Association of Botulinum Neurotoxin Serotypes A and B with Synaptic Vesicle Protein Complexes†

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ABSTRACT: Botulinum neurotoxins (BoNTs) elicit flaccid paralysis through cleavage of SNARE proteins within peripheral neurons. There are seven serotypes of the BoNTs, termed A−G, which differ in the SNARE protein and/or site that is cleaved. BoNTs are single-chain toxins that comprise an N-terminal zinc metalloprotease domain that is disulfide linked to the C-terminal translocation/receptor binding domain. SV2 and synaptotagmin have been identified as receptors for BoNT serotypes A and B, respectively. Using affinity chromatography, BoNTs A and B were observed to bind synaptic vesicle protein complexes in synaptosome lysates. Tandem LC−MS/MS identified SV2, synaptotagmin I, synaptophysin, vesicle-associated membrane protein 2 (VAMP2), and the vacuolar proton pump as components of the BoNT−receptor complex. Density gradient analysis showed that BoNT serotypes A and B exhibited unique interactions with the synaptic vesicle protein complexes. The association of BoNT serotypes A and B with synaptic vesicle protein complexes implicates a physiological role for protein complexes in synaptic vesicle biology and provides insight into the interactions of BoNT and neuronal receptors.

Botulinum neurotoxins (BoNTs),[1] the most potent protein toxins for humans, block acetylcholine release at the neuromuscular junction (NMJ) through the cleavage of SNARE proteins involved in synaptic vesicle exocytosis (*1*). BoNTs are divided into seven distinct serotypes (termed A−G) based on antigenic specificity (e.g., α-sera to BoNT/A do not neutralize toxin serotypes B−G). BoNTs can be further divided into subserotypes; for example, there are four subserotypes of BoNT/A (termed A1−A4) (*2*), which are approximately 90% identical at the amino acid level. BoNTs are produced as single-chain molecules that undergo post-translational cleavage to form a dichain protein that is composed of a catalytic light chain (LC, ∼50 kDa) and a heavy chain (HC, ∼100 kDa) linked through a single disulfide (*3*). BoNT LCs are zinc metalloproteases that cleave one or more components of a conserved SNARE protein complex consisting of syntaxin, SNAP25, and vesicle-associated membrane protein 2 (VAMP2), resulting in inhibition of synaptic vesicle exocytosis (*4*−*9*). BoNT HC contains two functional domains: an N-terminal translocation domain (HCT, ∼50 kDa) and a C-terminal receptor binding domain (HCR, ∼50 kDa) (*10*, *11*). The HCR domain (Figure 1) comprises two subdomains, HCR_N and HCR_C. While HCR_N has not been assigned a function, HCR_C appears to mediate binding of toxin to the cognate protein receptors (*12*, *13*).

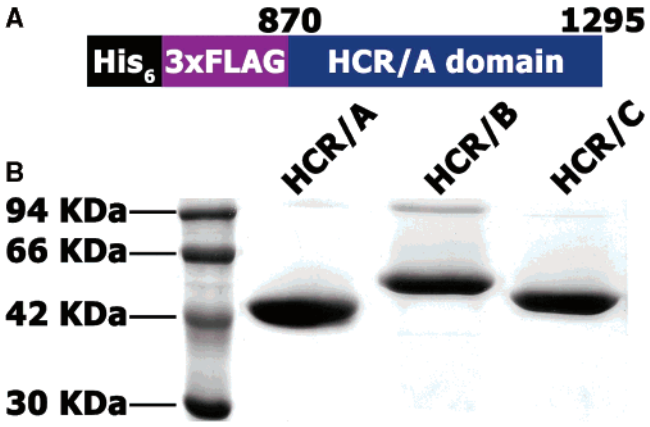

FIGURE 1: Purification of botulinum neurotoxin HCR domains. (A) Ribbon diagram of the BoNT/A HCR domain (residues 870−1295, PDB entry 3bta, ∼3 Å resolution). (B) Recombinant FLAG-tagged HCR domains were purified from *E. coli* by a three-column strategy using nickel affinity, gel filtration, and ion exchange chromatography. HCR/A, HCR/B, and HCR/C (∼5 μg) were separated by SDS−PAGE and visualized after being stained with silver.

BoNTs intoxicate neurons at femtomolar concentrations and specifically associate with the presynaptic membranes of α-motoneurons (*14*). BoNTs enter neurons via receptor-mediated endocytosis where neuronal tropism involves binding of BoNT to unique host cell receptors (*14*, *15*). While

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[1] Abbreviations: BoNTs, botulinum neurotoxins; VAMP2, vesicle-associated membrane protein 2; NMJ, neuromuscular junction; LC, botulinum neurotoxin light chain; HC, botulinum neurotoxin heavy chain; HCT, heavy chain N-terminal translocation domain; HCR, heavy chain C-terminal receptor binding domain; mPIC, mammalian protease inhibitor cocktail; Syt I, synaptotagmin I; Syt II, synaptotagmin II; v-ATPase, vacuolar type ATPase; proHB-EGF, heparin-binding EGF-like growth factor; LF, lethal factor; EF, edema factor; PA, protective antigen; TEM8, ATR/tumor endothelial marker 8; CMG2, capillary morphogenesis protein 2; LRP6, low-density lipoprotein receptor-related protein 6.

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the neuronal tropism of BoNTs is well-established, the molecular interactions mediating this association are only now being defined. By defining the entry process of BoNT/B-involved synaptic vesicle exocytosis, seminal studies by Chapman and colleagues proposed that synaptic vesicle proteins functioned as the physiological receptor for BoNT/B (*16*, *17*). Using luminal domain fragments of synaptic vesicle proteins fused to GST, synaptotagmin I (Syt I) and synaptotagmin II (Syt II) were shown to specifically mediate the binding and entry of BoNT/B into cultured neuroendocrine cells (*16*). A similar approach demonstrated that Syt I and Syt II also mediated the binding and entry of BoNT/G (*12*), whereas isoforms of synaptic vesicle protein 2 (SV2) mediated the binding and entry of BoNT/A into cultured neurons. Recently, the structures of BoNT/B (*18*) and HCR/B (*19*) bound to a synaptotagmin peptide have been determined.

Neuronal synaptic transmission is initiated when an action potential induces the opening of Ca^{2+} channels on the plasma membrane that stimulates synaptic vesicle exocytosis and neurotransmitter release. After plasma membrane fusion, synaptic vesicles undergo endocytosis, recycling to refill with neurotransmitters, and are ready for another round of exocytosis. Synaptic vesicles contain transporter proteins that are composed of the v-type ATPase to generate an electrochemical gradient, neurotransmitter transporter proteins that load neurotransmitters into the vesicle, and trafficking proteins. Trafficking proteins are responsible for synaptic vesicle exocytosis and include intrinsic membrane proteins, proteins associated via post-translational modifications, and peripherally bound proteins. While the individual role of vesicle proteins in exocytosis is beginning to emerge, the physical interactions among vesicle proteins remain largely unknown. A fundamental study by Scheller and colleagues demonstrated interactions between six major synaptic vesicle proteins (*20*). Solubilization of synaptic vesicle membranes in CHAPS resulted in the recovery of a large protein complex that included SV2, Syt I, synaptophysin (Syp), VAMP2, and the v-type ATPase. Solubilization in octyl glucoside preserved an SV2−Syt complex, while solubilization of synaptic vesicles with Triton X-100 preserved a Syt−SV2 complex and a Syp−VAMP2 complex. These protein interactions were proposed to underlie aspects of neurotransmitter secretion, vesicle trafficking, and spatial organization within the nerve terminus. While interactions between Syt I and SV2 and between Syp and VAMP2 have been confirmed by several laboratories, the physiological significance of these presynaptic protein receptor complexes remains unclear.

This study shows the physical association of BoNT serotypes A and B with synaptic vesicle protein complexes, which implicates a physiological role for these complexes in vesicle biology.

EXPERIMENTAL PROCEDURES

Materials. Unless otherwise stated, molecular biology-grade chemicals and reagents were obtained from Sigma-Aldrich Co. (St. Louis, MO). Restriction enzymes were from New England Biolabs. Antibodies against synaptotagmin I, synaptophysin, VAMP2, and the proton pump were obtained from Synaptic Systems. The SV2 monoclonal antibody developed by K. M. Buckley was obtained from the Developmental Studies Hybridoma Bank developed under the auspicies of the National Institute of Child Health and Human Development and maintained by Department of Biological Sciences, The University of Iowa (Iowa City, IA). Anti-synaptotagmin II monoclonal antibody clone 8G2 was purchased from MBL International. Anti-SNAP25 monoclonal antibody clone SMI-81 was purchased from Sternberger Monoclonals Inc. Anti-SNAP25 monoclonal antibody clone MC-6053 was purchased from R and D Antibodies. Sprague-Dawley rat embryonic day 18 cortical neurons were from Brainbits LLC (Springfield, IL) and cultured as described by the supplier.

Generation of the BoNT HCR Protein Expression Vector. pET-28a (Novagen) was modified to contain a 3×FLAG epitope directly downstream of the histidine tag. pET-28a was linearized by digestion with NdeI and NotI, gel purified, and ligated with the following cassette: 5′ T ATG GAC TAC AAA GAC CAT GAC GGT GAT TAT AAA GAT CAT GAC ATC GAT TAC AAG GAT GAC GAT GAC AAG GGT ACC ACT CTG CAG TAA GC 3′. DNA encoding HCR/A (residues 870−1295 of BoNT/A) was amplified and subcloned into the modified pET28-3XFLAG expression vector that contained unique *Kpn*I and *Pst*I sites. Correct insertion of the HCR DNA was confirmed by DNA sequencing. An identical cloning strategy was used to construct expression vectors for HCR/B and HCR/C. Competent *Escherichia coli* BL-21 RIL(DE3) cells were transformed with each pET28-HCR vector and grown overnight on LB agar with 50 μg/mL kanamycin and 100 μg/mL chloramphenicol at 37 °C. Transformants were stored at −80 °C in 12% (v/v) glycerol.

HCR Expression and Purification. *E. coli* (pET28-HCR) was grown overnight on LB agar with 50 μg/mL kanamycin and 100 μg/mL chloramphenicol. Cells were inoculated into LB medium containing the same antibiotics, grown at 30 °C for 2 h at 250 rpm to an OD_{600} of ∼0.6, induced by addition of 0.5 mM IPTG, and cultured overnight at 16 °C. Cells were harvested, lysed with a French press, and clarified by centrifugation and filtration (cellulose acetate). HCR/A was purified from the filtered lysate using sequential chromatography: Ni^{2+}-NTA resin, Sephacryl S-200HR, and DEAE Sephacryl. Fractions containing purified HCRs were dialyzed overnight against 25 mM Hepes-KOH buffer (pH 7.4), 145 mM NaCl, and 1 mM EDTA (HNE buffer). Purified proteins were then stored either at −20 °C in the presence of 40% (v/v) glycerol or undiluted at −80 °C. A typical purification from a 1 L culture yielded between 5 and 20 mg of 3×FLAG-HCR depending on the serotype (Figure 1B).

Partial Purification of Synaptic Vesicles from Rat Cerebral Cortex. Synaptic vesicles were prepared essentially as described with minor modifications (*21*). Rat cerebral cortices (PelFreez Bio) were thawed on ice and placed into ice-cold buffered sucrose [320 mM sucrose and 4 mM HEPES-KOH (pH 7.4)]. Materials were maintained at 4 °C throughout the purification. Five cerebral cortices were pooled and homogenized in the buffered sucrose solution with a glass−Teflon homogenizer using 12 up-and-down strokes at 900 rpm. Subsequent steps of differential centrifugation were performed as follows. The homogenate was centrifuged for 10 min at 3000 rpm ($800g_{av}$) to yield a loose pellet (P1) and a postnuclear supernatant (S1). S1 was decanted into fresh tubes, taking care not to transfer adipose tissue, and

centrifuged for 30 min at 20 000 rpm (45000g_{av}), the supernatant removed (S2), and the pellet (P2) washed via suspension in 0.8 M buffered sucrose and centrifugation to yield a supernatant (S2′) and pellet (P2′). The P2′ pellet was suspended in hypotonic lysis buffer [5 mM HEPES-KOH and 1 mM EDTA (pH 7.4)] and stirred for 30 min. The lysate was then centrifuged for 30 min at 20 000 rpm (45000g_{av}) to pellet large membranes, and the supernatant was then centrifuged for 2 h in a Beckman Ti50.2 rotor at 48 000 rpm (∼150000g_{av}) to yield a pellet fraction (P3). The P3 pellet was suspended in HNE buffer [25 mM HEPES-KOH, 145 mM NaCl, and 1 mM EDTA (pH 7.4)] and loaded onto a 5 to 20% step gradient of Iodixanol prepared in HNE buffer. Gradient centrifugation was performed for 16 h in a Beckman SW28 rotor at 28 000 rpm (∼140000g_{av}). The gradient contained a band of turbidity at the 5−10% Iodixanol interface, which was enriched for synaptic vesicle markers as determined by Western blotting. The band was isolated from the gradient, diluted 10-fold with HNE buffer, and centrifuged for 1.5 h in a Beckman Ti50.2 rotor at 48 000 rpm to pellet the synaptic vesicles (P4). The partially purified synaptic vesicle pellet (P4) was then extracted for 1 h in 10 mL of HNE buffer containing 2% (w/v) CHAPS or 2% (v/v) Triton X-100, 1 mM PMSF, and 1× mammalian protease inhibitor cocktail (mPIC, Sigma-Aldrich). Following a brief centrifugation (30 min at 14000g_{av}), the clarified lysate (P4 extract) was either used immediately or rapidly frozen with dry ice and methanol and then stored at −80 °C. More than 90% of the synaptic vesicle markers were recovered in the supernatant upon CHAPS and Triton X-100 solubilization.

FLAG Immunoprecipitation. Interactions of synaptic vesicle membrane proteins and 3×FLAG-HCRs were analyzed by immunoprecipitation. Forty microliters of anti-FLAG M2 agarose beads (Sigma-Aldrich) was washed three times with 10 volumes of HNE buffer and then blocked for 30 min in 10 volumes of HNE buffer supplemented with 2% (w/v) BSA. Blocked beads were washed three times with 10 volumes of HNE buffer and added to 800 μL of synaptic vesicle extract (P4 extract, ∼1 mg/mL) with or without 2% (w/v) CHAPS or 2% (v/v) Triton X-100. 3×FLAG-HCR/A, 3×FLAG-HCR/B, or 3×FLAG-HCR/C was then added to the reaction mixture to a final concentration of 100 nM and the solution incubated for 2 h at 4 °C with rotation. Beads were recovered by centrifugation and washed three times with 850 μL of HNE buffer with detergent and protease inhibitors. 3×FLAG proteins were then released from the resin by incubation for 2 h at 4 °C with 100 μL of HNE buffer with detergent and protease inhibitors supplemented with 150 ng/μL 3×FLAG peptide (Sigma-Aldrich). Eluted proteins were separated from beads by brief centrifugation and mixed with 25 μL of 5× SDS−PAGE sample buffer. Proteins were resolved on an 11% (w/v) SDS−PAGE gel until the dye front had migrated ∼5 mm into the resolving gel. This step separated the excess 3×FLAG peptide, which migrates just ahead of the dye front, from proteins in the sample. The gel was fixed and stained with Coomassie Brilliant Blue and the sample lane excised, taking care not to remove the 3×FLAG peptide. The gel sample was then analyzed using LC−MS/MS. Independent samples under each condition were analyzed, and only proteins found at least twice were considered significant. In other experiments, eluted proteins were resolved on 12% (w/v) SDS−PAGE gels and either stained with silver or transferred to Immobilon-P membranes and subjected to Western blotting.

GST Pulldown Assays. GST fusion proteins (10 μg each) immobilized on 10 μL of glutathione−Sepharose beads were incubated with 3×FLAG-HCR domains (500 ng) in a total volume of 100 μL of HNE buffer with or without 2% (w/v) CHAPS or 2% (v/v) Triton X-100 for 90 min at 4 °C. Beads were collected by centrifugation and washed three times with 20 volumes of the same buffer. Washed pellets were extracted in sample buffer and analyzed by SDS−PAGE and Western blotting against GST and the 3×FLAG epitope (HCRs).

Buoyant Density Centrifugation. Detergent extracts (0.5 mL) were incubated for 2 h at 4 °C with rotation alone, or with 100 nM 3×FLAG-HCR/A. Following incubation, lysates were clarified by centrifugation (30 min at 14000g_{av}), and 0.4 mL was resolved on 5 to 40% Iodixanol gradients (11.2 mL) prepared in HNE buffer containing the same detergent. A sedimentation time (6 h at ∼200000*g* in a SW41 rotor) was selected to facilitate analysis of material with a wide range of buoyant densities. Fractions (1.3 mL) from each gradient were examined by SDS−PAGE and Western blotting for the presence of the vesicle markers: SV2, synaptotagmin I, synaptophysin, and Rab3a. Interactions involving the synaptic vesicle proteins would be expected to result in coelution of two or more markers on a single gradient and/or differences in the behavior of a single marker between the two detergents.

Neutralization of BoNT Activity in Rat Cortical Neurons. Rat cortical neurons were cultured on either laminin-coated glass coverslips (immunofluorescence assays) or plastic TC dishes in Neurobasal medium supplemented with 2 mM glutamine and B27 supplement for 10−14 days prior to use. Cells were treated with control solution [15 mM Hepes, 145 mM NaCl, 5.6 mM KCl, 2.2 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5.6 mM glucose, 0.5 mM ascorbic acid, and 0.1% BSA (pH 7.4)] or high-K^+ solution (same as the control solution but adjusted to 95 mM NaCl and 56 mM KCl) for 15 min at 37 °C, in the presence of 10 nM BoNTs with or without 1 μM 3×FLAG-HCR. Cells were washed with PBS and incubated for a further 48 h at 37 °C in fresh Neurobasal medium and conditioned medium (1:1). Following the treatment, cells were rinsed in PBS and analyzed by SDS−PAGE and immunoblotting using mouse anti-SNAP25 (clone SMI-81), anti-VAMP2 (clone 69.1), and anti-SV2 (DSHB). For immunofluorescence studies, cells were washed three times with PBS, fixed with 4% (w/v) paraformaldehyde in PBS (15 min at room temperature), permeabilized with 0.1% Triton X-100 and 4% formaldehyde in PBS (for 10 min at room temperature), stained with either mouse α-SNAP25-C (clone MC-6053) or α-VAMP2, and costained with mouse anti-SV2. Staining was visualized using an anti-mouse Zenon kit containing Alexa488 or Alexa568. Images were captured at room temperature using a Nikon TE2000 microscope equipped with a CFI Plan Apo VC 60X Oil, NA 1.4 type lens and a Photometrics CoolSnap EZ camera. Image acquisition and the subsequent analysis were performed using Metamorph version 6. Figures were compiled using Photoshop CS (Adobe).

Hazardous Procedures. BoNTs used in this study were below the maximum amounts excluded from regulation as defined by the CDC Select Agent Program.

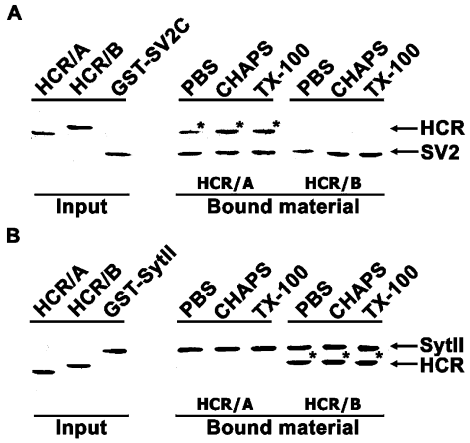

FIGURE 2: Binding of HCR/A and HCR/B to SV2 and Syt II is independent of detergents. GST fusion proteins (10 μg each) immobilized on glutathione−Sepharose beads were incubated with 80 nM HCR in a total volume of 100 μL of HNE buffer with or without 2% (w/v) CHAPS or 2% (v/v) Triton X-100 for 90 min at 4 °C. Bound material was analyzed by SDS−PAGE and immunoblotting using anti-FLAG (HCR domains) and anti-GST antibodies. The positions of the HCR domains are denoted with an asterisk.

RESULTS

Interactions of BoNT HCR/A and HCR/B with SV2 and Synaptotagmin II. Identification of neuronal protein receptors for BoNTs has been limited by the availability of suitable protocols and reagents. To characterize the interaction of BoNTs with putative neuronal receptors, recombinant HCR domains containing the FLAG epitope were generated (Figure 1). The interaction of HCR/A and HCR/B with GST fusion proteins containing the luminal domains of SV2 and synaptotagmin II was tested (*16*, *17*). Isolation of the GST−SV2C fusion protein (SV2C residues 454−580) on glutathione beads resulted in the recovery of HCR/A, but not HCR/B (Figure 2A). Conversely, HCR/B, but not HCR/A, bound to the GST−Syt II fusion protein (Syt II residues 1−267) (Figure 2B). The small amount of HCR/A recovered in the GST−Syt II immunoprecipitation relative to the input is a property of HCR/A and has been previously reported (*12*). To determine whether detergents disrupt these interactions, the reactions were performed in the presence of either the zwitterionic detergent CHAPS or the nonionic detergent Triton X-100. The efficiency of binding of HCR/A to SV2C and binding of HCR/B to Syt II was not affected by either 2% (w/v) CHAPS or 2% (v/v) Triton X-100. These observations showed that the direct interactions of HCR/A with SV2 and HCR/B with synaptotagmin II were not affected by CHAPS or Triton X-100 at the concentrations used in these studies.

BoNT HCR Domains Compete with BoNT Holotoxins for Binding to Primary Cortical Neurons. To further validate the use of HCR domains as a tool for studying BoNT receptors, their ability to inhibit the intracellular activity of the native holotoxins in cultured primary rat cortical neurons (DIV 10−14) was determined. As reported previously, cleavage of SNAP25 and VAMP2 by BoNT/A and BoNT/B, respectively, was activity-dependent (Figure 3) (*13*, *16*, *17*). Exposure of neurons to holotoxins in the presence of a 100-fold excess of the homologous, but not the heterologous, HCR domain reduced the extent of substrate cleavage by severalfold as determined by Western blotting and immunofluorescence (Figure 3).

Copurification of a Presynaptic Protein Complex with HCR/A. Potential interactions involving synaptic vesicle membrane proteins and BoNT HCRs were analyzed by detergent solubilization and subsequent immunoprecipitation and protein identification by nanoLC−MS/MS. A synaptic vesicle fraction (P4 extract) prepared from rat brain cortex was solubilized in either the zwitterionic detergent CHAPS or the nonionic detergent Triton X-100, since earlier studies showed that CHAPS extraction preserved a synaptic vesicle membrane protein complex, while Triton X-100 extraction dissociated several components of the complex (*22*). HCRs were immunoprecipitated from P4 extracts using a 3×FLAG epitope, with a recovery rate of >90%. Moreover, the use of excess 3×FLAG peptide to selectively release immune complexes significantly reduced the levels of nonspecific proteins associated with the beads.

Immunoprecipitation of HCR/A from P4 extracts prepared in CHAPS resulted in the copurification of at least six synaptic vesicle membrane proteins (Table 1) as determined by mass spectrometry. The association of this protein complex with HCR/A was specific since elution from control reactions (3×FLAG-GFP, beads alone) did not contain these proteins (Figure 4). The analysis was repeated using a P4 extract prepared in Triton X-100. Under these conditions, HCR/A copurified with synaptic vesicle protein 2 (SV2) and to a lesser extent synaptotagmin I; other components of the neurotransmitter protein complex were not detected (Figure 4). These data demonstrate that BoNT HCR/A associates with synaptic vesicle membrane proteins and suggest the BoNT protein receptor may be a component of a larger protein complex.

Interaction of HCR/A and HCR/B with a Presynaptic Vesicle Protein Complex. Immunoprecipitation of HCR/B from P4 extracts prepared in CHAPS resulted in copurification of a protein complex similar to HCR/A (Table 1 and Figure 4). While the core components of the complexes were conserved (SV2, synaptotagmin I, synaptophysin, synaptogyrin 3, VAMP, and the vacuolar proton pump), subtle differences in the protein complexes or isoforms were detected. These differences may reflect the heterogeneity of the synaptic vesicles within the cerebral cortex and may contribute to the targeting of individual BoNT serotypes to a subset of neurons. Immunoprecipitation of HCR/B from P4 extracts prepared in Triton X-100 resulted in the copurification of HCR/B bound to synaptotagmin I (Figure 4). The absence of Syt II as determined by LC−MS/MS was unexpected given its reported high-affinity binding to BoNT/B (*16*) and may result from the reported differential expression of Syt I and Syt II in the cerebral cortex (*23*). Immunoblotting using an anti-Syt II specific monoclonal antibody (clone 8G2) confirmed the presence of Syt II in TCA precipitates of the cortex lysates (data not shown). However, due to the low sensitivity of the antibody, the possibility that HCR/B immunoprecipitates contained Syt II at levels below the detection limit could not be ruled out. To further address the specificity of the observed interactions, the ability of HCR/C to also bind to synaptic vesicle components was assessed. Under identical conditions, HCR/C failed to immunoprecipitate either the isolated SV2−synap-

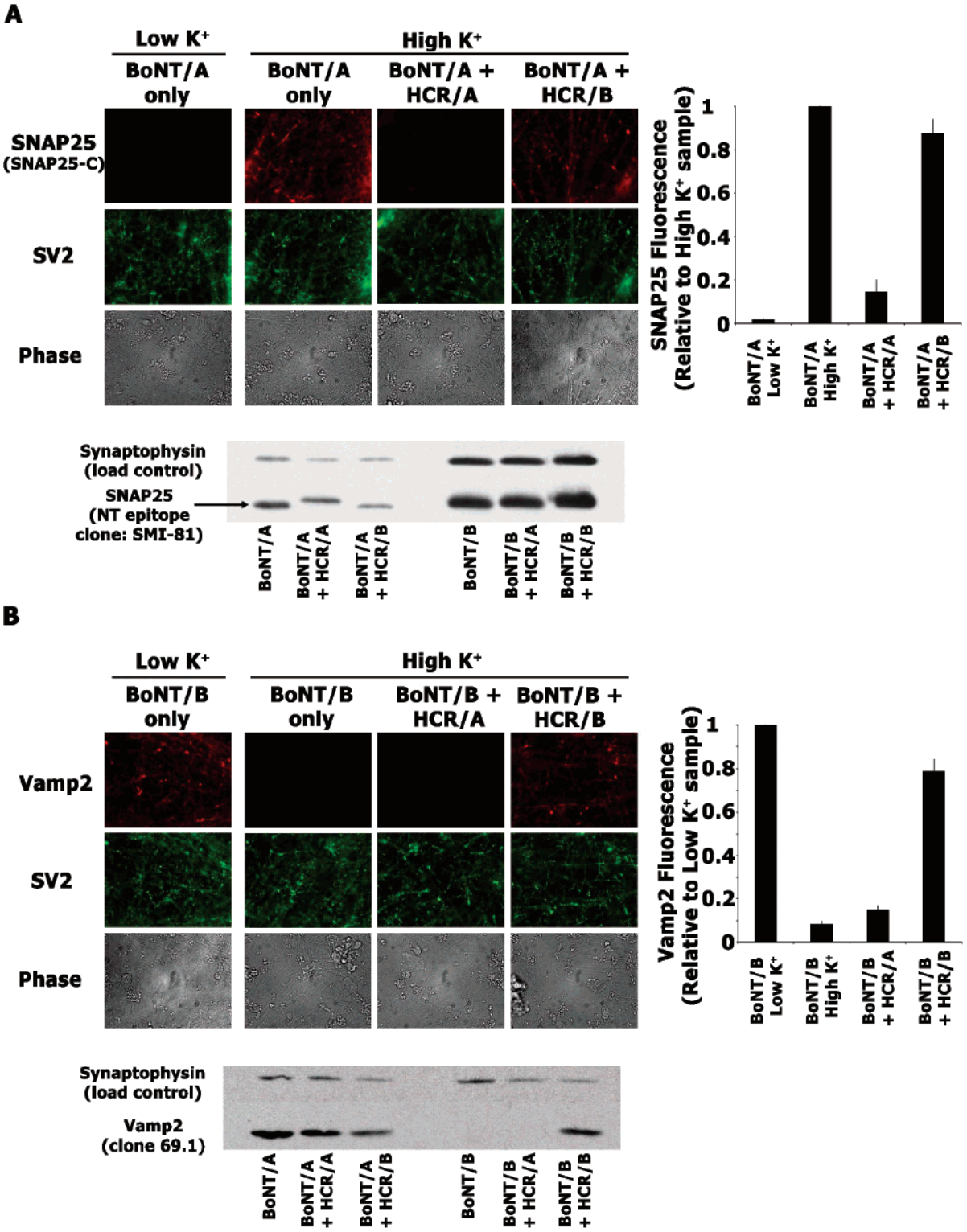

FIGURE 3: BoNT HCR domains inhibit binding of BoNT holotoxins to and entry of BoNT holotoxins into primary cortical neurons. Cortical neurons were exposed to 10 nM BoNT/A (A) or BoNT/B (B) in the presence or absence of 1 μM HCR/A or HCR/B for 15 min. Cells were then rinsed and incubated for a further 48 h at 37 °C. Cleavage of SNAP25 by BoNT/A was detected using anti-SNAP25-C that recognizes only the cleaved form of SNAP25. The level of cleavage of SNAP25 by BoNT/A was reduced ~85% by HCR/A ($P < 0.001$, *t*-test, $n = 24$) but not by HCR/B ($P > 0.05$, *t*-test, $n = 36$). Cleavage of VAMP2 by BoNT/B was assessed using an anti-VAMP-2 antibody (clone 69.1) that does not recognize the cleaved protein. The level of cleavage of VAMP2 by BoNT/B was reduced ~80% by HCR/B ($P < 0.001$, *t*-test, $n = 52$) but not by HCR/A ($P > 0.05$, *t*-test, $n = 38$). Cells were counterstained using an anti-SV2 antibody to indicate the presence of synaptic vesicles. Error bars represent the standard error of the mean. In parallel samples, cells were recovered and cleavage of SNAP25 (A) or VAMP2 (B) was analyzed by Western blotting. Cleavage of SNAP25 was detected using monoclonal SMI-81 directed against an N-terminal epitope. Cleaved SNAP25 is denoted with an arrow.

totagmin I complex from the Triton X-100 extract or the CHAPS-stabilized protein complex (Figure 4). To address the possibility that recombinant HCR/C was nonfunctional, the entry of HCR/C into cultured primary neurons was investigated. Both native BoNT/C and recombinant HCR/C were seen to enter cultured neurons (data not shown). Thus,

Table 1: Copurification of Synaptic Vesicle Proteins with HCR/A and HCr/B[a]

	accession number	identification	no. of peptides sequenced/score[b] HCR/A	HCR/B
vesicle proteins	139352	vATPase, V0 subunit a1-I	20/957	18/853
	3955100	vATPase, V1 subunit D1	12/423	12/423
	34869154	vATPase, V1 catalytic subunit A1	7/380	4/154
	19705578	vATPase, V1 catalytic subunit B2	8/312	8/312
	37589624	vATPase, V1 subunit E1	5/183	5/183
	190460	vATPase, V1 catalytic subunit B1	5/141	5/141
	55631030	vATPase, V0 subunit d2	3/204	2/138
	31981588	vATPase, V1 subunit H	2/111	2/111
	74229928	synaptotagmin I	21/828	23/928
	17901861	SV2B	18/411	18/411
	17105366	SV2A	6/210	4/175
	34870526	synaptogyrin 3	4/163	4/163
	6678195	synaptophysin	3/136	2/110
	16758726	vGlut1	4/108	4/108
	55635643	vGlut2	4/81	2/104
	13928804	SV2C	3/65	n/a[c]
	4894188	VAMP 2B	3/210	2/144
others	13591882	SNAP 25	1/68	1/68
	1587842	syntaxin-1A	n/a[c]	2/88
	51704841	GAPDH	4/177	4/177
	76624485	Unc-13B1 like	3/59	3/59
	13591908	AP-2, alpha 2 subunit	n/a[c]	2/74

[a] CHAPS-extracted neurotransmitter proteins that copurified with HCR/A or HCR/B were identified by LC−MS/MS (only proteins identified in two independent analyses are listed). [b] Number of observed peptides/number of total peptides. [c] Not observed.

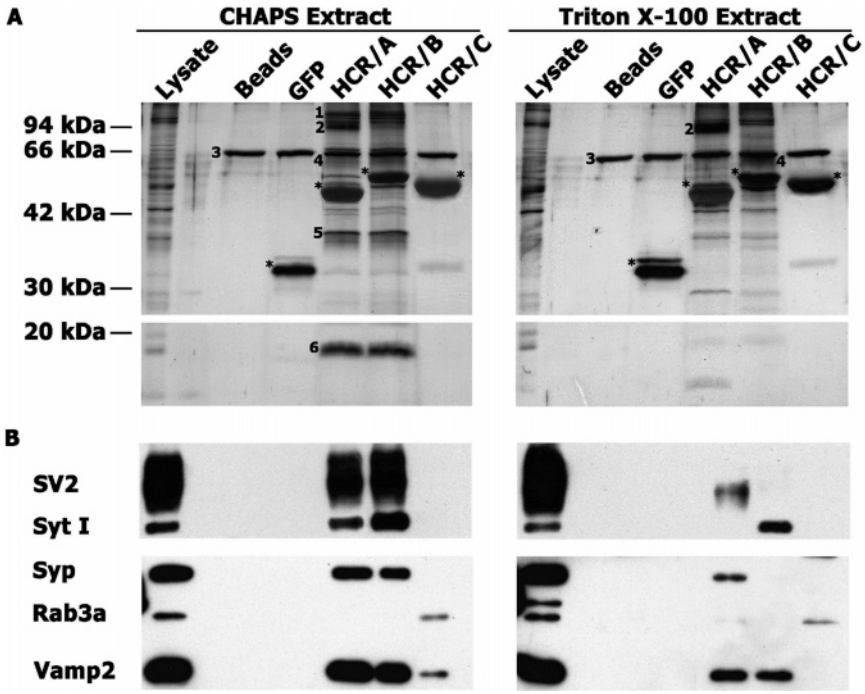

FIGURE 4: Immunoprecipitation analysis of detergent-solubilized synaptic vesicles. Synaptic vesicle proteins were solubilized with either 1% (w/v) CHAPS (left) or 1% (v/v) Triton X-100 (right) and subjected to FLAG immunoprecipitation with the following: 100 nM GFP, HCR/A, HCR/B, or HCR/C. The immunoprecipitates were analyzed by staining with silver (A) or Western blotting (B) for the presence of SV2, synaptotagmin I (Syt I), synaptophysin (Syp), Rab3a, and VAMP2. The "beads" lane displays the background contributed by FLAG M2 agarose beads. Numbers in panel A represent the following: (1) v-ATPase, V0 subunit a1-I, (2) SV2, (3) BSA (FLAG M2 agarose bead blocker), (4) synaptotagmin I (asterisk), HCRs/GFP, (5) synaptophysin, and (6) VAMP2.

HCR/A and HCR/B, but not HCR/C, interact with synaptic vesicle protein complexes.

Native BoNT/A Associates with the Presynaptic Vesicle Protein Complex. To determine the specificity of the protein complex associated with HCRs, the association of native holotoxin BoNT/A and synaptic vesicle proteins was investigated. Immunoprecipitation of BoNT/A from CHAPS extracts resulted in the coprecipitation of SV2, Syt I, and Syp (Figure 5A). Controls showed that the SV2, Syt I, and Syp coprecipitation was BoNT/A-dependent, indicating the coprecipitation was specific. Immunoprecipitation of BoNT/A from Triton X-100 extracts resulted in the coprecipitation of SV2 and to a lesser extent Syt I, but not Syp. This is consistent with the earlier observations that SV2 and Syt I

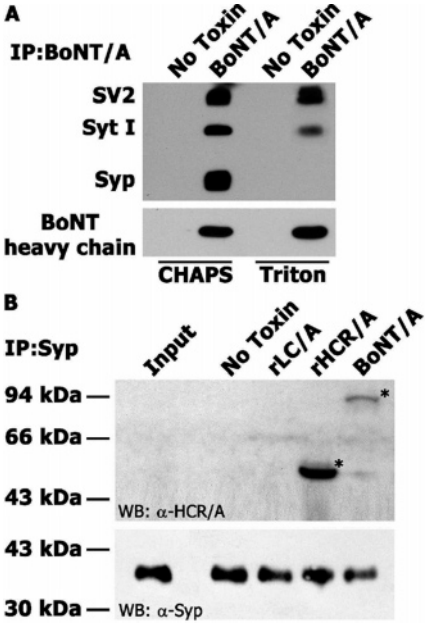

FIGURE 5: Co-immunoprecipitation of synaptophysin with native BoNT/A. (A) Synaptic vesicle proteins solubilized with 2% (w/v) CHAPS or 2% (v/v) Triton X-100 were incubated alone or in the presence of 25 nM native BoNT/A and subjected to immunoprecipitation using a rabbit polyclonal serum against the light chain (rLC/A, residues 1−425). Copurified material was separated on an 8% (w/v) SDS−PAGE gel and visualized by Western blotting against SV2, Syt I, and Syp (top) or rHCR/A (bottom). (B) Synaptic vesicle proteins solubilized with 2% (w/v) CHAPS were incubated alone or in the presence of 25 nM rLC/A, rHCR/A, or native BoNT/A and subjected to immunoprecipitation with an anti-synaptophysin antibody. Immunoprecipitates were resolved by SDS−PAGE and subjected to Western blotting using an anti-HCR/A serum (top) or an anti-synaptophysin antibody (bottom). The positions of rHCR/A and native BoNT/A HC are denoted with asterisks.

stably associate in Triton X-100 (*24*, *25*). In the complementary experiment, immunoprecipitation of synaptophysin from CHAPS extracts coprecipitated BoNT/A and HCR/A (Figure 5B). As reported previously, immunoprecipitation of synaptophysin from Triton X-100 lysates did not coprecipitate either BoNT/A or HCR/A (data not shown and ref *17*). These data indicate that both native BoNT/A and recombinant HCR/A interact with synaptic vesicle protein complexes.

Iodixanol Gradient Centrifugation Resolves HCRs Bound to Detergent-Extracted Protein Complexes. The results of the immunoprecipitation analysis indicate that the association of the HCR/A and HCR/B domains with synaptic vesicle protein complexes and that these associations are differentially preserved by detergents. Buoyant density centrifugation was used to further define the interactions between synaptic vesicle proteins and HCR domains. HCR/A associated with dense fractions of the CHAPS gradient, which indicated the presence of synaptic vesicle protein−HCR/A complexes (Figure 6A). Of the synaptic vesicle proteins that were examined, only Rab3a was excluded from the complex (data not shown). Since Rab3a is not a component of the identified synaptic vesicle protein−HCR/A complex, this indicated that intact synaptic vesicles were not present in the gradient fractions. The rapidly migrating synaptic vesicle protein−HCR/A complex was not observed in Triton-extracted vesicle preparations (Figure 6B). In both detergent gradients, a

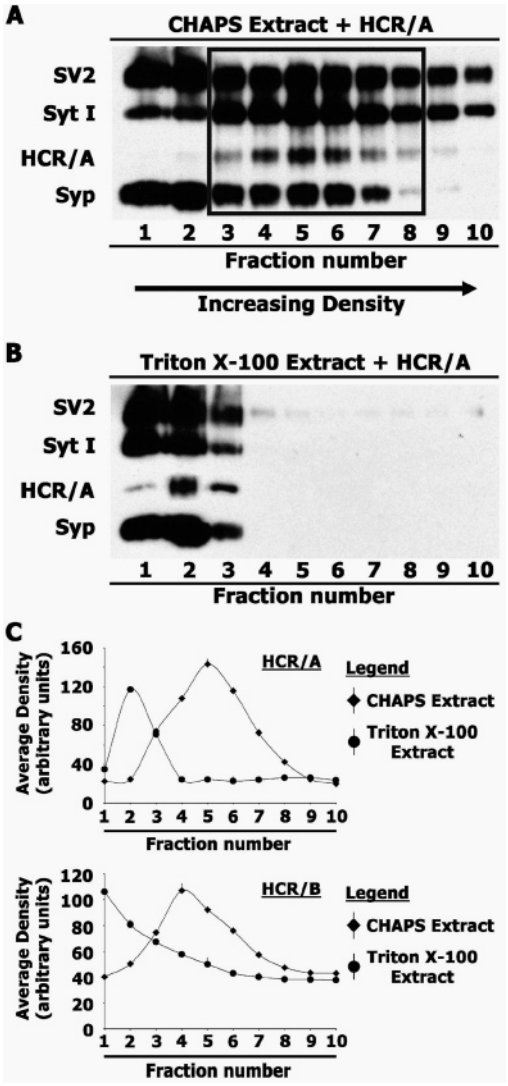

FIGURE 6: Buoyant density gradient analysis of detergent-solubilized synaptic vesicles. Synaptic vesicle fractions were solubilized with either 1% (w/v) CHAPS (A) or 1% (v/v) Triton X-100 (B) and incubated for 2 h at 4 °C in the presence of 100 nM HCR/A. Clarified lysates were subsequently resolved on 5 to 40% (w/v) linear Iodixanol gradients prepared in the corresponding detergent (as described above) for 6 h. Fractions from the gradients (starting from the top, fractions 1−10) were collected and analyzed by Western blotting for the presence of SV2, Syt I, Syp, and HCR/A. (C) HCR/A (top) and HCR/B (bottom) signal intensity was quantified by scanning densitometry using the FluorChem 8900 Imaging System and plotted relative to gradient fraction number.

fraction of the vesicle proteins, including SV2, were detected in the light fractions of the gradient (fractions 1−3). This could result from complex dissociation or represent distinct protein complexes. The observation that HCR/A fractionated with the larger complexes in CHAPS and was not detected in the light fractions suggested that HCR/A has a preferred association with the synaptic vesicle protein complex (Figure 6A, boxed region, and Figure 6C). HCR/A did not migrate into the CHAPS gradient in the absence of synaptic vesicle proteins or in the presence of Triton X-100 vesicle preparations, suggesting the observed fractionation pattern was due the association of HCR/A with the protein complex (data not shown). HCR/B exhibited a unique fractionation pattern, relative to HCR/A, in the presence of either CHAPS or Triton X-100 vesicle extracts. HCR/B associated with light and

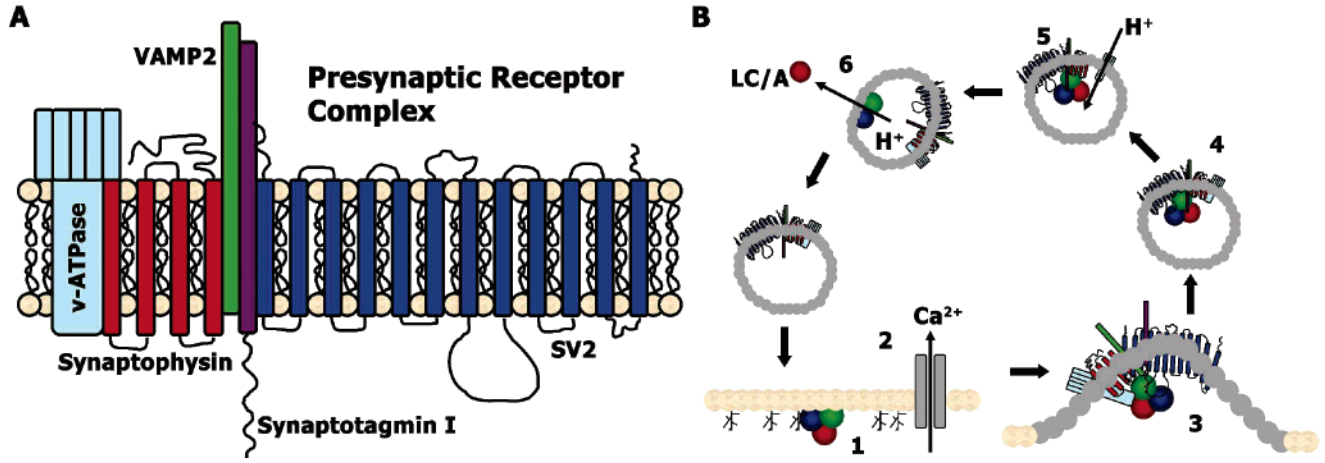

FIGURE 7: Model for binding and entry of BoNTs at the neuromuscular junction. (A) Interaction of synaptic vesicle proteins forms a large protein complex that includes synaptic vesicle protein 2 (SV2), synaptotagmin I (Syt I), synaptophysin (Syp), vesicle-associated membrane protein 2 (VAMP 2), and the vacuolar proton pump (v-ATPase). The presynaptic receptor complex is stable following solubilization with CHAPS but partially dissociates following solubilization with Triton X-100. (B) BoNT/A associates with the presynaptic membrane of α-motor neurons through interactions with oligosaccharides such as ganglioside G_{T1b} (1). Calcium influx stimulates synaptic vesicle membrane fusion (2). BoNT/A interacts with the exposed receptor complex, stimulating synaptic vesicle recycling (3). Recycled synaptic vesicles are acidified through the activity of the v-ATPase (4). Vesicle acidification drives the translocation of the BoNT/A light chain (LC/A) into the cytosol (5), completing the cycle (6).

dense fractions in both gradients (Figure 6C) but was detected only in the lightest fraction 1 in the absence of synaptic vesicle proteins. This suggests HCR/B can associate with synaptotagmin I in both the CHAPS stable protein complex and in distinct complexes or with synaptotagmin monomers.

DISCUSSION

In this study, a model system was developed for the identification and analysis of potential neuronal receptors. The association of HCR/A and HCR/B with a presynaptic vesicle protein complex was demonstrated that included SV2, synaptotagmin I, synaptophysin, synaptogyrin 3, VAMP2, and multiple subunits of the vacuolar type ATPase (v-ATPase) (Table 1). The CHAPS stable complex resembled a previously identified complex formed in synaptic vesicles (Figure 7A). Scheller and colleagues reported that complex formation was CHAPS-dependent and that Triton X-100 disrupted the complex (*20*). Estimates of the protein:lipid ratios suggested that the complex formed the major protein component of the intact vesicle. Moreover, shadowing and immunogold electron microscopy suggested the complex may exist in native synaptic vesicles. Subsequent studies have confirmed interactions between specific components of the complex and demonstrated how these interactions regulate synaptic vesicle activities (*24*−*28*). While these studies represented the initial identification of the complex, the observation that BoNTs are able to bind the complex provides the first support for a physiological function for the complexes in synaptic vesicle biology.

The complex identified by Scheller and colleagues was comprised of SV2, synaptotagmin I, synaptophysin, VAMP2, and the V0 d1 subunit (previously named Ac39) of the vacuolar type ATPase (v-ATPase) (*20*). The composition of the complexes was shown to be detergent sensitive, suggesting that certain protein−protein interactions were direct while others were indirect. Sucrose gradient and gel filtration analysis indicated that the CHAPS complex was heterogeneous in size. These results suggest that the largest complexes may be unstable under the conditions used for isolation. Alternatively, the heterogeneity may represent smaller complexes containing a different subset of vesicle proteins or protein monomers. The BoNT receptor complexes contained several additional proteins (SNAP25, synaptogyrin 3, GAPDH, and vGLUT1/2) as well as the core components of the complex described by Scheller. In addition to the v-ATPase V0 subunit d isoform 2, V0 subunit a and V1 subunits A1, B1, B2, D1, E1, and H were identified. A recent report has demonstrated that rat neurons express three different V0 a subunits (a1-I, a1-IV, and a2), which are sorted to different neuronal membrane compartments. The a1-I isoform identified in this study was the only a subunit shown to be specifically concentrated in nerve terminals (*29*). This suggests that the BoNT receptor complex isolated in this study was derived from synaptic vesicles and supports the hypothesis that individual V0 a subunits have specific roles in neural function. The direct interaction between the v-ATPase and other vesicle components is not known, but previous reports showed that V0 d1 associates with the synaptophysin−VAMP2 complex in the presence of Triton X-100 (*28*). The complex associated with HCR/A and HCR/B also contained synaptogyrin 3. Synaptogyrins comprise a family of tyrosine-phosphorylated proteins with two neuronal (synaptogyrins 1 and 3) and one ubiquitous (cellugyrin) isoform (*30*). In contrast to the ubiquitous distribution of synaptogyrin 1, the expression pattern of synaptogyrin 3 was more restricted (*31*). While the functions of synaptogyrins are unclear, the association of synaptogyrin 3 with the BoNT receptor complex indicates specific isoforms may represent functionally distinct populations of synapses and/or synaptic vesicles. The BoNT receptor complexes also contained vesicular glutamate transporter 2 (vGLUT-2) in addition to the main transporter, vGLUT-1. While exhibiting similar transport kinetics, the two transporters are expressed in different parts of the brain. In adult mice, the distribution of the transporters is mostly nonoverlapping with vGLUT1 expressed in the cortex and vGLUT2 expressed in the brain stem (*32*−*34*). It is not yet clear why vGLUT-1 and vGLUT-2 are differentially expressed, but the association

of two vGLUTs with the BoNT receptor complexes suggests subsets of glutamatergic neurons may be targeted by the BoNTs. Furthermore, preliminary data suggest that the BoNT/A HCR domain preferentially associates with vesicle complexes containing vGLUTs and not vGAT (M. R. Baldwin and J. T. Barbieri, unpublished observation). However, due to the amounts of protein obtained by immunoprecipitation, there is limited information about the stoichiometry of the proteins in the complex.

The results of the immunoprecipitation analysis indicated that a number of interactions may occur between synaptic vesicle proteins and HCR domains. To further define interactions between synaptic vesicle proteins and HCR domains, Iodixanol gradient analysis was performed. The HCR/A−CHAPS receptor complex rapidly migrated into the gradient (Figure 6A). In contrast, the rapidly migrating HCR/A−receptor complex was not observed in Triton X-100 extracts (Figure 6B). This suggests the fractionation pattern observed was due to association of HCR/A with the CHAPS receptor complex and correlates with the immunoprecipitation analysis. In agreement with the Scheller data, heterogeneity was observed in CHAPS complex size in either the presence or absence of HCR/A. Interestingly, HCR/A fractionated with the larger CHAPS complexes and was not detected in the light fractions (Figure 6A, boxed). This suggested that HCR/A associated with SV2 in the context of a large vesicle protein complex rather than as a protein monomer.

The immunoprecipitation analysis established that HCR/B, like HCR/A, also associated with synaptic protein complexes (Table 1 and Figure 4). Protein identification using LC−MS/MS and Western blotting indicated the CHAPS complexes bound by both HCR/A and HCR/B were similar. However, while HCR/B competed with BoNT/B for binding and entry into rat cortical neurons, HCR/B did not compete with BoNT/A (Figure 3). To address this apparent discrepancy, HCR/B was subjected to gradient analysis under conditions identical to those used in the HCR/A studies (Figure 6C). In the presence of either CHAPS or Triton X-100, HCR/B was associated with vesicle proteins in both light and dense fractions. The differences between HCR/A and HCR/B binding could result from several possibilities. First, the structural arrangement of the vesicle proteins within the CHAPS receptor complex may allow simultaneous binding of both HCR/A and HCR/B via SV2 and synaptotagmin I, respectively. Second, HCR/B may bind to a distinct subset of protein complexes which contain overlapping vesicle proteins. Third, HCR/B may associate with synaptotagmin I as part of a protein complex or as a free monomer. In support of the third possibility, Kozaki and colleagues (*35*−*38*) previously reported that BoNT/B bound both rat synaptosomes and purified rat synaptotagmin II with similar affinities ($K_d \approx 0.4$ nM).

The interaction of BoNTs with protein complexes containing putative receptor molecules parallels recent observations that diphtheria (DT) and anthrax toxins bind to receptors composed of at least two protein components. Expression cloning identified the precursor of heparin-binding EGF-like growth factor (proHB-EGF) as being necessary and sufficient for conferring sensitivity to DT in resistant cell populations (*39*). Subsequently, Mekada and colleagues demonstrated that direct interaction of DRAP 27/CD9 with Monkey proHB-EGF formed a DT receptor complex with increased DT binding affinity and consequently increased toxin sensitivity (*40*−*43*). Consequently, CD9 was proposed as a coreceptor for DT and set a precedent for the concept of toxins using protein complexes as high-affinity protein receptors. Anthrax toxin is comprised of three separate proteins: lethal factor (LF), edema factor (EF), and protective antigen (PA). PA is responsible for the import into cells (*44*). The initial recognition between PA and the host receptors, ATR/tumor endothelial marker 8 (TEM8) and/or capillary morphogenesis protein 2 (CMG2), is critical for entry of the toxin (*45*, *46*). However, binding of PA to the CMG2 receptor alone is not sufficient for endocytosis of the toxin into cells (*47*). Recently, Cohen and colleagues demonstrated low-density lipoprotein receptor-related protein 6 (LRP6) is required for anthrax toxin endocytosis (*48*). Further experiments suggested a model in which the extracellular domain of LRP6 initially binds to the extracellular domains of either CMG2 or TEM8. When PA binds, the signal for internalization of the toxin−receptor complex is mediated through the extracellular domain of LRP6 which is propagated by the LRP6 intracellular C-terminal domain. This leads to the internalization of the toxin−receptor complex into an endosomal compartment where LF and EF are translocated to the cytoplasm.

The data presented in this study are consistent with the long-standing double-receptor model proposed by Montecucco (*49*) (Figure 7B). First, BoNTs interact with lipid- and/or protein-linked oligosaccharides such as ganglioside G_{T1b}, concentrating the toxin on the presynaptic membrane. This initial interaction appears critical, since disruption of ganglioside synthesis in mice reduces their sensitivity to CNTs (*50*−*52*). Similarly, preincubation of BoNTs with gangliosides reduces the level of binding to cultured neurons. Subsequent to initial capture, calcium influx resulting from an action potential stimulates synaptic vesicle membrane fusion. This exposes synaptic protein complexes to the extracellular milieu, allowing neurotoxin binding and subsequent uptake through clathrin-mediated endocytosis. BoNT/A and BoNT/B associate with a synaptic vesicle protein complex on the exposed cell surface. Endocytosis of the toxin−synaptic protein vesicle complex initiates the internalization of BoNT into cells. Future studies will elucidate the role of individual proteins in the binding and entry process. While earlier studies have revealed the physical presence of these synaptic vesicle protein complexes, their association with BoNT/A and BoNT/B implicates a physiological role in the biology of neuronal vesicle trafficking.

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