Synaptic Vesicle Proteins: Targets and Routes for Botulinum Neurotoxins

Gudrun Ahnert-Hilger, Agnieszka Münster-Wandowski and Markus Höltje

Abstract Synaptic vesicles (SV) are key organelles of neuronal communication. SV are responsible for the storage of neurotransmitters, which are released by Ca^{2+}-dependent exocytosis. After release and interaction with postsynaptic receptors, transmitters rapidly diffuse out of the synaptic cleft and are sequestered by plasma membrane transporters (in some cases following enzymatic conversion). SVs undergo endocytosis and are refilled by specific vesicular transmitter transporters different in the various neuronal subtypes. Besides these differences, SVs in general are equipped with a remarkable common set of proteins. Botulinum neurotoxins (BoNTs) inhibit neurotransmitter release from almost all types of neurons by cleaving proteins required for membrane fusion localized either to SVs (synaptobrevin) or to the plasma membrane (SNAP-25 and syntaxin) depending on the BoNT serotype. To enter the neuronal cytoplasm, BoNTs specifically interact with the luminal domain of SV proteins (synaptotagmin or SV2, depending on serotype) transiently exposed during exocytotic membrane fusion and occurring in almost every neuron. Thus, the highly specific interaction with luminal domains of SV proteins commonly expressed on all SV types is one reason why BoNTs exhibit such a high neuronal specificity but attack almost every neuron type.

Keywords Synaptic vesicle (SV) · SV2 · Synaptotagmin (Syt) · Synaptobrevin/VAMP · SNAP-25 · Syntaxin

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Abbreviations

BoNTs	Botulinum neurotoxin
ClC3	Chloride exchanger 3
Habc	N-terminal a-helical domain of syntaxin
HC	Heavy chain
LC	Light chain
SNAP	Soluble N-ethylmaleimide-sensitive factor attachment protein
SNARE	SNAP receptor) complex
SNAP-25	Synaptosomal-associated protein of 25 kDa
SV	Synaptic vesicle
SV2	Synaptic vesicle glycoprotein 2
Syt	Synaptotagmin
TI-VAMP	Tetanus toxin insensitive VMAP
VAMP	Vesicle-associated membrane protein
V-ATPase	Vacuolar proton ATPase
VGLUT	Vesicular glutamate transporter
VGAT	Vesicular GABA transporter
VMAT	Vesicular monoamine transporter

Contents

1 Introduction

Communication between neurons in the central nervous system and between neurons and muscles in the periphery mainly occurs at specialized structures, the synapses. Functional variations at these sites by either modulating the postsynaptic or the presynaptic function will change input and output at synapses. In a physiological context, this describes synaptic plasticity. Damage of pre- and post-synaptic structures and functions is the initial cause of a variety of diseases affecting the central nervous system. The postsynaptic answer mainly depends on the postsynaptic receptors and ion channels and, presynaptically, on the availability and fusion competence of synaptic vesicles (SV). Furthermore, the amount of

neurotransmitter stored in an individual vesicle contributes in determining the synaptic strength. Botulinum neurotoxins (BoNTs) serotypes A–G and tetanus neurotoxin produced by six *Clostridium* species specifically and exclusively attack presynaptic nerve terminals. BoNTs consist of a heavy chain (HC) responsible for internalization and a light chain (LC) that harbors protease activity. Intoxication exerts a long-lasting block of neurotransmitter release and therefore prevents communication between the pre- and the post-synaptic site. This review will summarize the abundance, structure, interaction, and function of synaptic proteins that play a role in the pathophysiology of the different BoNTs.

2 Protein Equipment of Synaptic Vesicles

SV are key organelles of neuronal communication. They are found at the end of neuronal axons in presynaptic terminals. Their main function is to concentrate, store, and release neurotransmitters into the synaptic cleft. SV undergo repeated rounds of exo- and endocytosis. From the cytoplasmic space, SV first have to dock, followed by a priming step that renders them ready for fusion. Finally, mediated by an increase of the cytoplasmic free Ca^{2+} concentration, SV fuse with the presynaptic membrane and release their transmitter content into the synaptic cleft (for review see (Südhof 2012)). Thus, vesicular exocytosis of transmitter is one of the most important steps for neuronal communication. The specific protein equipment ensures the high reliability of these processes spatiotemporally. Due to their rather uniform biophysical properties, SV can be highly purified. This has opened the possibility for proteome analysis of SV from brain by mass spectrometry. The outcome was a rather complex assembly of proteins, most of them involved in membrane trafficking (Takamori et al. 2006).

The majority of these proteins appears to be common to most SV populations irrespective of the type of neuron they are hosted. In addition to the brain, this may also apply to the peripheral nervous system at least for some of the major SV proteins. Besides these common proteins, subpopulations of SV differ in vesicular transmitter transporters, which are highly specific for the various transmitters occurring in the nervous system. The various BoNTs preferentially affect cholinergic neurons under pathophysiological conditions, but in general prevent neurotransmission from almost every type of neuron suggesting common targets present in the majority of neurons. Regarding the molecular mechanism of how BoNTs affect neurotransmission, two molecular events have to be distinguished. The first involves the traffic routes by which the high molecular weight toxins reach their intracellular target. This includes the endocytic uptake, the translocation of the LC to the cytoplasm, and a cytosolic reduction of the S–S bridge linking the heavy and the light chain. While the proteins involved in endocytic uptake have been mostly identified, the further processing of the internalized toxin is still poorly understood. Second, the intracellular target molecules that include synaptic proteins necessary

for vesicular exocytosis are proteolytically cleaved directly leading to an inhibition of neurotransmission. This review will mainly focus on vesicular and synaptic proteins being either targets or relevant for BoNT uptake.

2.1 Common and Differential Protein Makeup of SV

SV are equipped with a great variety of integral and associated proteins. More than 80 different integral membrane proteins were identified by proteomics. The integral membrane proteins may be grouped into those responsible for trafficking and those required for transmitter loading and its modulation.

One of the key events in vesicular exocytosis is the formation of the core or SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein (SNAP) receptor) complex consisting of the vesicular protein synaptobrevin and the preferentially plasma membrane-localized proteins syntaxin and SNAP-25 (synaptosomal-associated protein of 25 kDa) (Sutton et al. 1998).

Synaptobrevin-2 or vesicle-associated membrane protein (VAMP) -2 is one of the most abundant proteins on SV but is also present on other secretory vesicles outside the central nervous system (see also Table 1). VAMPs cover a family of proteins which are characterized by a C-terminal integral membrane domain. The N-terminus (aa 1–90 or more depending on isoform and species) faces the cytosol and comprises a SNARE motif for the interaction with SNAP-25 and syntaxin. About 70 copies of VAMP-2 are present per SV. Besides VAMP-2, other family members occur on SV including synaptobrevin-1/VAMP-1, which is the second abundant VAMP isoform (Raptis et al. 2005; Schoch et al. 2001), VAMP-4, and VAMP-7/Ti-VAMP. According to a proteomic comparison of glutamatergic (VGLUT1) and GABAergic SV isolated from whole rat brain, VAMP-1, VAMP-2, and VAMP-7 appear to be more abundant on VGLUT1 compared to VGAT expressing SV (Gronborg et al. 2010). In cholinergic terminals, both VAMP-1 and VAMP-2 have been identified (see Table 1) All VAMPs are engaged in membrane fusion processes. VAMPs interact by their N-terminal chain with syntaxin and SNAP-25 family members to form the fusion core or SNARE complex as a prerequisite for SV exocytosis. The fundamental role of VAMP for vesicle fusion has been shown when analyzing VAMP-2 knockout animals. The mutants die at birth and lack almost completely the fast Ca^{2+}-dependent fusion. Other VAMP isoforms appear not to reconstitute for the lack of VAMP-2. Interestingly, the mutants show no morphological sign of neuronal degeneration at birth and the majority of other SV proteins was not changed compared to wild-type littermates suggesting that only at the end of prenatal development will VAMP-2 take over from other VAMP isoforms (Schoch et al. 2001). Besides its interaction with syntaxin and SNAP-25, VAMP-1 and VAMP-2 have been shown to form a transient complex with synaptophysin. Both interactions of VAMP with either synaptophysin or the SNARE proteins are mutually exclusive (Becher et al. 1999; Yelamanchili et al. 2005). Other members of the VAMP family are engaged in fusion processes outside the nervous system (see Table 1).

Table 1 Vesicle-associated membrane proteins (VAMP)

	Function	Distribution	Reference
Synaptobrevin-1/VAMP-1	v-SNARE essential for SV fusion	All types of central neurons but more abundant in spinal cord compared to VAMP-2; neuromuscular junction; brain nuclei, and motor neurons	(Raptis et al. 2005; Liu et al. 2011) 2011 (Aguado et al. 1999)
Synaptobrevin-2/VAMP-2	Most abundant v-SNARE essential for SV fusion and endocytosis	All types of central neurons; chromaffin cells; endocrine and exocrine pancreatic islet cells; mast cells; platelets; parietal cells; neuromuscular junction	(Schoch et al. 2001; Deak et al. 2004)
Cellubrevin/VAMP-3	v-SNARE essential for SV fusion	Neurons and nonneuronal cells	(Yang et al. 2001)
VAMP-4	v-SNARE also found on other organelles besides SV	Subset of central neurons secretory granules, SV	(Raingo et al. 2012)
VAMP-5	v-SNARE in skeletal muscle	Skeletal muscle	(Rose et al. 2009)
TI-VAMP /VAMP-7	v-SNARE nonsensitive to TeNT	Developing central neurons; adult all brain regions	(Coco et al. 1999; Danglot et al. 2012)
VAMP-8	v-SNARE	Endosome fusion	(Antonin et al. 2000)
Synaptotagmins			
Synaptotagmin-I	Ca^{2+} sensor of SV fusion	All types of central neurons; some sensory neurons; sympathetic peripheral neurons; PC12 cells; chromaffin cells; subpopulation of neuromuscular junction; autonomic and sensory neurons	(Aguado et al. 1999; Berton et al. 1997; Li et al. 1994; Pang et al. 2006; Perin et al. 1991; Xue et al. 2010)
Synaptotagmin-II	Ca^{2+} sensor of SV fusion	Preferential in spinal cord neurons; Motor neurons, and neuromuscular junction	(Aguado et al. 1999; Berton et al. 1997; Geppert et al. 1991; Pang et al. 2006)
Synaptotagmin-IV	Ca^{2+} sensor of SV fusion preferential during development	Subsets of brain and spinal cord neurons	(Berton et al. 1997)
Synaptotagmin-VII	Ca^{2+} sensor of SV fusion	Chromaffin cells pancreatic β-cells	(Gustavsson et al. 2008)

(continued)

Table 1 (continued)

	Function	Distribution	Reference
Synaptotagmin-IX	Ca^{2+} sensor of SV fusion	Preferential in neurons of the limbic system and the striatum	(Xu et al. 2007)
Synaptotagmin-V, -XII, and -XVII	Ca^{2+} sensor of SV fusion	SV with a preference for VGLUT1	(Gronborg et al. 2010)
SV2 proteins			
SV2A	Membrane glycoprotein 12 membrane spanning domains	All brain areas, less in spinal cord neuromuscular junction according to functional analysis	(Bajjalieh et al. 1993; Dong et al. 2006)
SV2B	Membrane glycoprotein 12 membrane spanning domains	All brain areas, less in spinal cord neuromuscular junction according to functional analysis	(Dong et al. 2006; Bajjalieh et al. 1993)
SV2C	Membrane glycoprotein 12 membrane spanning domains	More distinct distribution, preferential in basal ganglia neuromuscular junction according to functional analysis	(Dardou et al. 2011; Dong et al. 2006; Rummel et al. 2009)
SNAP-25 family members			
SNAP-25A	Membrane-associated protein, member of the SNARE complex	All brain areas before birth	(Bark et al. 2004)
SNAP-25B	Splice isoform of SNAP-25A	All brain areas and peripheral nervous system	(Bark et al. 2004)
SNAP-23	Membrane-associated protein, member of the SNARE complex resistant to BoNT	Cortex preferential GABAergic neurons	(Bragina et al. 2007)
SNAP-29	Membrane-associated protein, also other intracellular membranes, inhibits dissociation of SNARE complex	Ubiquitously, including neurons	(Pan et al. 2005)
SNAP-47	Membrane-associated protein, member of the SNARE complex, also other intracellular membranes besides SV, resistant to BoNT	All brain areas	(Holt et al. 2006)
Syntaxin			

(continued)

Table 1 (continued)

	Function	Distribution	Reference
Syntaxin-1A	Member of the SNARE complex	Ubiquitously expressed in brain preferentially present in sensory neurons and autonomic fibers	(Ruiz-Montasell et al. 1996); (Aguado et al. 1999; Bennett et al. 1993)
Syntaxin-1B	Member of the SNARE complex	Ubiquitously expressed in brain preferentially present in motor neurons	(Ruiz-Montasell et al. 1996); (Aguado et al. 1999)
Syntaxin-2 (Epimorphin)	Member of SNARE complex	Present in mesenchymal cells, fibroblasts, and myofibroblast adjacent to epithelia of lung, gut liver	(Wang et al. 2006)
Syntaxin-3, Syntaxin-4	Plasma membrane	Epithelial cells	(Watson and Pessin 2001; Karvar et al. 2005)
Syntaxin-5	Cis golgi	Heart, lung, liver, spleen, kidney	(Bennett et al. 1993; Watson and Pessin 2001)

The plasma membrane SNARE proteins syntaxin and SNAP-25 also reside on SV (Holt et al. 2006). Since SNARE complexes do not dissociate in their membrane bridging (trans-) configuration, every cycling of SV leaves some syntaxin and SNAP-25 in the vesicular membrane. Syntaxin-1 consists of two closely related isoforms A and B (over 80 % of sequence homology) which are the main isoforms involved in SV exocytosis (Bennett et al. 1993). Other members of the large syntaxin family are involved in all types of membrane fusion in different cells (see Table 1). Syntaxin is composed of an N-terminal α-helical domain (Habc), a C-terminal SNARE motif H3, followed by a transmembrane region. In an unbound stage, the Habc motif folds back to the SNARE motif keeping syntaxin in a closed conformation thereby preventing an interaction with SNAP-25 and VAMP. Before entering the SNARE complex, the Habc domain frees the SNARE motif and syntaxin is in an open conformation now available for an interaction with SNAP-25. The presynaptic matrix protein munc-18—a key modulator of exocytosis—binds to syntaxin in its closed conformation which is probably necessary during sorting to the plasma membrane, thereby preventing uncontrolled interactions with other SNARE proteins on ER or Golgi membranes (Christie et al. 2012). In addition, munc-18 binds to syntaxin engaged in the SNARE complex formation and this binding is mediated by the N-terminal N-peptide of syntaxin. Probably, munc-18 either promotes the syntaxin SNARE protein interaction by a controlled release of the bound Habc motif or stabilizes the interaction with the SNARE partners (Christie et al. 2012) for review see (Han et al. 2010). Expression of syntaxin-1A and 1B overlaps in most brain areas but in some parts of the central and peripheral nervous system, the isoforms are differentially distributed. In this respect, syntaxin-1B is mainly found in fibers from motor neurons whereas syntaxin-1A appears to be preferentially expressed in perivascular fibers belonging to the autonomic nervous system (Aguado et al. 1999). Deletion of syntaxin-1A has no severe impact on mice survival indicating that syntaxin-1B probably functionally replaces syntaxin-1A (Han et al. 2010; Gerber et al. 2008). When expressing syntaxin-1B in a predominantly locked open conformation, the resulting homozygous mutants developed lethal epileptic seizures and exhibited decreased syntaxin-1B levels (Gerber et al. 2008). These data suggest that syntaxin-1 by its isoforms may gradually interfere with the various steps involved in SV exocytosis.

SNAP-25 occurs in the two splice variants SNAP-25 A and B that differ in only nine amino acids out of the 39 residues encoded by the alternatively spliced exon. The expression of the splice variants appears to be switched from “A” to “B” after birth (Bark et al. 2004). SNAP-23 represents another isoform and is also present in nonneuronal cells (see Table 1). Members of the SNAP-25 family are stably associated with the plasma membrane by palmitoylation of four cysteine residues in between the two SNARE motifs. Deletion of SNAP-25A/B in mice does not prevent prenatal development but mutants die perinatally. SNAP-25 deletion mutants do not show stimulated transmitter release indicating the importance of SNAP-25 for neuronal communication (Delgado-Martinez et al. 2007; Washbourne et al. 2002).

All three SNARE proteins (VAMP, SNAP-25, and syntaxin) are involved in vesicular exocytosis and termed v-SNARE (VAMP) or t-SNAREs (syntaxin and

SNAP-25) signifying their location on vesicular or target plasma membrane. As can be deduced from the various deletion mutants, SNARE proteins are mandatory for stimulated vesicular release from neurons. They are also targets for the different serotypes of BoNT proteases (see below). SNARE complex formation involves the N-terminal cytoplasm facing part of VAMP, the C-terminal SNARE motif of syntaxin adjacent to the transmembrane domain, and the N- and C-terminal domains of SNAP-25. These four α-helical chains tether to a supercoiled string whose formation provides the energy to overcome the rejecting forces between the vesicular and the plasma membrane, allowing them to fuse (Sutton et al. 1998).

Besides the SNARE proteins, synaptotagmins (Syt), further SV proteins are involved in membrane fusion. Syt are the Ca^{2+} sensor of vesicle exocytosis. So far, 17 isoforms have been described with Syt-I and -II mainly responsible for neuronal SV exocytosis. Syt contain a single transmembrane domain and two C2 domains (C2A and C2B) that both bind Ca^{2+} with different affinity. In addition, Syt also bind to negatively charged membrane phospholipids like the highly abundant phosphatidylserine and phosphatidyl inositol 4,5-biphosphate, thereby accelerating membrane fusion by more than four orders of magnitude (Rhee et al. 2005; Vennekate et al. 2012). Syts have been shown to be essential for exocytotic membrane fusion (Chapman 2008; de Wit et al. 2009; Xue et al. 2010). Syt-I is a presynapse-specific isoform that regulates synaptic vesicle trafficking. Syt-I is upregulated during postnatal development mainly in the brain, while Syt-II appears to be dominantly expressed in spinal cord neurons probably including motor neurons (Berton et al. 1997). The various Syt isoforms exhibit a differential distribution. Syt-I is present in all types of brain neurons as well as in terminals of the autonomic nervous system. By contrast, Syt-II that is more expressed in the spinal cord preferentially occurs in α-motor neurons and neuromuscular endplates (Li et al. 1994; Pang et al. 2006). Other isoforms also confer Ca^{2+} sensitivity to exocytotic membrane fusion in nonneuronal cells (see Table 1). Besides Syt-I and -II, Syt-IV and -V are also highly expressed in brain. Expression of Syt-IV in neurons is regulated during development and is induced by neuronal activity (Berton et al. 1997; Ibata et al. 2002). Null mutants for Syt-IV exhibit deficits in motor coordination and hippocampus-dependent memory formation (Ferguson et al. 2000). Syt-IV is the first Syt isoform that has been found to exhibit random distribution in neurons not preferentially localized to SV release sites and has only 16 amino acids facing the vesicular lumen (Ibata et al. 2002). Whether SNARE complex formation primes vesicles for fusion or executes it is still a matter of debate. Recent data with Syt-I may shed some light on this question. In an elegant study using liposome fusion approaches, it was convincingly shown that Syt overcomes the electrostatic forces and bridges the vesicular and the plasma membrane. Following an action potential, the inflowing Ca^{2+} binds to Syt thereby changing its conformation and decreasing the space between the two membranes. Now the three SNARE proteins come close enough to tether into the SNARE complex and to execute the final fusion. In this scenario Syt acts upstream of core complex formation. This effect appears to be mediated by the C2B domain and is characteristic for Syt-I (van den Bogaart et al. 2011).

SV are equipped with a great variety of membrane proteins, many of them occurring in several copy numbers. Synaptophysin is the most abundant synaptic vesicle protein with respect to protein mass. It occurs in almost every type of neuron in the central and the peripheral nervous system. With about 30 copies per vesicle, it is only outnumbered by VAMP. Synaptophysin is a member of the tetraspan family including synaptoporin and synaptogyrin that all have four transmembrane domains and an N- and C-terminus facing the cytosol. Synaptophysin is X-chromosomal linked while the other tetraspan proteins are located on other genes. Despite these facts, little is known about its physiological functions. Synaptophysin knockout animals show no severe phenotypical changes. Synaptophysin is developmentally upregulated and the synaptophysin/VAMP interaction represents a hallmark of synaptic maturation (Becher et al. 1999). In addition, the synaptophysin/VAMP interaction appears to correlate with synaptic activity. It is increased after prolonged (Hinz et al. 2001) and spent during short-term stimulation (Reisinger et al. 2004). Synaptophysin appears to be relevant for some steps during endocytosis (Kwon and Chapman 2011; Spiwoks-Becker et al. 2001) and especially for VAMP retrieval following exocytotic membrane fusion (Gordon et al. 2011), but the slowed down endocytosis does not severely impair the animals. Thus, synaptophysin appears to modulate special, higher functions and consequences of synaptophysin deletion are only obvious when analyzing complex behavioral tasks (Schmitt et al. 2009).

All SV are equipped with an oligomeric vacuolar proton ATPase (V-ATPase) that is essential for proton pumping. The resulting acidification of the lumen of SV provides the energy required for transmitter loading. Surprisingly, the copy number per vesicle has been estimated to be between one and two, making it possible that an SV may also fail to incorporate a copy of this enzyme during recycling. Such an SV would remain silent but has the chance to get a copy of the proton pump during its next excursion to the plasma membrane (Takamori et al. 2006).

2.2 Vesicular Transmitter Transporter and SV2

Despite the rather uniform protein equipment of SV regulating their traffic, exocytosis and endocytic retrieval, SV differ in the transmitter phenotype and therefore in the transporters they use to fill SV with transmitters. BoNT preferentially target cholinergic neurons but prevent neurotransmission from almost every neuron irrespective of its transmitter phenotype with varying sensitivity (Foran et al. 2003). Classical (i.e., nonpeptide) neurotransmitters are synthesized in the synaptic cytoplasm and then loaded into SV by means of vesicular transporters that are driven by a proton electrochemical gradient across the vesicle membrane built up by a V-ATPase. It appears that the vesicular transporters, in conjunction with the biosynthetic enzymes and (at least in some cases) the plasma membrane transporters, are mainly responsible for determining the neurotransmitter that is released from a particular neuron. For the main transmitters involved in neuronal

communication, vesicular transporters have been identified for glutamate (VGLUT, three isoforms), GABA and glycine (VGAT or VIAAT), acetylcholine (VAChT), and catecholamines and serotonin (VMAT, two isoforms) (Hnasko et al. 2010). VAChT, VMAT, and mammalian VGLUTs are characterized by 12 membrane domains. An exception is VGAT which has nine membrane domains and the C-terminus facing the lumen of the SV (Martens et al. 2008). Generally, transport involves the exchange of one or two protons per molecule of neurotransmitter depending on the transmitter transporter type.

SV usually contain only transporters for a single neurotransmitter, however, exceptions from this rule increase. Expression of VGLUT in GABAergic or aminergic neurons probably results in stimulus-dependent corelease of glutamate but may also improve loading of the "home" transmitter by increasing the electrochemical gradient due to the negatively charged glutamate that have to be compensated by additional protons. Examples of such scenarios are VGLUT3 on cholinergic vesicles (Gras et al. 2008; Holt et al. 2006), VGLUT2 (Zander et al. 2010) or VGLUT3 (Seal et al. 2008) on subpopulations of GABAergic vesicles, or VGLUT2 on dopaminergic (Hnasko et al. 2010) and VMAT2 vesicles (Zander et al. 2010). Other proteins such as ion channels or ion exchangers may be required in addition to the transporters in order to load vesicles efficiently with high concentrations of transmitter. In this context, the vesicle-associated chloride exchanger ClC3 appears to modulate transmitter loading by increasing ΔpH (Ahnert-Hilger and Jahn 2011; Riazanski et al. 2011; Stobrawa et al. 2001).

Although overlapping expression between the various vesicular transmitter transporters can no longer be totally excluded, immunoisolation techniques using transporter-specific antibodies allowed to purify SV subpopulation and to compare their protein equipment by mass spectroscopy. Surprisingly, the differences between VGLUT1 and VGAT SV subpopulations are small suggesting only subtle differences between SV subpopulations besides the transporter equipment. One of the main differences includes MAL, a tetraspan protein distantly related to synaptophysin which is only present on VGLUT1-positive SV (Gronborg et al. 2010).

SV glycoprotein 2 is characterized by a 12 membrane spanning structure reminiscent of sugar transporter proteins. However, so far no transporter function has been described. There are three isoforms SV2A, B, and C transcribed by different genes (Bajjalieh et al. 1992; Janz and Sudhof 1999). While SV2A is present in all types of neurons, SV2B and C have a more differentiated distribution (Bajjalieh et al. 1994; Janz and Sudhof 1999). Indeed, SV2B and SV2C appear to preferentially reside on VGLUT1 or VGAT SV, respectively (Gronborg et al. 2010) All SV2 proteins directly interact with Syt-I and appear to influence its expression, trafficking, or endocytosis (Yao et al. 2010). SV2A deletion mutants have severe seizures and animals die a few weeks after birth, while SV2B knockouts are phenotypically almost normal but exhibit reduced neurotransmission at rod photoreceptor synapses (Morgans et al. 2009). SV2A knockout neurons exhibit decreased transmitter release and the epileptic phenotype in SV2A mutants is probably due to the reduced GABA release (Chang and Sudhof 2009). Another feature of SV2A is its selective

interaction with the antiepileptic drug levetiracetam not shared by the other SV2 isoforms (Lynch et al. 2004). Although looking like a transporter, current models argue for SV2 proteins being mainly involved in Syt homeostasis (Yao et al. 2010).

3 SNARE Proteins: Targets of BoNTs

Neuronal SNARE proteins are cleaved by the zinc-dependent endoprotease activity of the light chains of the various clostridial neurotoxin serotypes in a SNARE protein-specific way. BoNT/A and E cleave SNAP-25, BoNT/B, D, F, G, and TeNT the SV protein VAMP, and BoNT/C hydrolyzes the integral plasma membrane protein syntaxin and at higher concentrations of the LC also SNAP-25 (Schiavo et al. 2000, Binz 2012). Generally, neurotoxin-mediated cleavage sites for VAMP and syntaxin are located between the C-terminal membrane anchors and the ionic '0' layer. Only uncomplexed or partially assembled SNARE proteins can be proteolyzed by neurotoxins while the fully assembled SNARE complex is resistant to cleavage (Sutton et al. 1998). Cleavage of VAMP 2 by BoNT/G yields the shortest and by BoNT/F the longest C-terminal peptide. Also, BoNT/C1, A, and E cleave SNAP-25 at different sites (Binz 2012). Cleavage of either of these proteins prevents neurotransmission underscoring the importance of SNARE proteins for neuronal communication. All BoNT serotypes also affect human neurons (Humeau et al. 2000).

Besides the overall effects of BoNTs on Ca^{2+}-dependent neurotransmitter release, there are distinct kinetic differences indicating that it matters which of the SNARE proteins is cleaved and at what site. Although it may be difficult to analyze these differences at conventional synapses, large terminals with several release sites exhibiting larger pools of SV may be suitable to discriminate kinetic differences. The Calyx of Held is a large glutamatergic terminal in the auditory pathway which can be voltage-clamped and manipulated with respect to its intracellular Ca^{2+} concentration. This experimental arrangement allows a direct application of the light chains of clostridial neurotoxins. By this approach, the acute effects of various toxins can be studied and kinetic differences be worked out in a limited time window. It could be shown that cleavage of syntaxin by BoNT/C yields a complete block without changing the kinetics of the remaining SV and their release site so far not affected by the toxin. The same applied to VAMP cleaved by the LC of tetanus neurotoxin and probably also for the LC of BoNT/B which uses the identical cleavage site. Manipulating the intracellular Ca^{2+} concentrations by caged Ca^{2+} (thereby mimicking Ca^{2+} influx through channels), however, revealed that cleaved VAMP modifies the coupling between Ca^{2+} channels and release-competent vesicles. This may be either due to a loss of a postulated interaction between vesicles and the special release site or by vesicles which are delayed in endocytosis and therefore occlude release sites (Sakaba et al. 2005). By contrast, BoNT/A produced a strong reduction in the Ca^{2+} sensitivity of neurotransmitter release which can be

overcome by increasing the presynaptic free Ca^{2+} concentration (Sakaba et al. 2005). Given the extension of medical applications for BoNTs, deeper insight into the differences of their effects on neurotransmission at individual synapses are helpful to shape their therapeutic profiles.

Generally, BoNTs affect all types of neurons with the highest preference for cholinergic ones. Regarding central excitatory and inhibitory neurons, it appears that especially both BoNT/A and BoNT/E differ in their potency in glutamatergic versus GABAergic neurons. The reason for this phenomenon may be the reduced or almost absent expression of SNAP-25 in GABAergic neurons, which is replaced by another member of the SNAP-25 family, i.e., SNAP-23 especially in the adult nervous system. Overexpression of SNAP-25 increases the sensitivity of GABAergic neurons for BoNT/A suggesting that resistance may be primarily caused by the target and not mediated by the protein receptor required for internalization (Matteoli et al. 2009; Verderio et al. 2006; Verderio et al. 2007). Expression of BoNT/A cleavage-resistant isoforms of the SNAP-25 family in these neurons may be an explanation. These include SNAP-23 (Ravichandran et al. 1996; Galli et al. 1998), SNAP-47 (Holt et al. 2006), or SNAP-29 (Schiavo et al. 2000; Steegmaier et al. 1998). Probably, resistance to BoNT/A relies more on the reduced amount of SNAP-25 which may be overcome by increased SNAP-25 expression (even in its BoNT/A cleaved from) as well as on the special Ca^{2+} dynamics in GABAergic terminals than on the presence of a different SNAP-25 isoform (Grumelli et al. 2010). In contrast to these reports, peripheral neurons which differ only in their amount of SNAP-25 exhibit an almost identical sensitivity toward BoNT/A treatment with respect to fast synaptic transmission (Gibbins et al. 2003). In addition, it is also conceivable that subpopulations of inhibitory neurons differ in their BoNT/A resistance either on the basis of SNAP-25 family member isoforms or on the equipment with gangliosides and vesicular proteins necessary for internalization (Rummel 2012).

4 Synaptotagmin and SV2: Routes for BoNTs into Nerve Terminals

Internalization of the various BoNTs involves binding and subsequent transfer into the neuron by the toxins' HC (Rummel 2012). A double receptor concept has been developed which applies to all BoNTs. It is worth notifying that the two closely interacting proteins, SV2 and Syt-I and -II, also represent the tracking molecules for almost all BoNTs. Thus, it may be speculated that the protein receptor for BoNT/C that escaped detection so far could also be SV2, Syt, or represent complexed heterodimers of these SV proteins (Yao et al. 2010).

5 Variations of Vesicular Properties: Modulation of BoNT Intoxication?

All BoNTs are taken up via SV that upon cycling transiently expose their luminal face to the extracellular surface allowing the heavy chains to bind via their C-terminal half to their protein receptor, thereby initiating the uptake of the whole toxin. Thus, variation in the equipment with these protein receptors or their interaction with other SV proteins may have an impact on the efficiency of BoNTs. The trapped BoNT forms a pore by its N-terminal part of the heavy chain, which allows the LC to enter the cytosol (Fischer 2012). This translocation step depends on acidic pH inside the vesicle and the preceding/progression of intoxination can be stopped by inhibiting the vacuolar proton pump, i.e., via bafilomycin. Immediately after endocytosis, the SV is empty and has an increased pH compared to a ready-to-fuse SV. The low H^+ concentration is then increased by the proton pump, and once the luminal pH is low enough it then allows the translocation of the LC into the cytosol. As mentioned above, the vacuolar proton pump resides with one or two copies on average per SV opening the possibility that proton pump-free SV will be endocytosed. In these, trapped BoNT will not have the chance to be translocated to the cytosol and eventually released again by another round of exocytosis or end up in the endosomal compartment. Given the variation between different neuronal subtypes, SV depending on their equipment with transmitter transporter and additional exchangers may differ in the building up and maintenance of a low luminal pH. Besides the putative absence of a proton pump, other examples of such scenario are the chloride exchangers or VGLUTs on GABAergic SV, which slightly change luminal pH and consequently transmitter storage. Depending on the changes in the luminal pH, the effects of BoNTs may be either slowed down or accelerated. Although these effects may be small, they may add to the differences observed for the different neuronal subtypes and their sensitivity toward the various BoNTs.

6 Concluding Remarks

All BoNTs are taken up by neurons. Their internalization is best described by a double-receptor concept that involves an initial binding to neuron-specific gangliosides. A second protein receptor appears during neuronal stimulation. SV fuse with the plasma membrane and transiently expose their intravesicular luminal site to the extracellular surface. These protein receptors include the SV proteins Syt and SV2. The acidification of SV mediated by the vacuolar proton pump and sustained by vesicular transporters and ion exchangers mediate translocation of the BoNT LC to the cytosol. The light chains of all BoNTs harbor a protease specific for one of the SNARE proteins. Cleavage of SNARE proteins prevents SV fusion with the plasma membrane and arrests neuronal communication (Fig. 1). This has

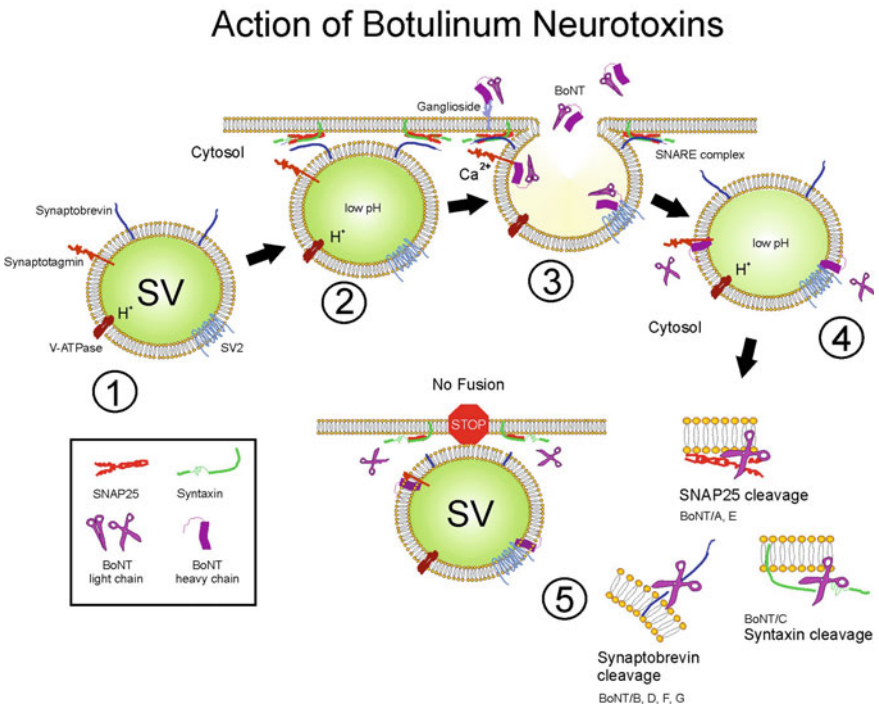

Fig. 1 Botulinum neurotoxins (BoNT) prevent exocytosis by cleaving synaptic proteins. Synaptic vesicles (SV) approach the synaptic membrane (*1, 2*) and form the SNARE complex as a prerequisite for the fusion pore and exocytotic transmitter release (Geppert et al. 1991). During this step, BoNT bound to gangliosides at the extracellular side can be taken up into the vesicle by binding of the heavy chain to specific target proteins synaptotagmin or SV2. Following retrieval from the plasma membrane, recycling vesicles become acidified again by means of the vacuolar proton pump (V-ATPase). This results in incorporation of the N-terminus of the heavy chain into the vesicular membrane, partial unfolding, and translocation of the light chain into the cytosol (*4*) to target the specific synaptic proteins involved in the exocytotic machinery (*5*)

deleterious effects on the organism without severely damaging the toxin hosting neuron. Variable equipment of SV with protein isoforms responsible for either internalization or SNARE complex formation (see Table 1) may explain the differences between neuron subpopulations regarding variations in their sensitivity toward BoNT serotypes.

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