Identification of the SV2 protein receptor-binding site of botulinum neurotoxin type E

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INTRODUCTION

The seven serotypes of BoNT (botulinum neurotoxin; BoNT/A–G) are highly poisonous bacterial di-chain protein toxins of the AB-type that cause the disease botulism. Their extreme potency is, among other factors, generated by the exceptional neurospecific binding to target cells, in which peripheral cholinergic motoneurons turn them into the most poisonous substances known. Interaction with gangliosides accumulates the neurotoxins on the plasma membrane and binding to a synaptic vesicle membrane protein leads to neurotoxin endocytosis. SV2 (synaptic vesicle glycoprotein 2) mediates the uptake of BoNT/A and /E, whereas Syt (synaptotagmin) is responsible for the endocytosis of BoNT/B and /G. The Syt-binding site of the former was identified by co-crystallization and mutational analyses. In the present study we report the identification of the SV2-binding interface of BoNT/E. Mutations interfering with SV2 binding were located at a site that corresponds to the Syt-binding site of BoNT/B and at an extended surface area located on the back of the conserved ganglioside-binding site, comprising the N- and C-terminal half of the BoNT/E-binding domain. Mutations impairing the affinity also reduced the neurotoxicity of full-length BoNT/E at mouse phrenic nerve hemidiaphragm preparations demonstrating the crucial role of the identified binding interface. Furthermore, we show that a monoclonal antibody neutralizes BoNT/E activity because it directly interferes with the BoNT/E–SV2 interaction. The results of the present study suggest a novel mode of binding for BoNTs that exploit SV2 as a cell surface receptor.

Key words: botulinum neurotoxin E, C-terminal fragment of the heavy chain (Hc–fragment), synaptic vesicle glycoprotein 2 (SV2), synaptotagmin.

The highly specific binding and uptake of BoNTs (botulinum neurotoxins; A–G) into peripheral cholinergic motoneurons turns them into the most poisonous substances known. Interaction with gangliosides accumulates the neurotoxins on the plasma membrane and binding to a synaptic vesicle membrane protein leads to neurotoxin endocytosis. SV2 (synaptic vesicle glycoprotein 2) mediates the uptake of BoNT/A and /E, whereas Syt (synaptotagmin) is responsible for the endocytosis of BoNT/B and /G. The Syt-binding site of the former was identified by co-crystallization and mutational analyses. In the present study we report the identification of the SV2-binding interface of BoNT/E. Mutations interfering with SV2 binding were located at a site that corresponds to the Syt-binding site of BoNT/B and at an extended surface area located on the back of the conserved ganglioside-binding site, comprising the N- and C-terminal half of the BoNT/E-binding domain. Mutations impairing the affinity also reduced the neurotoxicity of full-length BoNT/E at mouse phrenic nerve hemidiaphragm preparations demonstrating the crucial role of the identified binding interface. Furthermore, we show that a monoclonal antibody neutralizes BoNT/E activity because it directly interferes with the BoNT/E–SV2 interaction. The results of the present study suggest a novel mode of binding for BoNTs that exploit SV2 as a cell surface receptor.

It is well established that PSGs (polysialogangliosides) play an important role in the binding of BoNTs to neuronal cells [9]. PSGs are abundant constituents of the neuronal plasma membrane. Interaction with PSGs is thought to accumulate the BoNTs in the plane of the plasma membrane leading to an increased local concentration of the toxin. Binding to PSGs is mediated by a conserved binding site [GBS (ganglioside-binding site)] containing an SxWY...G motif as shown for BoNT/A, /B [10,11], /E, /F [12–14], /G [15–17] and one of two sites of the closely related TeNT (tetanus neurotoxin) [18,19]. The structure and composition of the PSG-binding sites of BoNT/C [20–22] and /D [23,24] are different.

In agreement with the proposed dual-receptor model [25] it was demonstrated that luminal parts of synaptic vesicle transmembrane proteins operate as BoNT receptors. Syt (synaptotagmin)-I and -II, membrane proteins of synaptic vesicles, were first identified as receptors for BoNT/B and /G [26–29]. Later it was demonstrated that the large luminal domain 4 of all three isoforms of the SV2 (synaptic vesicle glycoprotein 2 (SV2A–C)) act as receptor for BoNT/A [30,31]. Subsequently, it could be shown that a single N-glycosylation site in the large luminal domain 4 is requisite for BoNT/E to bind to SV2A and SV2B, but not SV2C [32]. It is still under debate whether SV2 also serves as a receptor for BoNT/D [23,33] and BoNT/F [12,14,33,34]. Only recently, it has been reported that in addition to BoNT/B and /G, the mosaic type BoNT/D–C also exploits Syt as a receptor [35].

Crystal structures of Hc–B in complex with the luminal domain of its protein receptor Syt-II [36,37], as well as mutual
analysis of H₂-B and H₂-G [17], revealed the molecular details of these interactions. It was shown that the Syt-II-binding site is located in close proximity to the PSG-binding site allowing for the simultaneous binding of PSG and the protein receptor. Furthermore, it was demonstrated that the luminal domain of Syt-II, which is unstructured in solution [36], adopts an α-helical conformation upon contact with H₂-B. The interaction is mainly governed by hydrophobic contacts (e.g. Phe<sup>37</sup> and Phe<sup>34</sup> of Syt-II and Phe<sup>190</sup> and Phe<sup>194</sup> of H₂-B), but a major salt bridge is also involved (Glu<sup>57</sup> of Syt-II and Lys<sup>192</sup> of H₂-B). Furthermore, a mutational analysis revealed that the binding interface between H₂-D-C and Syt is distinct, but partially overlaps with that of H₂-B and H₂-G [35]. In contrast with BoNT/B, G and D-C detailed information is currently unavailable for BoNTs which exploit the interaction with SV2 to gain access to target cells.

In the present study we demonstrate that residues mediating the BoNT/E–SV2 interaction are located at a position that is comparable with the Syt-binding site of BoNT/B and G and at an expanded surface area which is located on the back of the PSG-binding site comprising residues of H₂-C and of the adjacent region of H₂-G. In support of these data, we show that the binding site of a neutralizing monoclonal antibody to BoNT/E partially overlaps with the SV2-binding site. These findings illustrate a new mode of binding for BoNTs which exploits SV2 as a cell surface receptor.

**EXPERIMENTAL**

**Structure analysis**

Structure analyses and superimposition were done using the Discovery Studio Visualizer 2.5 software (Accelrys). Prediction of potential protein-binding sites was done applying the ODA (optimal docking area) algorithm (http://www.molsoft.com/oda.cgi) [38].

**Construction of plasmids**

The plasmid pH6F3H₂-ES, facilitating the bacterial expression of H₂-E fused to N-terminal His<sub>6</sub> and 3×FLAG tags and a C-terminal Strep-tag (IBA), was generated by cloning a synthetic DNA sequence encoding N-terminal His<sub>6</sub> and 3×FLAG tags and a BamHI/Strep-linker into the EcoRI and HindIII sites of pQE-3 (Qiagen) to yield the plasmid pH6F3E. Subsequently, a DNA sequence encoding H₂-E (amino acids 846–1251) was isolated from pS3H₂-EH<sub>6</sub> [12] and ligated into the BamHI and Smal sites of pH6F3E to form the plasmid pH6F3H₂-ES. The same DNA segment was also cloned into pSP73 (Promega) to form the plasmid pSP73-H₂-E facilitating the in vitro transcription and translation of unfused H₂-E. A plasmid encoding full-length BoNT/E (pH6BoNTES-Thro) has been described previously [12]. The plasmids pSM-SV2ALDS3 and pSM-SV2CLDS3, facilitating the eukaryotic expression of the glycosylated luminal domain 4 of SV2A and SV2C, were transfected into HEK (human embryonic kidney)–293 cells using Lipofectamine<sup>™</sup> 2000 (Life Technologies) according to the manufacturer’s instructions. Stably expressing cells were selected using G418 (Carl Roth). Following limiting dilution, clones H8E3 (SV2A) and H11 (SV2C) were chosen for the production and purification of the luminal domains of SV2A and SV2C respectively. For this purpose, confluent cells of a total area of 2250 cm<sup>2</sup> were harvested by rinsing and collected by centrifugation at 500 × g for 5 min at 4 °C. The cell pellets were washed once with ice-cold PBS and either shock-frozen in liquid nitrogen and kept frozen at −70 °C until use. Protein concentrations were determined by densitometry following SDS/PAGE (12.5 % gel) and Coomassie Blue staining using various known concentrations of BSA as standards. The luminal domains were purified using lysates of Escherichia coli strain M15 [pREP4] cells (Qiagen), following 16–18 h of induction at 21 °C. Proteins were consecutively purified on nickel-nitrilotriacetic acid (Macherey & Nagel) and Streptactin-Superflow (IBA) columns according to the manufacturers’ instructions. Full-length neurotoxins were eluted using 100 mM Tris/HCl (pH 8.0) and 10 mM desthiobiotin, whereas H₂ fragments were prepared in 100 mM Tris/HCl (pH 7.4), 100 mM NaCl and 10 mM desthiobiotin. Fractions containing high amounts of protein were pooled, shock-frozen in liquid nitrogen and kept frozen at −70 °C until use. Protein concentrations were determined by densitometry following SDS/PAGE (12.5 % gel) and Coomassie Blue staining using various known concentrations of BSA as standards. For the production of glycosylated luminal domain 4 of SV2A and SV2C, pSM-SV2ALDS3 and pSM-SV2CLDS3 were transfected into HEK (human embryonic kidney)–293 cells using Lipofectamine<sup>™</sup> 2000 (Life Technologies) according to the manufacturer’s instructions. Stably expressing cells were selected using G418 (Carl Roth). Following limiting dilution, clones H8E3 (SV2A) and H11 (SV2C) were chosen for the production and purification of the luminal domains of SV2A and SV2C respectively. For this purpose, confluent cells of a total area of 2250 cm<sup>2</sup> were harvested by rinsing and collected by centrifugation at 500 × g for 5 min at 4 °C. The cell pellets were washed once with ice-cold PBS and either shock-frozen in liquid nitrogen and kept frozen at −70 °C until use or directly dissolved in lysis buffer [20 mM Tris (pH 7.4), 80 mM NaCl and 0.5 % Triton X-100]. Subsequently to ultrasonic disruption of the cells, lysates were centrifuged at 4100 × g for 30 min at 4 °C and the supernatants were transferred on to Streptactin-Superflow agarose beads. To account for different expression levels of SV2A and SV2C luminal domain fusion proteins in the respective cell lines, 125 μl (SV2A) or 62.5 μl (SV2C) of beads were used respectively. The luminal domains were allowed to bind overnight at 4 °C with gentle shaking. Following three washing steps using lysis buffer the immobilized luminal domains were used as baits in SV2-binding assays.

**In vitro transcription and translation**

H₂ fragments were synthesized in vitro from pSP73 derivatives using the reticulocyte lysate system (Promega) and L-[<sup>35</sup>S]methionine (370 kBq, >37 TBq/mmol; Hartmann Analytic)
according to the manufacturer’s instructions, in a total volume of 12.5 μl (H₄E) or 25 μl (H₄A).

**SV2-binding assay**

Binding of wild-type and mutant H₄ fragments to SV2 was carried out in binding buffer [20 mM Tris (pH 7.4), 80 mM NaCl, 0.5 % Triton X-100 and 0.1 % BSA]. A total of 10 μl of Streptactin-Superflow agarose beads (IBA) coated with approximately 3 μg of glycosylated luminal domain 4 of SV2 was incubated with in vitro-translated radiolabelled H₄ fragments for 3 h at 4°C either in the presence or absence of 125 μg of mixed bovine brain gangliosides (Calbiochem) in a total volume of 200 μl. In order to analyse the capability of monoclonal antibodies to interfere with the binding of H₄ fragments to SV2, IgG was added to the reaction mixture at a final concentration of 200 nM. Following incubation, beads were collected by centrifugation at 2000 g for 3 min at 4°C and washed three times using lysis buffer. Washed pellet fractions were incubated in SDS sample buffer for 20 min at 37°C and analysed by SDS/PAGE (12.5 % gel) and phosphorimaging. The signals of the bound H₄ fragments were quantified by means of densitometry using AIDA (Advanced Image Data Analyzer) software (Raytest, version 2.11) with the signals of HC fragments binding to uncoated beads (beads only) being subtracted from the specific signals of H₄ fragments binding to SV2.

**Antibody production**

The monoclonal antibody 4E13 directed against H₄E was derived from a yeast-displayed scFv (single chain Fv) antibody library constructed from the V₉ (variable HC) and V₅ (variable LC) region genes of a human volunteer immunized with pentavalent botulinum toxoid (results not shown and [39]). The 4E13 antibody was isolated by selection of the library on BoNT/E holotoxin using previously described methods [39]. The variable chain genes were recloned into a mammalian expression vector and the resulting human IgG/k antibody was purified by Protein G chromatography [40]. Recombinant yeast-displayed BoNT/E H₄, H₅ and LC domains were used to identify the 4E13 bound to the BoNT/E H₄ domain [41]. To map the fine epitope of 4E13, a library of 2.16 x 10⁷ random yeast-displayed BoNT/E HC mutants were generated using error prone PCR as previously described [42] and the library was sorted for loss of binding 4E13 to the yeast displayed domain. Individual clones that had lost binding to 4E13 were sequenced to identify the epitope region. A total of 20 single alanine residue mutants were then generated spanning the putative epitope, their affinity for 4E13 measured and the change in free energy [ΔΔG_mut-set, ΔΔG_mut-set = RT ln(K_d,mut/K_d,wt)] between the HC alanine (Ala) mutant relative to that of the wild-type (wt) was calculated as described previously [42].

**Co-immunoprecipitation**

All centrifugation and incubation steps were carried out at 4°C, except where otherwise stated. Co-immunoprecipitation of native SV2A and SV2B from solubilized synaptosomal membranes was done in binding buffer [20 mM Tris (pH 7.4), 80 mM NaCl, 0.5 % Triton X-100 and 0.1 % BSA] in a total volume of 200 μl. The preparation of rat brain synaptosomes was performed as described previously [18]. After centrifugation at 5000 g for 5 min the collected synaptosomes were dissolved in lysis buffer [20 mM Tris (pH 7.4), 80 mM NaCl and 0.5 % Triton X-100]. Following 1 h of incubation the lysate was centrifuged at 21000 g for 10 min and the supernatant was transferred into a fresh tube. A total of 10 μl of Protein G–agarose beads (Amersham Biosciences) was incubated with 2 μg of the anti-FLAG M2 monoclonal antibody (Sigma–Aldrich), 6 μg of the indicated wild-type or mutant BoNT/E H₄ fragment, and 50 μg of synaptosomal proteins in the presence of 125 μg of mixed bovine brain gangliosides. Following 3 h of incubation, beads were collected by centrifugation at 2000 g for 3 min and washed three times using lysis buffer. Washed pellet fractions were incubated in SDS sample buffer for 20 min at 37°C and analysed by SDS/PAGE (12.5 % gel) and Western blotting using specific antibodies directed against SV2A (catalogue number 119 002, Synaptic Systems,) and SV2B (catalogue number 119 102, Synaptic Systems).

**Ganglioside-binding assay**

All incubation and washing steps were carried out using PBS supplemented with 0.1 % BSA at 4°C except where otherwise stated. Mixed bovine brain gangliosides (Calbiochem) dissolved in DMSO (20 μg/μl) were diluted in methanol and applied to 96-well PVC plates (20 μg/well; catalogue number 2595, Corning Costar). The solvent was allowed to evaporate overnight at room temperature (21°C). Wells were washed once before non-specific binding sites were blocked by incubation with 100 mM carbonate buffer (pH 9.4) supplemented with 2 % BSA for 1 h. The wells were washed once and the binding assays were performed in a total volume of 100 μl containing the indicated concentrations of wild-type and mutant H₄ fragments. Following 3 h of incubation the wells were washed twice and an anti-FLAG M2 monoclonal antibody (1:4000 dilution) was added for 1.5 h. Subsequently, the wells were washed twice and an HRP (horseradish peroxidase)-conjugated rabbit anti-mouse polyclonal antibody (1:16000 dilution, IBA) was added. Following 1 h of incubation the wells were washed three times and the bound H₄ fragments were detected by the addition of 100 μl of 1-Step Ultra-TMP Elisa (Thermo). After 40 min of incubation at room temperature the reaction was stopped by adding 100 μl of 2 M H₂SO₄. The absorbance at 450 nm was detected using a SpectraCount plate reader (Packard). The inflection points of the binding curves were determined by fitting the data to the sigmoidal dose–response equation using GraphPad Prism (GraphPad Software, version 4.03) and expressed as logEC₅₀ values in units of nM.

**MPN (mouse phrenic nerve) hemidiaphragm assay**

Recombinant wild-type and mutant full-length BoNT/E neurotoxins were proteolytically activated to form the active di-chain neurotoxins employing immobilized trypsin (0.001 units per μg of single-chain neurotoxin; Sigma–Aldrich) equilibrated in 100 mM Tris/HCl (pH 8.0) for 25 min at room temperature with gentle shaking. The reaction was stopped by three consecutive centrifugation steps, each including the transfer of the supernatant to a fresh tube to remove any residual trypsin. The activated neurotoxins were stored on blue ice until used. The MPN assay was performed as described previously [43] in compliance with the German animal protection law (TSchG). According to §4 Abs. 3 [killing of animals for scientific purposes (TSchG)] animals were killed by trained personnel before dissection of organs and its number reported yearly to the animal welfare officer of the Central Animal Laboratory and to the local authority, Veterinäramt Hannover. The phrenic nerve was continuously stimulated at 5–25 mA at a frequency of 1 Hz. Isometric contractions were recorded using the VITRODAT Online software.
Figure 1  SV2 binding of H2/E domains

(A) SDS/PAGE analysis of HEK-293 cell-expressed purified SV2A and C luminal domain fusion proteins which were used as baits in SV2-binding assays. (B and C) Binding assay of the H2 and H2/E (D) and H2/E and H2/E domains (C) to SV2A and SV2C. The purified immobilized glycosylated luminal domain of SV2A or SV2C was incubated with in vitro-translated radiolabelled derivatives of BoNT/A or E in the presence of mixed bovine brain gangliosides. After removal of unbound protein, bound H2 was visualized by autoradiography following SDS/PAGE. *Probable H2E in differing electrophoretic mobility. Arrowhead denotes bound H2E. In (A–C) M denotes molecular mass markers. (D) Superimposition of the structures of H2B (light blue) in complex with Syt-II (red, PDB code 2NM1) and H2E (pale green, PDB code 3FFZ). Selected residues mediating the interaction of H2B with Syt-II are highlighted in blue stick representation. Residues in the homologous position of H2E that were subjected to mutational analysis are shown as green sticks. (E) Structure of H2E (PDB code 3FFZ). Residues of H2E selected for mutation on the basis of ODA analysis are shown as green sticks. ODA sites are highlighted by black circles. (D and E) Residues that exhibit no significant effect when mutated are labelled in black.

(FMI). The time required to decrease the amplitude to 50% of the starting value (paralytic half-time) was determined. To estimate the altered neurotoxicity of the BoNT/E mutants, a concentration–response curve, consisting of three data points was compiled to which a power function could be ascribed:
y(BoNT/E wild-type, 30, 100 and 300 pM) = 335.81 × 10^{-0.3209}, R^2 = 0.9984. The resulting paralytic half-times were converted into the corresponding concentrations of wild-type BoNT/E, using the equation given above, and neurotoxicity was finally expressed as the percentage of the BoNT/E wild-type neurotoxicity.

Sandwich ELISA

All incubation and washing steps were carried out at room temperature except where otherwise stated. Monoclonal human antibody 4E13, directed against H2E, was diluted in 100 mM carbonate buffer (pH 9.4) and applied to 96-well high-binding plates (500 ng/well; catalogue number 655081, Greiner Bio One). Following an overnight incubation at 4°C, wells were washed once using PBS before the non-specific binding sites were blocked by incubation with 100 mM carbonate buffer (pH 9.4) supplemented with 2% BSA for 1 h. The wells were washed once using PBS and the binding assays were performed in a total volume of 50 μl containing the indicated amounts of wild-type and mutant H2 fragments. Following 1 h of incubation the wells were washed three times using PBS with 0.05% Tween 20 and an HRP-conjugated anti-Strep-tag monoclonal antibody (1:10000 dilution) was added for 1 h. Subsequently, the wells were washed three times using PBS with 0.05% Tween 20 and the bound H2 fragments were detected by the addition of 100 μl of 1-Step Ultra-TMP. After 45 min of incubation at room temperature the reaction was stopped by adding 100 μl of 2 M H2SO4. The absorbance at 450 nm was detected using a SpectraCount plate reader. The inflection points of the binding curves were determined by fitting the data to the sigmoidal dose–response equation using GraphPad Prism and expressed as logEC_{50} values in units of nM.

CD spectroscopy

CD data was collected with a Jasco J-810 spectropolarimeter using a 1-mm path-length cuvette with 10 μM wild-type or mutant H2E diluted in PBS. Spectra were recorded from 195 to 250 nm with 100 nm/min, a response of 1 s, standard sensitivity, a bandwidth of 1 nm and five accumulations. Temperature-induced denaturation was performed by monitoring the CD signal at 210 nm from 21°C to 60°C every 1–5°C.

RESULTS

An expanded interface mediates the BoNT/E–SV2 interaction

Previous studies demonstrated that the glycosylation of Asn573 in the large luminal domain of SV2A is prerequisite for its interaction with BoNT/E [32]. To meet this requirement and to be able to study the binding of H2/E to SV2, we expressed and purified the luminal domains of SV2 as fusion proteins in HEK-293 cells according to a previously described method [32]. Purified SV2A and SV2C fusion proteins were detected by SDS/PAGE and Coomassie Blue staining as a set of bands predominantly migrating between 60–70 kDa and 70–85 kDa respectively (Figure 1A). The calculated molecular mass of the
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Figure 2 SV2 binding of HCE mutants

(A) Assays were performed as described in Figure 1. Bound radiolabelled protein was quantified by means of densitometry following SDS/PAGE and phosphor imaging. Binding of HCE mutants was calculated relative to the binding of wild-type HCE. Results are means ± S.D. (n = 3–9). (B) Co-immunoprecipitation (IP) of selected HCE mutants was carried out by incubating FLAG-tagged wild-type and mutant HCE with an anti-FLAG antibody and solubilized synaptosomal membranes. Bound SV2 was visualized by Western blotting (WB) using specific antibodies directed against SV2A and SV2B respectively. M denotes molecular mass markers.

To check whether HCE is capable of binding to the expressed SV2 luminal domain fusion proteins, we incubated in vitro-translated radiolabelled HCE with immobilized glycosylated SV2A and SV2C luminal domain fusion proteins in the presence of PSGs. HCE showed robust binding to the SV2A, but not the SV2C luminal domain (Figure 1B). In contrast, HCA, which served as a positive control, bound to both the SV2A and SV2C luminal domain fusion proteins (Figure 1B). This result is in agreement with the reported ability of BoNT/A to interact with all three isoforms of SV2 [30] and probably owing to a different glycosylation pattern caused by N-glyosylation of additional sites in the luminal domain of SV2C compared with SV2A.

To analyse which domain of HCE is mediating the interaction with SV2A, we analysed the binding of in vitro-translated radiolabelled HCNE and HCCG to immobilized glycosylated SV2A and SV2C luminal domain fusion proteins in the presence of PSGs. HCCG showed binding to the SV2A, but not the SV2C, luminal domain (Figure 1C). In contrast, HCE showed no interaction with SV2A or SV2C. However, HCE binding to the SV2A luminal domain was much stronger than that of HCCG. Since the difference in affinity to Syt-II between HCB and HCG compared with HCE and HCG respectively is far less pronounced [27], this suggests that residues of HCE are additionally involved in SV2A binding with HCE.

In order to identify the SV2A-binding site of BoNT/E, amino acids were selected for mutation on the basis of homology considerations. Initially, we assumed that the position of the protein receptor-binding site relative to the PSG-binding site is conserved throughout all BoNT serotypes. Therefore we superimposed the structure of HCE (PDB code 3FFZ) on to the structure of HCB in complex with its protein receptor Syt-II (PDB code 2NM1, Figure 1D) yielding a root mean square deviation of 3.6 Å (1 Å = 0.1 nm) over all 397 alignable Cα atoms. Residues of HCE corresponding to residues of HCB which...
mediate the interaction with Syt-II (hereafter referred to as the Syt-site homologue) were mutated. A total of three out of ten mutants analysed showed a significant decrease in SV2A binding (Figure 2A). Introduction of a negative charge by the replacement of Thr1174, which corresponds to Lys1192 of HCB, by a glutamate residue reduced the amount of bound H$_{3}$E to 60 $\pm$ 9% compared with the wild-type. In contrast, introduction of a positive charge by the replacement of Thr1174 by an arginine residue did not interfere with SV2A binding. Substitution of Lys1176, which is similarly positioned as Val1118 of H$_{3}$B, by a phenylalanine residue reduced the amount of bound H$_{3}$E to 45 $\pm$ 8% of the wild-type. Phe1184, which corresponds to Phe1204 of H$_{3}$B, showed a decrease in binding to 61 $\pm$ 15% of that of the wild-type when mutated to a serine residue. Remarkably, the exchange of Tyr1183, which corresponds to the important Phe1194 of H$_{3}$B, by an alanine residue showed no significant effect on the binding of H$_{3}$E to SV2A. Taken together the results show that the Syt-site homologue of BoNT/E is involved in the interaction with its protein receptor. However, compared with the corresponding mutations of H$_{3}$B [17] the extent of the observed effects on the interaction of H$_{3}$E with SV2A is low suggesting additional sites or residues are mediating the interaction with SV2A.

Hence we performed a computer-based surface analysis by applying the ODA algorithm [38], which mainly predicts protein–protein interaction sites. It correctly identified the Syt-binding protein interaction sites. It correctly identified the Syt-binding site of HCB (results not shown). By means of this algorithm three surface patches (ODA1–3, see Supplementary Figure S1 at http://www.biochemj.org/bj/453/bj4530037add.htm), but neither the PSG-binding site nor the Syt-site homologue were identified as potential protein-interaction sites. ODA1 was located on the back of the PSG-binding site, whereas ODA2, surprisingly, was found at the interface of H$_{3}$E and H$_{3}$C. ODA3 was excluded from further analysis because it is occluded by H$_{3}$N and thus not exposed in the BoNT/E holotoxin [4]. Subsequently, we generated a set of 14 mutants of the ODA1 site, seven of which significantly interfered with the interaction of H$_{3}$E and SV2A (Figures 1E and 2A). Replacement of Tyr1183 by a serine residue reduced the amount of bound H$_{3}$E to 40 $\pm$ 7% of the wild-type value. Binding of the H$_{3}$E F1160A mutant to SV2A was far more drastically reduced to 2 $\pm$ 1% of the wild-type. Replacement of Gly1181 by phenylalanine, methionine or valine residues decreased binding to 15 $\pm$ 4%, 59 $\pm$ 8% and 16 $\pm$ 3% of the wild-type respectively. Finally, binding of the H$_{3}$E R1183E and R1183S mutants to SV2A was reduced to 5 $\pm$ 2% and 21 $\pm$ 3% of the wild-type respectively. Next, we mutated three residues of the ODA2 site, all of which strongly reduced the binding to SV2A (Figures 1E and 2A). Replacement of Tyr1041 and Tyr1042, which are located in H$_{3}$C and facing H$_{3}$E, by an alanine residue reduced the amount of bound H$_{3}$E to 18 $\pm$ 4% and 24 $\pm$ 1% respectively. Binding of the E1246S mutant, the mutation in which is located close to the C-terminal end of H$_{3}$C and facing H$_{3}$E, was reduced to 11 $\pm$ 3%.

To check whether residues, which are located between the three identified binding sites, also contribute to the interaction with SV2A, we subsequently mutated four additional residues, Asp1083 and Lys1084 (which connect the ODA1 and ODA2 sites), and Asp1099 and Lys1126 (which connect the ODA2 site with the Syt-site homologue). The D1083S, K1084A and K1084E mutants reduced the amount of bound H$_{3}$E to 67 $\pm$ 6%, 18 $\pm$ 8% and 9 $\pm$ 3% of the wild-type level respectively. Replacement of Asp1099 and Lys1126 with serine and glutamic acid respectively, caused a decrease in affinity to 49 $\pm$ 3% and 18 $\pm$ 4% of the wild-type level respectively.

The observed effects of mutations of both the ODA1 and ODA2 sites exceed the effects observed with mutations at the Syt-site homologue and reached the degree of the effects observed with mutations of H$_{3}$B that affect its binding to Syt. The strong reductions in binding affinity to SV2A at these two newly identified regions suggest that mainly the residues of the ODA1 and ODA2 sites mediate the interaction of BoNT/E with SV2A. However, residues of the Syt-site homologue are probably additionally involved in the BoNT/E–SV2A interaction. Furthermore, residues located between the three sites also contribute to the H$_{3}$E–SV2 interaction, indicating the presence of an extended and complex binding interface between H$_{3}$E and SV2.

To verify these results and to be able to generalize the results to SV2B, we performed co-immunoprecipitation experiments employing E. coli-expressed FLAG-tagged wild-type H$_{3}$E and selected mutants and a detergent extract of synaptosomal membranes (Figure 2B). Mutants which exhibited a reduced binding to SV2A in the SV2-binding assay also precipitated less native SV2A from synaptosomal membranes, although the extent of effects in the pull-down compared with immunoprecipitation experiments were not entirely consistent regarding the H$_{3}$E mutants G1181V, R1183S and W1224L. Furthermore, H$_{3}$E mutants that exhibited weaker binding to SV2A or reduced precipitation of full-length SV2A from synaptosomal lysates also showed a decreased precipitation of SV2B, implying an overall similar binding mode, although the luminal domains of SV2A and SV2B exhibit only approximately 54% amino acid identity. However, the Y1149S and T1174E mutations affected binding to SV2B to a greater degree than to SV2A. This suggests subtle differences in the interaction with SV2A and SV2B. The reason for the substantial precipitation of SV2A by H$_{3}$E W1224L is currently not known.

**Neurotoxicity of mutated full-length BoNT/E**

To assess the impact of the various mutations on the biological activity, corresponding full-length BoNT/E mutants were produced in E. coli cells as single-chain proteins and subsequently activated using trypsin digestion. The activities of the obtained active di-chain neurotoxins were finally determined using an MPN hemidiaphragm toxicity test. Mutations of BoNT/E that decreased binding to SV2A also caused a decrease in neurotoxicity of MPN preparations (Figure 3). The neurotoxicity of the BoNT/E T1174E and K1176F mutants (mutation of the Syt-site homologue), was decreased to 47 $\pm$ 9% and 49 $\pm$ 3% of the wild-type respectively. As expected from the binding data, the S1180A mutant showed no significant change in neurotoxicity. The F1160A mutation, located at the newly identified ODA1 site, caused a decrease in neurotoxicity to 32 $\pm$ 1% compared with the wild-type. Replacement of Gly1181 by a phenylalanine, methionine or valine residue reduced the neurotoxicity to 46 $\pm$ 4%, 58 $\pm$ 1% and 36 $\pm$ 4% of the wild-type respectively. The neurotoxicities of the R1183E and R1183S mutants were reduced to 22 $\pm$ 1% and 47 $\pm$ 7% of the wild-type respectively. Replacement of Tyr1041 and Tyr1042, located at the newly identified ODA2 site, by an alanine residue reduced the neurotoxicity to 1 $\pm$ 0.1% and 0.6 $\pm$ 0.01% of the wild-type respectively. The neurotoxicity of the E1246S mutant was decreased to 19 $\pm$ 2% of the wild-type. Two out of four mutations of BoNT/E located between the three binding sites also significantly reduced the neurotoxicity (K1084A, 16 $\pm$ 1% and K1126E, 33 $\pm$ 3% of the wild-type activity). In accordance with the binding data the BoNT/E D1083S and D1099S mutants exhibited no and weak effects on neurotoxicity respectively. We noticed that the extent of effects did not always exactly match the respective reductions in SV2A.
Identification of the SV2-binding site of botulinum neurotoxin type E

To analyse the impact of HCE mutations on the neurotoxicity of BoNT/E, the neurotoxicity of correspondingly mutated full-length neurotoxins was determined using MPN preparations. The paralytic half-times were determined and converted into the corresponding concentrations of wild-type BoNT/E using a dose–response curve. The resulting toxicities were finally expressed relative to wild-type BoNT/E. Results are means ± S.D. for at least three experiments.

binding (e.g. F1160A exhibited a 98 % reduction in binding, but only a 68 % reduction in neurotoxicity, whereas Y879A caused only a 82 % reduction in binding, but a 99 % reduction in neurotoxicity). A possible explanation for this observation would be that the mutations, especially for residues in the ODA2 site, are not only interfering with SV2 binding, but also with later steps during intoxication (e.g. translocation of the LC), which are only considered by the MPN hemidiaphragm toxicity test and not by the SV2A-binding assay. Differences in glycosylation of the native and the recombinantly expressed SV2 might also explain these discrepancies. Taken together, these findings evidence that the Syt-site homologue, as well as the two newly identified SV2 sites (ODA1 and ODA2), contribute to the interaction of BoNT/E with SV2A and that this interaction is crucial for the biological activity of BoNT/E.

CD spectroscopic structure analysis of mutated H$_5$E

To check whether any of the introduced mutations had affected the structural stability of H$_5$E and to exclude that the observed effects on receptor binding and neurotoxicity were owing to substantial structural changes within mutated H$_5$E we recorded the melting curves of selected E. coli-expressed H$_5$E mutants by using far-UV CD spectroscopy. The melting temperatures ($T_m$) of the H$_5$E mutants, defined by the inflection points of the recorded steep sigmoidal melting curves, varied between 41.9 °C and 45.6 °C with wild-type H$_5$E melting at 44.1 °C (Supplementary Figure S2 at http://www.biochemj.org/bj/453/bj4530037add.htm). A total of ten out of the 13 mutants exhibited a deviation of $T_m$ of 1.5 °C or less, which does not indicate significant structural changes suggesting an unaltered secondary structure of these H$_5$E mutants. In contrast the K1126E, G1181F and R1183E mutants displayed a reduction in $T_m$ value by 1.9 °C, 2.2 °C and 1.8 °C respectively, indicating that the structures of these three mutants might be affected and that at least parts of the observed effects have to be attributed to a change in structure. However, since G1181V and R1183S mutations exerted the same effects on SV2 binding and BoNT/E neurotoxicity as the G1181F and R1183E mutations respectively, it is evident that these two residues are crucial for SV2 binding and neurotoxicity.

PSG-binding ability of mutated H$_5$E

The carbohydrate portion of SV2 plays an essential role for the interaction of BoNT/E with SV2. Since E. coli-expressed and hence non-glycosylated SV2 luminal domain shows no detectable affinity for H$_5$E [31], the effect of mutations on the binding to the bare peptide of SV2 could not be studied. To be able to discriminate between residues involved in binding the peptide backbone or carbohydrate portion of SV2, we assessed interaction of selected H$_5$E mutants by using PSGs as surrogate for the carbohydrate portion of SV2. To this end a mixture of PSGs was immobilized on microtitre plates and incubated with E. coli-expressed wild-type and mutant H$_5$E. In this experiment H$_5$E W1224L served as the control, because it contains a deactivated PSG-binding site. Consequently, binding of this mutant to immobilized PSGs was strongly reduced (Figure 4). The H$_5$E Y1041A, K1084A, K1126E and F1160A mutants showed an unchanged ability to bind to immobilized PSGs, whereas a slight decrease in binding to PSGs was observed for H$_5$E R1183S. H$_5$E T1174E showed a more pronounced reduction in PSG affinity, but still less pronounced than the W1224L mutant of the GBS. Since the T1174E mutation is located in the Syt-site homologue, it indicates that the Syt-site homologue is possibly involved in the interaction with the carbohydrate portion of SV2.

H$_5$E–SV2A interaction is blocked by monoclonal antibody 4E13

The human monoclonal antibody 4E13 binds BoNT/E holotoxin with a $K_d$ value of $1.8 \times 10^{-10}$ M as determined by kinetic-exclusion analysis [43b]. 4E13 is specific for H$_5$E as shown by...
using yeast-displayed BoNT/E domains [41]. The 4E13 epitope on H\textsubscript{CE} was determined by sorting a library of yeast-displayed BoNT/E HC mutants for loss of binding to 4E13 and the fine epitope identified by measuring the affinity of 4E13 for single alanine residue mutants of the BoNT/E HC. Of the 20 alanine residue mutants evaluated, seven reduced the affinity of 4E13 for H\textsubscript{CE} by more than 2-fold (R1100A, R1101A, K1102A, S1179A, Y1149A, G1181A and F1184A, Supplementary Table S1 at http://www.biochemj.org/bj/453/bj4530037add.htm). The G1181A mutation completely eliminated binding of 4E13 and the K1102A mutation reduced binding 100-fold. This epitope overlaps with both the ODA1 site and the Syt-site homologue. However, it was not clear whether binding of the antibody interferes with the neurotoxicity of BoNT/E. Therefore we analysed the neutralizing activity of 4E13 in MPN preparations. Pre-incubation of 100 pM wild-type BoNT/E with 67 nM 4E13 resulted in a loss of neurotoxicity of about 90\% (Figure 5B), indicating that 4E13 interferes with the neurotoxicity of BoNT/E. Because 4E13 recognizes H\textsubscript{CE} and neutralizes BoNT/E neurotoxicity it was feasible to assume that binding of 4E13 to BoNT/E interferes with the interaction of BoNT/E with one of its cell surface receptors, either PSGs or SV2. To check this hypothesis we incubated \textit{in vitro}-translated radiolabelled H\textsubscript{CE} with immobilized glycosylated SV2A luminal domain fusion protein in the presence or absence of 4E13. The antibody efficiently blocked the interaction of wild-type H\textsubscript{CE} in the presence of PSGs, whereas an unrelated monoclonal antibody directed against H\textsubscript{CE} (CR2; [44]) did not (Figure 5A, left-hand panel). This finding indicates that 4E13 indeed interferes with the interaction of H\textsubscript{CE} with either PSG or SV2A. Furthermore, 4E13 is capable of blocking the residual SV2A-binding of wild-type H\textsubscript{CE} in the absence of PSGs and of H\textsubscript{CE} W1224L in the presence of PSGs (Figure 5A). This data is consistent with the epitope-mapping data and suggests that binding of 4E13 interferes only with the SV2–H\textsubscript{CE} and not the PSG–H\textsubscript{CE} interaction.

Interestingly, 4E13 was not able to block the residual binding of H\textsubscript{CE} G1181V in the presence of PSGs (Figure 5A, right-hand panel). This finding also excludes the possibility of 4E13 interfering with PSG binding. Instead, it was reasonable to assume that 4E13 might not be able to efficiently recognize the H\textsubscript{CE} mutant G1181V. Hence, to analyse the ability of 4E13 to recognize H\textsubscript{CE} mutants, we employed a conventional sandwich ELISA setup using 4E13 as the capture antibody. 4E13 was capable of efficiently capturing wild-type H\textsubscript{CE} and H\textsubscript{CE} W1224L, but not H\textsubscript{CE} G1181V (Figure 5C) indicating that the latter mutation destroyed at least part of the 4E13 epitope and prevented the antibody from blocking the residual binding of H\textsubscript{CE} G1181V to SV2A. Taken together, these findings indicate that the binding of 4E13 to BoNT/E directly interferes with the BoNT/E–SV2 interaction, thereby preventing binding and uptake of BoNT/E into motoneurons. Furthermore, these findings suggest that the 4E13 epitope and one of the newly identified SV2 sites (ODA1) partially overlap, because mutation of Phe\textsuperscript{1160}, Gly\textsuperscript{1181} and Arg\textsuperscript{1183} interfere both with the interaction of SV2 and H\textsubscript{CE} and the binding of 4E13 to H\textsubscript{CE} (Figures 1F and 4C). These data are consistent with the yeast epitope-mapping data.

DISCUSSION

Binding and uptake of BoNTs into target cells is thought to proceed via a dual-receptor model involving a PSG and a protein receptor [25]. To gain access to neuronal cells, BoNT/B and /G exploit binding to Syt [26–28]. Residues of BoNT/B and /G mediating the interaction with Syt have been identified [17] and molecular details underlying this interaction have also been acquired [36,37]. It was shown that the spatial arrangement of PSG- and Syt-binding sites relative to each other allows for the simultaneous binding to PSG and Syt, resulting in specific high-affinity binding of BoNT/B and /G to the neuronal plasma membrane. In contrast, information about the location of the

Figure 4 Ganglioside binding of H\textsubscript{CE} mutants

To assess the ability of selected H\textsubscript{CE} mutants to bind to carbohydrates, mixed bovine brain gangliosides were immobilized on microtiter plates and incubated with varying concentrations of selected purified wild-type and mutant H\textsubscript{CE}. After removal of the unbound protein, bound H\textsubscript{CE} was detected using an anti-FLAG antibody. Half-maximal binding of each H\textsubscript{CE} is shown as logEC\textsubscript{50} values +− standard error. The results of the plot are representative of one experiment performed in duplicate (means +− S.D.).
SV2-binding sites of BoNT/A and /E and the specific residues mediating the interaction was lacking.

In the present study, we provide evidence for the presence of an expanded interface mediating the BoNT/E–SV2 interaction. The mutational analysis of surface residues demonstrates that a newly identified SV2 site of H$_C$E, located on the back of the GBS, is essential for the BoNT/E–SV2 interaction. However, a second region, which is surprisingly located at the interface of H$_C$E and H$_N$E, also contributes to the interaction. The Syt-site homologue, which we first assumed to represent the SV2-binding site of BoNT/E, additionally contributes to the interaction, but to a lesser extent, compared with the other two sites.

Furthermore, employing a monoclonal antibody (4E13) directed against H$_C$E we provide evidence that the neutralizing activity of 4E13 is based upon a direct interference with the BoNT/E–SV2 interaction and that the 4E13 epitope and the ODA1 and Syt-site homologue of the newly identified SV2-binding interface are partially overlapping.

The identification of more than one interaction site possibly reflects the fact that binding of BoNT/E requires glycosylation of the luminal domain of SV2A and SV2B [32] and perhaps involves carbohydrate and peptide interactions each in separate binding pockets of H$_C$E. The observation that H$_C$E T1174E, a Syt-site homologue mutant, showed a reduced binding to immobilized PSGs (Figure 4) indicated that this region of H$_C$E is possibly capable of interacting with carbohydrates and hence with the N-glycan of the luminal domain of SV2. However, further investigation, such as co-crystallization approaches using carbohydrate portions of glycan structures, is required to confirm that carbohydrates are directly interacting with the Syt-site homologue of H$_C$E.

It was demonstrated that double anchorage of BoNT/B to PSG and Syt is required for LCB (LC of BoNT/B) to undergo efficient pH-induced translocation into the target cell cytosol [45]. The location of the Syt-binding site of BoNT/B at the distal tip and in close proximity to the PSG-binding site of H$_C$B allows for
the simultaneous adherence to both receptors [36,37]. In the present study, we demonstrate that the major residues of BoNT/E mediating the interaction with SV2 are not located at the distal tip of H$_4$E and not close to the PSG-binding site. Because interaction with PSGs is in addition to the interaction with SV2 required for the high-affinity binding of BoNT/E to target cells (Figures 2 and 5A; [32]), this finding raises the question whether the location of the newly identified expanded SV2-binding interface also allows for the double anchorage of BoNT/E to PSG and SV2. The large luminal domain of SV2 consists of approximately 125 amino acids and probably exhibits a complex fold. We therefore assume that the large luminal domain provides enough flexibility to facilitate the simultaneous binding of BoNT/E to both PSG and SV2. Certainly, further investigation is required to elucidate the actual structure of the SV2 luminal domain. It also remains to be shown whether the SV2-binding interface of BoNT/A is similar with respect to position and dimensions.

**AUTHOR CONTRIBUTION**

Stefan Mahnhold, James Marks, Andreas Rummel and Thomas Binz conceived the experiments. Stefan Mahnhold, Jasmin Strotmeier, Consuelo Garcia-Rodriguez and Jianlong Lou performed the experiments. Stefan Mahnhold, Andreas Rummel, Consuelo Garcia-Rodriguez and Jianlong Lou analysed the data. Stefan Mahnhold, James Marks, Andreas Rummel and Thomas Binz wrote the paper.

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SUPPLEMENTARY ONLINE DATA
Identification of the SV2 protein receptor-binding site of botulinum neurotoxin type E

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Figure S1 Result of the ODA analysis performed on H2E

Large red balls indicate a high probability of the respective surface patch to interact with a protein, whereas small blue balls indicate a low probability. Three surface patches (ODA1–3) were identified as potential SV2-interacting sites. Whereas ODA3 was excluded from further analysis, ODA1 and ODA2 were subjected to mutational analysis.

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Melting curves of selected HcE mutants were determined by recording their thermal denaturation by means of far-UV CD spectroscopy. In order to find the inflection point of the curves and hence the melting temperature, \( T_m \), data was fitted to the Boltzmann sigmoidal model using GraphPad Prism software. The data of the plot are representative of one experiment (each five accumulations) for the individual mutants. The data for wild-type HcE are means ± S.D. for three experiments.

### Table S1 Affinities and \( \Delta \Delta G \) values of alanine residue-substituted BoNT/E HcE mutants for binding to the monoclonal antibody 4E13

The dissociation equilibrium constant (\( K_d \)) was calculated for each alanine residue mutant. The difference in free energy (\( \Delta \Delta G_{\text{Ala-wt}} \)) between the alanine-substituted and wild-type (wt) HcE was calculated according to the formula 
\[
\Delta \Delta G = RT \ln \left( \frac{K_d_{\text{Ala}}}{K_d_{\text{wt}}} \right).
\]

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