Published in final edited form as:

Toxicon. 2013 December 1; 0: 101–107. doi:10.1016/j.toxicon.2013.01.007.

Molecular dissection of botulinum neurotoxin reveals interdomain chaperone function

Audrey Fischer¹ and Mauricio Montal^{*}

Section of Neurobiology, Division of Biological Sciences, University of California San Diego, La Jolla, CA 92093-0366, USA

Abstract

Clostridium botulinum neurotoxin (BoNT) is a multi-domain protein made up of the approximately 100 kDa heavy chain (HC) and the approximately 50 kDa light chain (LC). The HC can be further subdivided into two halves: the N-terminal translocation domain (TD) and the C-terminal Receptor Binding Domain (RBD). We have investigated the minimal requirements for channel activity and LC translocation. We utilize a cellular protection assay and a single channel/single molecule LC translocation assay to characterize in real time the channel and chaperone activities of BoNT/A truncation constructs in Neuro 2A cells. The unstructured, elongated belt region of the TD is demonstrated to be dispensable for channel activity, although may be required for productive LC translocation. We show that the RBD is not necessary for channel activity or LC translocation, however it dictates the pH threshold of channel insertion into the membrane. These findings indicate that each domain functions as a chaperone for the others in addition to their individual functions, working in concert to achieve productive intoxication.

Keywords

Protein translocase; Biological weapons; Botulism; Antibotulinal agents; Modular nanomachine

1. Introduction

Botulinum neurotoxin (BoNT) inhibits synaptic exocytosis in peripheral cholinergic synapses causing botulism, a disease characterized by descending flaccid paralysis. *Clostridium botulinum* cells secrete seven BoNTs isoforms designated as BoNT/A to G (Schiavo et al., 2000). All BoNT isoforms are synthesized as a single polypeptide chain and then cleaved to form a disulfide linked di-chain molecule by either clostridial or host cell proteases (Sathyamoorthy and DasGupta, 1985). The mature holotoxin consists of a 50 kDa light chain (LC) protease and a 100 kDa heavy chain (HC). The HC is comprised of the translocation domain (TD) (the N-terminal half), a long four-helix bundle, and the receptor-binding domain (RBD) (the C-terminal half), consisting of a single α -barrel and a single β -trefoil motif (Lacy and Stevens, 1999; Lacy et al., 1998; Schiavo et al., 2000; Swaminathan and Eswaramoorthy, 2000).

© 2013 Elsevier Ltd. All rights reserved.

^{*}Corresponding author. mmontal@ucsd.edu (M. Montal).

¹Current address: Johns Hopkins University, Applied Physics Lab, Laurel, MD 20723, USA.

Ethical statement: The authors declare that the research reported in the above mentioned article was conducted according to the ethical guidelines pertaining to our profession and underscored by the institution.

Conflict of interest: The authors declare that there are no conflicts of interest.

BoNT enter neurons by receptor-mediated endocytosis, initiated by the interactions between the BoNT RBD and a specific ganglioside, GT_{1B} (Ginalska et al., 2000; Nishiki et al., 1996; Rummel et al., 2009; Tsukamoto et al., 2005; Yowler et al., 2002) and protein co-receptor, SV2 for BoNT/A (Dong et al., 2006; Mahrhold et al., 2006), /D (Peng et al., 2011), /E (Dong et al., 2008) and /F (Fu et al., 2009) and synaptotagmins I and II for BoNT/B and BoNT/G (Rummel et al., 2004). Exposure of the BoNT-receptor complex to the acidic environment of endosomes induces a conformational change whereby the HC inserts into the endosomal bilayer membrane (Finkelstein, 1990; Gambale and Montal, 1988; Schiavo et al., 2000). Previously, we demonstrated that the HC forms a protein-conducting channel under endosomal conditions (Fischer and Montal, 2006), and translocates the LC protease into the cytosol (Fischer and Montal, 2007b; Koriazova and Montal, 2003), colocalizing it with its substrate SNARE (soluble NSF attachment protein receptor) protein (Blasi et al., 1993; Schiavo et al., 1992, 2000). Effectively, the HC functions as a protein translocase.

The LC and the C-terminal half of the RBD crystals have been solved for multiple serotypes and their functions clearly defined (Agarwal et al., 2005, 2004; Arndt et al., 2006, 2005; Breidenbach and Brunger, 2004; Chai et al., 2006; Hanson and Stevens, 2000; Jin et al., 2006; Segelke et al., 2004); however the central motifs of the protein are less well understood. The recent crystal structures of the BoNT/B RBD C-terminal half interacting with its ganglioside and protein co receptors raises queries regarding the role of the N-terminal half of the RBD (Chai et al., 2006; Jin et al., 2006). Potentially, the N-terminal motif of the RBD could serve a function in priming the TD to an insertion competent orientation with respect to the membrane (Muraro et al., 2009), or function as part of the protein-conducting channel itself. The elongated, unstructured belt region of the TD is another domain with an elusive function (Brunger et al., 2007; Galloux et al., 2008); potentially acting as a pseudo-substrate/chaperone for the LC during the majority of the intoxication process (Brunger et al., 2007).

Molecular dissection allowed us to investigate the domain requirements for BoNTchannel activity and protein translocation. By utilizing the single molecule LC translocation assay previously developed in our lab, we were able to determine the minimal module required to: 1. elicit BoNT ion channel activity, and 2. productively translocate LC. The assay allowed us to examine the pH threshold necessary for each of these functions as well as the pivotal role of the inter-chain disulfide bridge in the translocation process.

2. Minimal channel forming unit

We examined the channel activity of several sub-domains of BoNT/A holotoxin: HC, N-terminal half of the HC called the translocation domain (TD), and the TD without the N-terminal 100 amino acids known as the belt region (BTD). Protein insertion and channel formation were monitored on excised membrane patches from Neuro 2A cells under conditions which recapitulate those across endosomal membranes: the *cis* compartment, containing BoNT/A protein, was held at pH 5.3 and the *trans* compartment was maintained at pH 7.0 and supplemented with the membrane nonpermeable reductant TCEP. Channel activity of the TD and BTD modules exhibited similar characteristics to HC, as illustrated in Fig. 1. Single channel conductance () was determined to be approximately 65 pS as measured from the transition from the closed (C) to open (O) states; channel activity occurred in bursts between quiescent periods. The channel transitions through a 10 pS subconductance state at the onset and exit from a burst; within a burst the channel undergoes quick transitions between the intermediate state and the open state. The probability to reside in the open state (P_o), measured from the ms timescale transitions between the open and closed states, of each protein were all indistinguishable from that of BoNT/A HC as illustrated by the time expansion of current records shown in Fig. 1. The TD and BTD have

similar voltage dependence to the unoccluded state exhibited by the BoNT/A HC channel after completion of translocation (Fischer and Montal, 2006, 2007a, 2007b; Fischer et al., 2008, 2012); $V_{1/2}$, the voltage at which $P_o = 0.5$, is approximately -67 mV for holotoxin, approximately -64 mV for TD, and approximately -65 mV for BTD. Therefore, the BTD embodies a minimum channel forming entity (Fischer et al., 2012).

3. Channel insertion is pH independent in the absence of the RBD

Previous studies have shown that BoNT forms pH dependent and voltage dependent channels in lipid bilayers (Blaustein et al., 1987; Donovan and Middlebrook, 1986; Hoch et al., 1985) and neuronal cells (Fischer and Montal, 2007b; Sheridan, 1998); however, the pH threshold for channel formation of the TD and BTD was undetermined. In contrast to HC, channel activity of similar characteristics was monitored for the TD and BTD when the *cis* compartment solution was adjusted to more neutral pH values; representative records are shown in Fig. 2. Bursting patterns and τ was determined to be pH gradient independent (Fig. 2). The voltage dependence of the P_o was modulated by pH 6 but not pH 7 in the *cis* compartment as shown in the lower panel graphs of Fig. 2. The TD interaction with the RBD therefore alters the pH threshold for membrane insertion; in the absence of the RBD the TD and BTD readily form channels at neutral pH.

4. Minimal protein translocation unit

Translocation of BoNT/A LC and BoNT/E LC through the HC channel has been consistently monitored in real time and at the single molecule level in excised membrane patches from Neuro 2A cells (Fischer and Montal, 2007b). Similar results were recently reported for BoNT/B (Sun et al., 2011). For BoNT/A, LC translocation is observed as a time-dependent increase in Na^+ conductance through the HC channel; the initial 14 pS channel activity rapidly transitions through several intermediate τ before reaching a stable approximately 68 pS (Fischer and Montal, 2007a, 2007b). The steady-state τ is also the characteristic conductance of isolated HC recorded under identical conditions; therefore it represents the conductance of the unoccluded HC in holotoxin experiments after translocation is complete. We interpret the intermediate conductance states as reporters of discrete transient steps during the translocation of the LC across the membrane. During protease translocation, the protein-conducting channel progressively conducts more Na^+ around the polypeptide chain before entering an exclusively ion-conductive state. This typical pattern of channel activity for holotoxin proceeds under conditions which mimic those across endosomes and lead to LC translocation and retrieval of protease activity after completion of translocation.

We examined the role of the disulfide bridge during translocation by modulating the redox gradient across the membrane during LC translocation. In the absence of a reducing environment in the *trans* compartment, BoNT/A holotoxin channels were arrested in an intermediate conductance state, never achieving the unoccluded state (Fischer and Montal, 2007a, 2007b). We concluded that without the disulfide bridge reduction, the LC remains locked within the HC channel, unable to escape and refold within the cytosol. In contrast, pre-reduction of holotoxin before assay resulted in HC like channels without intermediates associated with LC translocation: The LC loses association and does not undergo translocation across the membrane (Fischer and Montal, 2007a). If the disulfide bridge is required for initiation of productive translocation, at what stage during translocation must reduction occur in order to productively release the LC into the cytosol? Addition of the membrane permeable beta-mercaptoethanol (β -ME) to the *trans* compartment allowed us to rapidly reduce both the *cis* and *trans* compartments during each step of LC translocation. Addition of β -ME at any of the intermediate conductance states resulted in a persistent

intermediate state, never achieving the unoccluded HC like state (Fischer and Montal, 2007a). The disulfide bridge therefore must remain intact during LC translocation; entry of the disulfide bridge into the reducing environment of the cytosol serves as the final step required for productive release. Recently, Montecucco et al. have confirmed the crucial role of the disulfide link (Simpson et al., 2004) using an elegant assay in cerebellar granular neurons (Pirazzini et al., 2011).

To investigate the potential role of the receptor binding domain in LC translocation we analyzed the LC-TD channel at early time points of channel activity (Fischer et al., 2008). Under endosomal conditions, LC-TD channel activity was similar to holotoxin; low conductance intermediate states were visualized prior to the unoccluded state (Fig. 3). At the onset of LC-TD channel activity, small, discrete events with a approximately 17 pS are clearly discerned. Progressively, undergoes a continuous increase until reaching a stable value of 69 pS (Fig. 3A and C red circle). The average time course of occurs with a $t_{1/2}$ approximately 130 s and displays multiple discrete transient intermediate conductances before achieving the steady state 69 pS. Productive translocation by LC-TD across cellular membranes was confirmed using a cellular intoxication assay (Eubanks et al., 2007). LC-TD exhibited similar growing patterns at pH 6 cis/pH 7 trans (pH 6/7) (Fig. 3A, middle panel). The $t_{1/2}$, measured from the average time course of change of after insertion, is increased from that of pH 5 cis conditions to 190 ± 10 s (Fig. 3A, middle panel, and 3C purple circle). The longer time required to complete LC translocation may correlate with the extent of fold the LC maintains at pH 6. Previous work has demonstrated that bafilomycin, a proton pump inhibitor which neutralizes endosomal compartments, effectively aborts BoNT intoxication (Keller et al., 2004; Simpson et al., 1994; Wang et al., 2008). A more complex set of requirements may underlie the adoption of a conformation of the LC compatible with productive entry and translocation through the channel. Consistent with this model are the results for LC-TD channel activity elicited at symmetric neutral pH; channel activity initiated and maintained at the unoccluded state of 64 pS (Fig. 3A, lower panel, and 3C blue circle). Circular dichroism analysis of the LC at pH 7 indicates a high α -helical content implying that the protein would remain folded rather than adopt a translocation competent conformation (Korazova and Montal, 2003). We interpret that LC does not unfold at pH 7 and therefore cannot be translocated through the approximately 15 Å pore of the TD channel (Hoch et al., 1985; Smart et al., 1997). Together, these findings substantiate the notion that the LC-TD is a minimum protein translocation unit (Fischer et al., 2008).

5. Productive intoxication requires several steps of interdomain chaperone activity

The findings summarized here illustrate the modular nature of BoNT, a protein in which each component functions individually yet the tight interplay governs that each domain serves as a chaperone for the others. The RBD insures LC unfolding to a translocation competent conformation occurs in synchrony with TD channel formation (Fig. 3) (Fischer et al., 2008). A similar requirement for BoNT/B has recently been presented by Sun et al. (2011). Sun et al. (2011) further suggested that, for BoNT/B, RBD association with its ganglioside receptor promotes channel formation through oligomerization. The LC chaperones the HC by maintaining it in a soluble conformation at neutral pH until residence within the acidic endosome where the LC serves to gate the TD channel in order to initiate LC translocation (Fischer and Montal, 2007b). The TD belt protects the LC from premature cleavage of non-specific substrates until localization within the cytosol (Brunger et al., 2007). Although the belt is not required for reconstitution of ion channel activity it may facilitate productive LC unfolding to a translocation competent conformation within the acidic compartment of the endosome, a hypothesis supported by the requirement for an intact disulfide bridge between the belt and the LC during LC translocation (Fischer and

Montal, 2007a). Finally, the TD protects the LC within the acidic environment of the endosome, chaperones the LC to the cytosol, and releases the LC in an enzymatically active conformation to act on the substrate SNARE proteins (Korazova and Montal, 2003).

This modular function makes BoNT and similar modular toxins a tool for biomolecule delivery to predetermined target tissues (Bade et al., 2004; Dobrenis et al., 1992; Duggan et al., 2002; Francis et al., 1995; Ichinose et al., 2002; Liu et al., 1999). Proteins of choices have been tethered to enzymatically inactive BoNT and demonstrated to translocate and function within the neuronal cell (Bade et al., 2004). While these studies have limited themselves to modulation of the enzymatic domain, replacement of the neuronal targeting receptor binding domain with one that recognizes a unique cellular surface protein could make BoNT an attractive delivery system for fold permissive cargo proteins to the scientist's target tissue of choice (Edupuganti et al., 2012; Stancombe et al., 2012).

Acknowledgments

The research summarized here was conducted in collaboration with Darren Mushrush, Shilpa Sambashivan, Borden Lacy and Axel Brunger. The original publications have been accordingly cited. This work was supported by the Pacific Southwest Regional Center of Excellence NIH AI065359 and, in part, by NIH-GM49711.

Abbreviations

ME	beta-mercaptoethanol
pH	pH gradient across the <i>cis</i> and <i>trans</i> compartments
	single channel conductance
BoNT	botulinum neurotoxin
BTD	beltless translocation domain
HC	heavy chain
LC	light chain
LC-TD	light chain-translocation domain
P_o	channel open probability
RBD	receptor binding domain
$t_{1/2}$	the half time for completion of translocation
TCEP	tris-(2-carboxyethyl) phosphine
TD	translocation domain
$V_{1/2}$	the voltage at which $P_o = 0.5$

References

- Agarwal R, Eswaramoorthy S, Kumaran D, Binz T, Swaminathan S. Structural analysis of botulinum neurotoxin type E catalytic domain and its mutant Glu212→Gln reveals the pivotal role of the Glu212 carboxylate in the catalytic pathway. *Biochemistry*. 2004; 43:6637–6644. [PubMed: 15157097]
- Agarwal R, Binz T, Swaminathan S. Structural analysis of botulinum neurotoxin serotype F light chain: implications on substrate binding and inhibitor design. *Biochemistry*. 2005; 44:11758–11765. [PubMed: 16128577]

- Arndt JW, Yu W, Bi F, Stevens RC. Crystal structure of botulinum neurotoxin type G light chain: serotype divergence in substrate recognition. *Biochemistry*. 2005; 44:9574–9580. [PubMed: 16008342]
- Arndt JW, Chai Q, Christian T, Stevens RC. Structure of botulinum neurotoxin type D light chain at 1.65 Å resolution: repercussions for VAMP-2 substrate specificity. *Biochemistry*. 2006; 45:3255–3262. [PubMed: 16519520]
- Bade S, Rummel A, Reisinger C, Karnath T, Ahnert-Hilger G, Bigalke H, Binz T. Botulinum neurotoxin type D enables cytosolic delivery of enzymatically active cargo proteins to neurones via unfolded translocation intermediates. *Journal of Neurochemistry*. 2004; 91:1461–1472. [PubMed: 15584922]
- Blasi J, Chapman ER, Link E, Binz T, Yamasaki S, De Camilli P, Sudhof TC, Niemann H, Jahn R. Botulinum neurotoxin A selectively cleaves the synaptic protein SNAP-25. *Nature*. 1993; 365:160–163. [PubMed: 8103915]
- Blaustein RO, Germann WJ, Finkelstein A, DasGupta BR. The N-terminal half of the heavy chain of botulinum type A neurotoxin forms channels in planar phospholipid bilayers. *FEBS Letters*. 1987; 226:115–120. [PubMed: 2446925]
- Breidenbach MA, Brunger AT. Substrate recognition strategy for botulinum neurotoxin serotype A. *Nature*. 2004; 432:925–929. [PubMed: 15592454]
- Brunger AT, Breidenbach MA, Jin R, Fischer A, Santos JS, Montal M. Botulinum neurotoxin heavy chain belt as an intramolecular chaperone for the light chain. *PLoS Pathogens*. 2007; 3:1191–1194. [PubMed: 17907800]
- Chai Q, Arndt JW, Dong M, Tepp WH, Johnson EA, Chapman ER, Stevens RC. Structural basis of cell surface receptor recognition by botulinum neurotoxin B. *Nature*. 2006; 444:1096–1100. [PubMed: 17167418]
- Dobrenis K, Joseph A, Rattazzi MC. Neuronal lysosomal enzyme replacement using fragment C of tetanus toxin. *Proceedings of the National Academy of Sciences of the United States of America*. 1992; 89:2297–2301. [PubMed: 1532255]
- Dong M, Yeh F, Tepp WH, Dean C, Johnson EA, Janz R, Chapman ER. SV2 is the protein receptor for botulinum neurotoxin A. *Science*. 2006; 312:592–596. [PubMed: 16543415]
- Dong M, Liu H, Tepp WH, Johnson EA, Janz R, Chapman ER. Glycosylated SV2A and SV2B mediate the entry of botulinum neurotoxin E into neurons. *Molecular Biology of the Cell*. 2008; 19:5226–5237. [PubMed: 18815274]
- Donovan JJ, Middlebrook JL. Ion-conducting channels produced by botulinum toxin in planar lipid membranes. *Biochemistry*. 1986; 25:2872–2876. [PubMed: 2424493]
- Duggan MJ, Quinn CP, Chaddock JA, Purkiss JR, Alexander FC, Doward S, Fooks SJ, Friis LM, Hall YH, Kirby ER, Leeds N, Moulds HJ, Dickenson A, Green GM, Rahman W, Suzuki R, Shone CC, Foster KA. Inhibition of release of neurotransmitters from rat dorsal root ganglia by a novel conjugate of a *Clostridium botulinum* toxin A endopeptidase fragment and *Erythrina cristagalli* lectin. *Journal of Biological Chemistry*. 2002; 277:34846–34852. [PubMed: 12105193]
- Edupaganti OP, Ovsepian SV, Wang J, Zurawski TH, Schmidt JJ, Smith L, Lawrence GW, Dolly JO. Targeted delivery into motor nerve terminals of inhibitors for SNARE-cleaving proteases via liposomes coupled to an atoxic botulinum neurotoxin. *The FEBS Journal*. 2012; 279:2555–2567. [PubMed: 22607388]
- Eubanks LM, Hixon MS, Jin W, Hong S, Clancy CM, Tepp WH, Baldwin MR, Malizio CJ, Goodnough MC, Barbieri JT, Johnson EA, Boger DL, Dickerson TJ, Janda KD. An in vitro and in vivo disconnect uncovered through high-throughput identification of botulinum neurotoxin A antagonists. *Proceedings of the National Academy of Sciences of the United States of America*. 2007; 104:2602–2607. [PubMed: 17293454]
- Finkelstein A. Channels formed in phospholipid bilayer membranes by diphtheria, tetanus, botulinum and anthrax toxin. *Journal of Physiology*. 1990; 84:188–190.
- Fischer A, Montal M. Characterization of Clostridial botulinum neurotoxin channels in neuroblastoma cells. *Neurotoxicity Research*. 2006; 9:93–100. [PubMed: 16785104]

- Fischer A, Montal M. Crucial role of the disulfide bridge between botulinum neurotoxin light and heavy chains in protease translocation across membranes. *Journal of Biological Chemistry*. 2007a; 282:29604–29611. [PubMed: 17666397]
- Fischer A, Montal M. Single molecule detection of intermediates during botulinum neurotoxin translocation across membranes. *Proceedings of the National Academy of Sciences of the United States of America*. 2007b; 104:10447–10452. [PubMed: 17563359]
- Fischer A, Mushrush DJ, Lacy DB, Montal M. Botulinum neurotoxin devoid of receptor binding domain translocates active protease. *PLoS Pathogens*. 2008; 4:e1000245. [PubMed: 19096517]
- Fischer A, Sambashivan S, Brunger AT, Montal M. Beltless translocation domain of botulinum neurotoxin A embodies a minimum ion-conductive channel. *The Journal of Biological Chemistry*. 2012; 287:1657–1661. [PubMed: 22158863]
- Francis JW, Hosler BA, Brown RH Jr, Fishman PS. CuZn superoxide dismutase (SOD-1):tetanus toxin fragment C hybrid protein for targeted delivery of SOD-1 to neuronal cells. *The Journal of Biological Chemistry*. 1995; 270:15434–15442. [PubMed: 7797532]
- Fu Z, Chen C, Barbieri JT, Kim JJ, Baldwin MR. Glycosylated SV2 and gangliosides as dual receptors for botulinum neurotoxin serotype F. *Biochemistry*. 2009; 48:5631–5641. [PubMed: 19476346]
- Galloux M, Vitrac H, Montagner C, Raffestin S, Popoff MR, Chenal A, Forge V, Gillet D. Membrane interaction of botulinum neurotoxin A translocation (T) domain. The belt region is a regulatory loop for membrane interaction. *The Journal of Biological Chemistry*. 2008; 283:27668–27676. [PubMed: 18693250]
- Gambale F, Montal M. Characterization of the channel properties of tetanus toxin in planar lipid bilayers. *Biophysical Journal*. 1988; 53:771–783. [PubMed: 2455552]
- Ginalski K, Venclovas C, Lesyng B, Fidelis K. Structure-based sequence alignment for the beta-trefoil subdomain of the clostridial neurotoxin family provides residue level information about the putative ganglioside binding site. *FEBS Letters*. 2000; 482:119–124. [PubMed: 11018534]
- Hanson MA, Stevens RC. Cocystal structure of synaptobrevin-II bound to botulinum neurotoxin type B at 2.0 Å resolution. *Nature Structural & Molecular Biology*. 2000; 7:687–692.
- Hoch DH, Romero-Mira M, Ehrlich BE, Finkelstein A, DasGupta BR, Simpson LL. Channels formed by botulinum, tetanus, and diphtheria toxins in planar lipid bilayers: relevance to translocation of proteins across membranes. *Proceedings of the National Academy of Sciences of the United States of America*. 1985; 82:1692–1696. [PubMed: 3856850]
- Ichinose M, Liu XH, Hagihara N, Youle RJ. Extracellular bad fused to toxin transport domains induces apoptosis. *Cancer Research*. 2002; 62:1433–1438. [PubMed: 11888916]
- Jin R, Rummel A, Binz T, Brunger AT. Botulinum neurotoxin B recognizes its protein receptor with high affinity and specificity. *Nature*. 2006; 444:1092–1095. [PubMed: 17167421]
- Keller JE, Cai F, Neale EA. Uptake of botulinum neurotoxin into cultured neurons. *Biochemistry*. 2004; 43:526–532. [PubMed: 14717608]
- Koriatova LK, Montal M. Translocation of botulinum neurotoxin light chain protease through the heavy chain channel. *Nature Structural Biology*. 2003; 10:13–18.
- Lacy DB, Stevens RC. Sequence homology and structural analysis of the clostridial neurotoxins. *Journal of Molecular Biology*. 1999; 291:1091–1104. [PubMed: 10518945]
- Lacy DB, Tepp W, Cohen AC, DasGupta BR, Stevens RC. Crystal structure of botulinum neurotoxin type A and implications for toxicity. *Nature Structural Biology*. 1998; 5:898–902.
- Liu XH, Castelli JC, Youle RJ. Receptor-mediated uptake of an extracellular Bcl-x(L) fusion protein inhibits apoptosis. *Proceedings of the National Academy of Sciences of the United States of America*. 1999; 96:9563–9567. [PubMed: 10449732]
- Mahrhold S, Rummel A, Bigalke H, Davletov B, Binz T. The synaptic vesicle protein 2C mediates the uptake of botulinum neurotoxin A into phrenic nerves. *FEBS Letters*. 2006; 580:2011–2014. [PubMed: 16545378]
- Muraro L, Tosatto S, Motterlini L, Rossetto O, Montecucco C. The N-terminal half of the receptor domain of botulinum neurotoxin A binds to microdomains of the plasma membrane. *Biochemical and Biophysical Research Communications*. 2009; 380:76–80. [PubMed: 19161982]
- Nishiki T, Tokuyama Y, Kamata Y, Nemoto Y, Yoshida A, Sato K, Sekiguchi M, Takahashi M, Kozaki S. The high-affinity binding of *Clostridium botulinum* type B neurotoxin to synaptotagmin

- II associated with gangliosides GT1b/GD1a. *FEBS Letters*. 1996; 378:253–257. [PubMed: 8557112]
- Peng L, Tepp WH, Johnson EA, Dong M. Botulinum neurotoxin D uses synaptic vesicle protein SV2 and gangliosides as receptors. *PLoS Pathogens*. 2011; 7:e1002008. [PubMed: 21483489]
- Pirazzini M, Rossetto O, Bolognese P, Shone CC, Montecucco C. Double anchorage to the membrane and intact inter-chain disulfide bond are required for the low pH induced entry of tetanus and botulinum neurotoxins into neurons. *Cellular Microbiology*. 2011; 13:1731–1743. [PubMed: 21790947]
- Rummel A, Karnath T, Henke T, Bigalke H, Binz T. Synaptotagmins I and II act as nerve cell receptors for botulinum neurotoxin G. *The Journal of Biological Chemistry*. 2004; 279:30865–30870. [PubMed: 15123599]
- Rummel A, Hafner K, Mahrhold S, Darashchonak N, Holt M, Jahn R, Beermann S, Karnath T, Bigalke H, Binz T. Botulinum neurotoxins C, E and F bind gangliosides via a conserved binding site prior to stimulation-dependent uptake with botulinum neurotoxin F utilising the three isoforms of SV2 as second receptor. *Journal of Neurochemistry*. 2009; 110:1942–1954. [PubMed: 19650874]
- Sathyamoorthy V, DasGupta BR. Separation, purification, partial characterization and comparison of the heavy and light chains of botulinum neurotoxin types A, B, and E. *The Journal of Biological Chemistry*. 1985; 260:10461–10466. [PubMed: 4030755]
- Schiavo G, Benfenati F, Poulain B, Rossetto O, Polverino de Laureto P, DasGupta BR, Montecucco C. Tetanus and botulinum-B neurotoxins block neurotransmitter release by proteolytic cleavage of synaptobrevin. *Nature*. 1992; 359:832–835. [PubMed: 1331807]
- Schiavo G, Matteoli M, Montecucco C. Neurotoxins affecting neuroexocytosis. *Physiological Reviews*. 2000; 80:717–766. [PubMed: 10747206]
- Segelke B, Knapp M, Kadkhodayan S, Balhorn R, Rupp B. Crystal structure of *Clostridium botulinum* neurotoxin protease in a product-bound state: evidence for noncanonical zinc protease activity. *Proceedings of the National Academy of Sciences of the United States of America*. 2004; 101:6888–6893. [PubMed: 15107500]
- Sheridan RE. Gating and permeability of ion channels produced by botulinum toxin types A and E in PC12 cell membranes. *Toxicon*. 1998; 36:703–717. [PubMed: 9655631]
- Simpson LL, Coffield JA, Bakry N. Inhibition of vacuolar adenosine triphosphatase antagonizes the effects of clostridial neurotoxins but not phospholipase A2 neurotoxins. *Journal of Pharmacology and Experimental Therapeutics*. 1994; 269:256–262. [PubMed: 8169833]
- Simpson LL, Maksymowych AB, Park JB, Bora RS. The role of the interchain disulfide bond in governing the pharmacological actions of botulinum toxin. *Journal of Pharmacology and Experimental Therapeutics*. 2004; 308:857–864. [PubMed: 14617695]
- Smart OS, Breed J, Smith GR, Sansom MS. A novel method for structure-based prediction of ion channel conductance properties. *Biophysical Journal*. 1997; 72:1109–1126. [PubMed: 9138559]
- Stancombe PR, Masuyer G, Birch-Machin I, Beard M, Foster KA, Chaddock JA, Acharya KR. Engineering botulinum neurotoxin domains for activation by toxin light chain. *The FEBS Journal*. 2012; 279:515–523. [PubMed: 22141396]
- Sun S, Suresh S, Liu H, Tepp WH, Johnson EA, Edwardson JM, Chapman ER. Receptor binding enables botulinum neurotoxin B to sense low pH for translocation channel assembly. *Cell Host & Microbe*. 2011; 10:237–247. [PubMed: 21925111]
- Swaminathan S, Eswaramoorthy S. Structural analysis of the catalytic and binding sites of *Clostridium botulinum* neurotoxin B. *Nature Structural Biology*. 2000; 7:693–699.
- Tsukamoto K, Kohda T, Mukamoto M, Takeuchi K, Ihara H, Saito M, Kozaki S. Binding of *Clostridium botulinum* type C and D neurotoxins to ganglioside and phospholipid. Novel insights into the receptor for clostridial neurotoxins. *The Journal of Biological Chemistry*. 2005; 280:35164–35171. [PubMed: 16115873]
- Wang J, Meng J, Lawrence GW, Zurawski TH, Sasse A, Bodeker MO, Gilmore MA, Fernandez-Salas E, Francis J, Steward LE, Aoki KR, Dolly JO. Novel chimeras of botulinum neurotoxins A and E unveil contributions from the binding, translocation, and protease domains to their functional

characteristics. The Journal of Biological Chemistry. 2008; 283:16993–17002. [PubMed: 18400760]

Yowler BC, Kensinger RD, Schengrund CL. Botulinum neurotoxin A activity is dependent upon the presence of specific gangliosides in neuroblastoma cells expressing synaptotagmin I. The Journal of Biological Chemistry. 2002; 277:32815–32819. [PubMed: 12089155]

Fig. 1.

A – BoNT/A HC (Fischer and Montal, 2006), **B** – TD (Fischer et al., 2008), and **C** – BTBD (Fischer et al., 2012) channel activity measured on excised patches of Neuro 2A cells. Representative single-channel currents at the indicated voltages; consecutive voltage pulses applied to the same patch for each experimental condition. Channel opening is indicated by a downward deflection; C and O denote the closed and open states. values for HC = 65.3 ± 0.4 pS, TD = 64.4 ± 0.4 pS, and BTBD = 69.2 ± 0.9 pS. The sections of the recordings obtained at -110 mV delimited by the red bars are shown in the bottom traces at a 10-fold higher time resolution; the prototypical square events which are characteristic of unitary channel currents are clearly discerned.

Fig. 2.

A – BoNT/A TD and **B** – BTBD channel activity is independent of a pH gradient across the membrane. Top panels illustrate representative channel activity monitored with pH 5 *cis*/7 *trans*, middle panel shows pH 6/7 and bottom panel shows no pH, symmetric pH 7, all measured at an applied voltage of -100 mV. After G_o seal formation, TD channel activity begins at 30 min (top), 14 min (middle), and 30 min (bottom) and BTBD channel activity begins at 14 min (top), 18 min (middle) and 29 min (bottom). Low conductance intermediates were not observed. Representative 200 ms segments of long recordings are illustrated; the single channel conductance for each condition is indicated. Corresponding

lower panels display analysis of the voltage-dependence of unoccluded channel activity for BoNT/A TD for pH 5/7 (●) ($V_{1/2} = -64.0 \pm 4.2$ mV), pH 6/7 (●) ($V_{1/2} = -46.8 \pm 3.1$ mV), pH 7/7 (●) ($V_{1/2} = -55.3 \pm 3.8$ mV), and for BTD for pH 5/7 (●) ($V_{1/2} = -64.9 \pm 2.2$ mV), pH 6/7 (●) ($V_{1/2} = -29.4 \pm 4.2$ mV), and pH 7/7 (●) ($V_{1/2} = -58.9 \pm 9.0$ mV) (Fischer et al, 2008, 2012).

Fig. 3. BoNT/A channel activity in excised patches of Neuro 2A cells over range of pH values in the *cis* compartment. **A** – BoNT/A LC-TD channel activity begins 10 min after G seal formation, $t = 0$ s, and transitions from low conductance intermediate state to the unoccluded state after completion of LC translocation. LC-TD channel activity begins 12 min after G seal formation for pH 6/7 and 45 min after seal formation for pH 7/7. **B** – BoNT/A holotoxin channel activity begins 10 min after G seal formation. Schematic representation of the time course of translocation are depicted under the experimental current recordings; the vertical bars denote interruption of the record to highlight the pattern of activity at the onset of translocation (occluded state) and completion of translocation (unoccluded state). **C** – Average time course of conductance change for BoNT/A holotoxin DpH 5/7 (), HC pH 5/7 (), LC-TD pH 5/7 (), LC-TD pH 6/7 (), and LC-TD pH 7/7 (). No channel activity was monitored for BoNT/A holotoxin under the following experimental conditions:

pH 6/7 () and pH 7/7 (). **D** –Analysis of unoccluded channel activity for BoNT/A holotoxin DpH 5/7 () ($V_{1/2} = -67.2 \pm 2.9$ mV), BoNT/A LC-TD for pH 5/7 (●) ($V_{1/2} = -59.0 \pm 9.1$ mV), BoNT/A LC-TD for pH 6/7 (●) ($V_{1/2} = -28.1 \pm 4.5$ mV), BoNT/A LC-TD for DpH 7/7 (●) ($V_{1/2} = -64.1 \pm 2.9$ mV).