein in PC12 cells and motor neurons (*21*). This protein later was identified as neuronal Thy-1 in NGF-differentiated PC12 cells (*34*); however, the role of Thy-1 as the physiological receptor for TeNT remains to be resolved. Jayaraman et al. (*33*) showed that a tripeptide, YEW, binds TeNT at the sialic acid binding site and competes with disialyllactose at this site. This implied that the sialic acid of gangliosides might mimic a peptide as a component of a protein receptor for TeNT or that a peptide sequence within a protein mimicked sialic acid in the sialic acid binding pocket. In this study, several candidates can be implicated as participating in the receptor component and include sialic acid of a ganglioside, a glycosylated protein, or a peptide that is a physical mimic of sialic acid.

Previous studies showed that TeNT and BoNTs had reduced toxicity in knockout mice lacking complex gangliosides (GM1a, GD1a, GD1b, GT1b, and GQ1b) as the result of disruption of the β-1,4 *N*-acetylgalactosaminyltransferase (GM2/GD2 synthase) (*35*). This is consistent with this study in which the TeNT HCR had high affinity for GT1b, GD1b, and GD1a. However, an α-2,8-sialyltransferase (GD3 synthase) gene knockout mouse that lacked "b" series gangliosides (GD1b, GT1b, and GQ1b) was only partially resistant to TeNT intoxication but vulnerable to BoNT/A, BoNT/B, and BoNT/E (*36*). This is inconsistent with a previous study which showed both GT1b and GQ1b in the "b" series of gangliosides have the strongest detoxification ability for BoNT/A in vitro (*17*) and with the current study, which showed the TeNT HCR can bind GD1a, since the GD3 knockout mice would be expected to possess GD1a. There

are several explanations for this apparent discrepancy. First, steady state ganglioside synthesis in the GD3 synthase gene knockout mice may be altered, changing the ganglioside composition to compensate for the loss of gangliosides that were "knocked out" genetically. This has been observed in GM2 synthase knockout murine embryonic fibroblasts (*37*). Second, the GD3 synthase gene knockout mice were challenged with a dose of ~1 million TeNT LD_{50}, which may not have mimicked the natural TeNT intoxication pathway. Third, differential localization of "a" and "b" series gangliosides within the mouse may contribute to the observed sensitivities of mice to TeNT (*38*).

Coreceptors may contribute to the serotype specificity of the clostridial neurotoxins. Rummel et al. (*39*) reported that, for BoNT/A and BoNT/B, several common mutations within the conserved ganglioside binding pocket yielded binding properties different from those of GT1b, while mutations within Syt-I and Syt-II influence the affinity of the proteins for BoNT/B and BoNT/G (*25, 30, 40*). The two-receptor model for BoNTs and TeNT was first proposed as a sequential binding process in neurons (*31*). This model is consistent with BoNT/A, BoNT/B, and BoNT/G, since their protein receptors are luminal domains of SV2 and Syt-I and Syt-II, respectively, and are surface exposed only after synaptic vesicle fusion with the plasma membrane (*25, 29, 30, 41*). However, TeNT may not follow this binding model, since the data acquired in the study presented here indicate that the coreceptor binding pockets are accessible independently where mutated forms of HCR retained a low affinity for gangliosides. These data are consistent with the high-affinity binding of TeNT to neurons requiring functional ganglioside and sialic acid binding pockets.

Utilizing GT1b, GD1b, GD1a, and GM1a along with mutated HCRs that lacked either ganglioside or sialic acid binding activities, additional resolution of two carbohydrate binding pockets within TeNT HCR was achieved. Quantifying the two individual binding pockets defined the ganglioside binding specificity of the ganglioside and sialic acid binding pockets of TeNT.

REFERENCES

1. Schiavo, G., Matteoli, M., and Montecucco, C. (2000) Neurotoxins affecting neuroexocytosis. *Physiol. Rev. 80*, 717–766.
2. Montecucco, C., and Schiavo, G. (1994) Mechanism of action of tetanus and botulinum neurotoxins. *Mol. Microbiol. 13*, 1–8.
3. Poulain, B. (1994) [Molecular mechanism of action of tetanus toxin and botulinum neurotoxins]. *Pathol. Biol. 42*, 173–182.
4. Pellizzari, R., Rossetto, O., Schiavo, G., and Montecucco, C. (1999) Tetanus and botulinum neurotoxins: Mechanism of action and therapeutic uses. *Philos. Trans. R. Soc. London, Ser. B 354*, 259–268.
5. Lacy, D. B., and Stevens, R. C. (1999) Sequence homology and structural analysis of the clostridial neurotoxins. *J. Mol. Biol. 291*, 1091–1104.
6. Humeau, Y., Doussau, F., Grant, N. J., and Poulain, B. (2000) How botulinum and tetanus neurotoxins block neurotransmitter release. *Biochimie 82*, 427–446.
7. Schiavo, G., Benfenati, F., Poulain, B., Rossetto, O., Polverino de Laureto, P., DasGupta, B. R., and Montecucco, C. (1992) Tetanus and botulinum-B neurotoxins block neurotransmitter release by proteolytic cleavage of synaptobrevin. *Nature 359*, 832–835.
8. Blasi, J., Chapman, E. R., Link, E., Binz, T., Yamasaki, S., Camilli, P. D., Sudhof, T. C., Niemann, H., and Jahn, R. (1993) Botulinum neurotoxin A selectively cleaves the synaptic protein SNAP-25. *Nature 365*, 160–163.
9. Foran, P., Lawrence, G. W., Shone, C. C., Foster, K. A., and Dolly, J. O. (1996) Botulinum neurotoxin C1 cleaves both syntaxin and SNAP-25 in intact and permeabilized chromaffin cells: Correlation with its blockade of catecholamine release. *Biochemistry 35*, 2630–2636.
10. Williamson, L. C., Halpern, J. L., Montecucco, C., Brown, J. E., and Neale, E. A. (1996) Clostridial neurotoxins and substrate proteolysis in intact neurons: Botulinum neurotoxin C acts on synaptosomal-associated protein of 25 kDa. *J. Biol. Chem. 271*, 7694–7699.
11. Blasi, J., Chapman, E. R., Yamasaki, S., Binz, T., Niemann, H., and Jahn, R. (1993) Botulinum neurotoxin C1 blocks neurotransmitter release by means of cleaving HPC-1/syntaxin. *EMBO J. 12*, 4821–4828.
12. Shone, C. C., Hambleton, P., and Melling, J. (1987) A 50-kDa fragment from the NH2-terminus of the heavy subunit of *Clostridium botulinum* type A neurotoxin forms channels in lipid vesicles. *Eur. J. Biochem. 167*, 175–180.
13. Montecucco, C. (1986) How do tetanus and botulinum toxins bind to neuronal membranes? *Trends Biochem. Sci. 11*, 314–317.
14. Karlsson, K. A. (1998) On the character and functions of sphingolipids. *Acta Biochim. Pol. 45*, 429–438.
15. Mocchetti, I. (2005) Exogenous gangliosides, neuronal plasticity and repair, and the neurotrophins. *Cell. Mol. Life Sci. 62*, 2283–2294.
16. Singh, A. K., Harrison, S. H., and Schoeniger, J. S. (2000) Gangliosides as receptors for biological toxins: Development of sensitive fluoroimmunoassays using ganglioside-bearing liposomes. *Anal. Chem. 72*, 6019–6024.
17. Kitamura, M., Iwamori, M., and Nagai, Y. (1980) Interaction between *Clostridium botulinum* neurotoxin and gangliosides. *Biochim. Biophys. Acta 628*, 328–335.
18. Tsukamoto, K., Kohda, T., Mukamoto, M., Takeuchi, K., Ihara, H., Saito, M., and Kozaki, S. (2005) Binding of *Clostridium botulinum* type C and D neurotoxins to ganglioside and phospholipid. Novel insights into the receptor for clostridial neurotoxins. *J. Biol. Chem. 280*, 35164–35171.
19. Rummel, A., Bade, S., Alves, J., Bigalke, H., and Binz, T. (2003) Two carbohydrate binding sites in the H(CC)-domain of tetanus neurotoxin are required for toxicity. *J. Mol. Biol. 326*, 835–847.
20. Pierce, E. J., Davison, M. D., Parton, R. G., Habig, W. H., and Critchley, D. R. (1986) Characterization of tetanus toxin binding to rat brain membranes. Evidence for a high-affinity proteinase-sensitive receptor. *Biochem. J. 236*, 845–852.
21. Herreros, J., Lalli, G., and Schiavo, G. (2000) C-Terminal half of tetanus toxin fragment C is sufficient for neuronal binding and interaction with a putative protein receptor. *Biochem. J. 347* (Part 1), 199–204.
22. Sutton, J. M., Chow-Worn, O., Spaven, L., Silman, N. J., Hallis, B., and Shone, C. C. (2001) Tyrosine-1290 of tetanus neurotoxin plays a key role in its binding to gangliosides and functional binding to neurones. *FEBS Lett. 493*, 45–49.
23. Fotinou, C., Emsley, P., Black, I., Ando, H., Ishida, H., Kiso, M., Sinha, K. A., Fairweather, N. F., and Isaacs, N. W. (2001) The crystal structure of tetanus toxin Hc fragment complexed with a synthetic GT1b analogue suggests cross-linking between ganglioside receptors and the toxin. *J. Biol. Chem. 276*, 32274–32281.
24. Jin, R., Rummel, A., Binz, T., and Brunger, A. T. (2006) Botulinum neurotoxin B recognizes its protein receptor with high affinity and specificity. *Nature 444*, 1092–1095.
25. Chai, Q., Arndt, J. W., Dong, M., Tepp, W. H., Johnson, E. A., Chapman, E. R., and Stevens, R. C. (2006) Structural basis of cell surface receptor recognition by botulinum neurotoxin B. *Nature 444*, 1096–1100.
26. Critchley, D. R., Habig, W. H., and Fishman, P. H. (1986) Reevaluation of the Role of Gangliosides as Receptors for Tetanus Toxin. *J. Neurochem. 47*, 213–222.
27. Angstrom, J., Teneberg, S., and Karlsson, K. A. (1994) Delineation and comparison of ganglioside-binding epitopes for the toxins of *Vibrio cholerae*, *Escherichia coli*, and *Clostridium tetani*: Evidence for overlapping epitopes. *Proc. Natl. Acad. Sci. U.S.A. 91*, 11859–11863.
28. Holmgren, J., Elwing, H., Fredman, P., and Svennerholm, L. (1980) Polystyrene-Adsorbed Gangliosides for Investigation of the Structure of the Tetanus-Toxin Receptor. *Eur. J. Biochem. 106*, 371–379.
29. Baldwin, M. R., and Barbieri, J. T. (2007) Association of botulinum neurotoxin serotypes A and B with synaptic vesicle protein complexes. *Biochemistry 46*, 3200–3210.

30. Rummel, A., Karnath, T., Henke, T., Bigalke, H., and Binz, T. (2004) Synaptotagmins I and II act as nerve cell receptors for botulinum neurotoxin G. *J. Biol. Chem. 279*, 30865–30870.
31. Montecucco, C., Schiavo, G., Brunner, J., Duflot, E., Boquet, P., and Roa, M. (1986) Tetanus toxin is labeled with photoactivatable phospholipids at low pH. *Biochemistry 25*, 919–924.
32. Swaminathan, S., and Eswaramoorthy, S. (2000) Structural analysis of the catalytic and binding sites of *Clostridium botulinum* neurotoxin B. *Nat. Struct. Biol. 7*, 693–699.
33. Jayaraman, S., Eswaramoorthy, S., Kumaran, D., and Swaminathan, S. (2005) Common binding site for disialyllactose and tri-peptide in C-fragment of tetanus neurotoxin. *Proteins 61*, 288–295.
34. Herreros, J., Ng, T., and Schiavo, G. (2001) Lipid rafts act as specialized domains for tetanus toxin binding and internalization into neurons. *Mol. Biol. Cell 12*, 2947–2960.
35. Kitamura, M., Takamiya, K., Aizawa, S., Furukawa, K., and Furukawa, K. (1999) Gangliosides are the binding substances in neural cells for tetanus and botulinum toxins in mice. *Biochim. Biophys. Acta 1441*, 1–3.
36. Kitamura, M., Igimi, S., Furukawa, K., and Furukawa, K. (2005) Different response of the knockout mice lacking b-series gangliosides against botulinum and tetanus toxins. *Biochim. Biophys. Acta 1741*, 1–3.
37. Shevchuk, N. A., Hathout, Y., Epifano, O., Su, Y., Liu, Y., Sutherland, M., and Ladisch, S. (2007) Alteration of ganglioside synthesis by GM3 synthase knockout in murine embryonic fibroblasts. *Biochim. Biophys. Acta 1771*, 1226–1234.
38. Gong, Y., Tagawa, Y., Lunn, M. P., Laroy, W., Heffer-Lauc, M., Li, C. Y., Griffin, J. W., Schnaar, R. L., and Sheikh, K. A. (2002) Localization of major gangliosides in the PNS: Implications for immune neuropathies. *Brain 125*, 2491–2506.
39. Rummel, A., Mahrhold, S., Bigalke, H., and Binz, T. (2004) The HCC-domain of botulinum neurotoxins A and B exhibits a singular ganglioside binding site displaying serotype specific carbohydrate interaction. *Mol. Microbiol. 51*, 631–643.
40. Dong, M., Richards, D. A., Goodnough, M. C., Tepp, W. H., Johnson, E. A., and Chapman, E. R. (2003) Synaptotagmins I and II mediate entry of botulinum neurotoxin B into cells. *J. Cell Biol. 162*, 1293–1303.
41. Dong, M., Yeh, F., Tepp, W. H., Dean, C., Johnson, E. A., Janz, R., and Chapman, E. R. (2006) SV2 is the protein receptor for botulinum neurotoxin A. *Science 312*, 592–596.

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