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Clostridial Neurotoxins in Synaptic Research

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Abstract

Research on clostridial neurotoxins provides a recent illustration of how medical research can spark fundamental breakthroughs in basic science. Clostridia are anaerobic bacilli that live in dust, soil, vegetation, and the gastrointestinal tract of animals and humans. Most are benign, but the pathogenic species produce potent neural toxins. In addition to their involvement in disease and germ warfare, interest in these toxins has increased within the basic research community with the appreciation that these agents can be used as precise experimental tools for dissecting the molecular basis of synaptic transmission, which is without question the most essential process in the nervous system.

Keywords

Tetanus toxin; Botulinum toxin; Synaptic transmission; VAMP; Syntaxin; Synaptotagmin

Tetanus and botulism are caused by two bacteria, *Clostridium tetani* and *Clostridium botulinum*, respectively (1, 2). Tetanus typically enters the body through a wound. The toxin is transported within peripheral motor nerves and enters the CNS. The toxin (tetanospasmin) binds to the ganglioside membranes of nerve synapses, becomes internalized, and ultimately blocks release of transmitter from inhibitory neurons in the spinal cord. This causes tonic spasticity, or rigidity of the abdominal, neck, and back muscles, leading to death by asphyxia. Three forms of botulism are distinguished: food-borne, infantile botulism, and wound-associated (3). These diseases result from seven different types of botulinum toxins produced by the bacterium *C. botulinum* (Table 1). Toxins type A and B survive the digestive process, and they are the cause of botulism induced by consuming contaminated food. In infant botulism, the bacterium from ingested spores grows in the digestive tract and toxin is released into the body. In wound-associated botulism, the bacterium propagates in infected tissue. Botulinum toxin interferes directly with the release of acetylcholine at peripheral nerve endings. Neurological symptoms of botulism include weakness and neuromuscular paralysis, which begins with the cranial nerves and proceeds to peripheral and respiratory musculature. Respiratory failure is the primary cause of death. Clostridial toxins are among the most potent biological toxins known (the dose lethal to 50% of the population [LD₅₀] is 0.1 ng/kg) (2). Even minute amounts of botulinum toxin entering the

body through inhalation, absorption through the eye, or a break in the skin can cause serious, painful illness.

All of the clostridial toxins share a similar structure. The precursor toxin is a polypeptide of about 150 kDa that must be cleaved to become functional. Reduction of a cysteine link under the low pH conditions inside endosomes releases a heavy chain (100 kDa) and a light chain (50 kDa) fragment. The light chain, which has catalytic activity, is responsible for poisoning synaptic transmission, and the heavy chain seems to confer specificity for targeting after internalization (2). A 20-amino-acid sequence in the amino-terminal domain of the light chain is a motif, common to all the clostridial toxins, that is shared by various zinc-dependent proteases (16).

These toxins exhibit both striking specificity of cellular targeting and precise proteolytic activity for specific molecules involved in exocytosis of synaptic vesicles. Tetanus toxin is transported retrogradely to the CNS, with subsequent secretion and reuptake at the central synapses. In contrast, botulinum toxin acts locally at the neuromuscular junction. Specific membrane receptors for these toxins have not been identified, but binding to charged lipids promotes recognition and internalization.

Proteins controlling synaptic vesicle exocytosis

Two key molecules are required both for intracellular and plasma membrane vesicular fusion. *N*-ethylmaleimide-sensitive fusion protein (NSF) is a cytosolic ATPase that is brought to the membrane by binding with another adapter molecule, soluble NSF attachment protein (SNAP) (10, 15). Both proteins are essential for intracellular vesicle fusion and exocytosis of synaptic vesicles, and they are highly conserved from yeast to man (10). For neurotransmission, specificity for synaptic vesicle exocytosis requires targeting appropriate vesicles to appropriate synapses and triggering their release by an influx of calcium. Clostridial neurotoxins have been instrumental in identifying the proteins that provide these essential aspects of exocytosis from presynaptic terminals.

Appropriate vesicles are targeted for release at precise points on the cell membrane; therefore, the vesicles and the release sites must be labeled with specialized proteins that recognize one another. These proteins must then interact with the NSF-SNAP complex to direct exocytosis of synaptic vesicles at the release sites. These two types of proteins are called *vesicle SNAP receptors* or SNAREs. They are delineated “v” or “t” -SNAREs depending upon whether they label the vesicle (v-SNARE) or the target presynaptic membrane (t-SNARE).

The specific proteolytic activity of clostridial neurotoxins has provided essential insight into the identity of the proteins that make up the v- and t-SNAREs, helped pin-point the active binding regions of these proteins, revealed how they are assembled into a three-dimensional complex, and provided clues to how calcium ions might trigger exocytosis. A very stable ternary complex of three synaptic proteins is isolated from brain (17). This consists of synaptic vesicle associated membrane protein (VAMP), and two proteins associated with the presynaptic membrane: syntaxin, and synaptosomal associated protein 25K (SNAP-25) (see

Table 1). Its association with the synaptic vesicle makes VAMP a putative v-SNARE, which could mediate exocytosis by binding to the putative t-SNAREs syntaxin and SNAP-25. A number of botulinum toxins cleave VAMP at specific residues, including TeTx, BoNT/B, BoNT/D, BoNT/F, and BoNT/G (4, 5, 8, 18). BoNT/A and BoNT/E do not affect VAMP but target SNAP-25 for proteolysis, releasing 9 and 26 amino acid fragments, respectively, from the carboxyl terminus (5, 19). Syntaxin is the substrate for only one clostridial toxin, BoNT/C, which cleaves syntaxin just inside the transmembrane region, producing a soluble cytoplasmic fragment (6). By testing binding interactions between fragments of these proteins after proteolysis with appropriate clostridial toxins, the relevant molecular interactions and binding domains have been revealed (Fig. 1).

Binary Interactions between t-SNARE Proteins

The two presynaptic membrane proteins SNAP-25 and syntaxin can bind one another in vitro. The carboxyl-terminal domain of SNAP-25 is not necessary for binding, because cleavage of the carboxyl terminal by BoNT/A or BoNT/E (see Table 1 and Fig. 1) has no effect on the binary interaction between it and the presynaptic membrane protein syntaxin (20). Deletion of 35 amino acids from the amino terminal of SNAP-25 will abolish binding. Similar experiments with proteolytic fragments (20) and protein constructs (21) show that the carboxyl-terminal third of syntaxin is required for binding to SNAP-25.

Binding of VAMP with Synaptic Membrane Proteins (t-SNAREs)

The synaptic vesicle protein VAMP binds both the presynaptic protein SNAP-25 and syntaxin in binary interactions in vitro. The binding between VAMP and syntaxin is weak but specific (20). The same region of syntaxin that is essential for binding to its partner membrane protein, SNAP-25, is also essential for binding the synaptic vesicle protein VAMP (i.e., the carboxyl terminal region of syntaxin).

VAMP also binds the other t-SNARE, SNAP-25, in a binary mixture. A very large domain of VAMP is involved in binding both of the two presynaptic proteins. Cleavage of VAMP with TeTX, BoNT/B, BoNT/D, or BoNT/G, which target this region of VAMP, impairs the binding of VAMP to both syntaxin and SNAP-25 (20).

Recent studies using fluorescence resonance energy transfer (FRET) analysis (22) and electron microscopy (23), show that the binding of VAMP to syntaxin occurs through a coiled-coil tertiary structure, as had been predicted from the amino acid sequence (20). This form of protein-protein interaction is found in transcription factors containing leucine zippers and in surface proteins of influenza virus that are involved in fusion and internalization of virus particles (24).

Ternary Interactions among VAMP, Syntaxin, and SNAP-25

The binding between the two presynaptic membrane proteins, SNAP-25 and syntaxin, is much stronger than the binding between either of these presynaptic proteins and the vesicle protein VAMP in binary interactions. However, binding of VAMP to SNAP-25 and syntaxin in a ternary complex forms a remarkably tight association that is resistant to sodium dodecyl

sulfate (SDS) treatment at 60°C (20). This suggests that the binary interactions between the two t-SNARE proteins form a high-affinity binding site for VAMP.

Cleaved fragments of VAMP can still form ternary complexes, but the stability of the complex is much weaker. Depending on where VAMP is cleaved, clostridial toxins either weaken the ternary complex (BoNT/D or BoNT/F) or sever it from the membrane (TeTX, BoNT/B, BoNT/G) (see Fig. 1). When syntaxin is cleaved by BoNT/C, which occurs just inside the transmembrane region, the ternary complex can still form, but it is no longer highly resistant to SDS treatment.

When SNAP-25 is cleaved by BoNT/A or BoNT/E, which severs terminal amino acids from the carboxyl terminal, the ternary complex can still form and it remains attached to the membrane. Why then, would these toxins inhibit neurotransmitter release? Recall that the proteolytically cleaved SNAP-25 can still bind its membrane partner, syntaxin, via the amino-terminal domain of SNAP-25. This allows the formation of the ternary complex, but the association is much weaker. Thus, the ternary complex can form without the carboxyl terminal domain of SNAP-25, but this weakened structure has impaired exocytosis.

Not shown in Figure 1 (because of difficulty representing this three-dimensional relationship in two dimensions) is recent evidence indicating that VAMP and syntaxin form parallel coiled-coil associations bridging the synaptic vesicle to the presynaptic membrane, rather than antiparallel associations, as implied by Figure 1 (22). The three-dimensional structure of the molecule must be such that VAMP is bent to bring the amino terminals (the ends that are free from membrane attachments) of both VAMP and syntaxin into parallel alignment (22). This structure is not apparent in electron microscopy of the ternary complex, however (23). The FRET analysis does not require fixing proteins onto a solid matrix, which might perturb the structural relationships as seen by electron microscopy (22). Lin and Scheller propose that after binding to the essential binding region on the carboxyl terminal of syntaxin, the α -helical coils “zip together” along their length to provide the highly stable ternary complex, and that this action drives membrane fusion by forcing the synaptic vesicle and presynaptic membranes into close apposition. This implies that the ubiquitous cytoplasmic NSF-SNAP complex might not drive fusion directly; instead, it would have a role in breaking apart the tightly bound ternary complex after fusion has occurred.

This differs from the previous thinking that NSF and SNAP served to recruit the vesicles to the presynaptic membrane for docking, and the energy liberated from hydrolysis of ATP (by NSF) would then drive vesicle fusion (10, 25). It is clear that NSF leads to the disassembly of SNARE complex (17, 26). However, in yeast, recent evidence shows that the NSF analog acts before fusion and docking (26, 27). An alternative hypothesis is that NSF could promote SNARE disassembly as a necessary precursor to fusion, but this ATP-dependent step would not drive the fusion step itself (22, 25).

Calcium Sensitivity

The calcium-sensitive mechanism that triggers exocytosis of synaptic vesicles rapidly in response to depolarization is the essential aspect of neurotransmitter release. Another protein

attached to synaptic vesicles, synaptotagmin, can also bind near the carboxyl terminal region of syntaxin, and this interaction occurs in a calcium-dependent manner (28, 29).

Synaptotagmin binding domains overlap the same sites on syntaxin required for binding VAMP and SNAP-25. While bound into the ternary complex, synaptotagmin could act like a tag of fabric stuck in a zipper formed by the syntaxin, VAMP, and SNAP-25 coiled coil structure. This would allow formation of the SNARE complex of proteins and synaptic vesicle docking, but synaptotagmin would prevent the coiled coils from zipping fully along their length and driving fusion of the vesicle with the presynaptic membrane (22). Thus, the action of calcium ions could be quite rapid, freeing synaptotagmin from the zipper and allowing the molecular forces between the coiled coils to fuse rapidly.

Previously unsuspected interaction between the calcium sensor synaptotagmin (12) with SNAP-25 has been described recently (11). Synaptotagmin is not a substrate for any of the clostridial toxins. Binding between synaptotagmin and SNAP-25 might explain why synaptic vesicles remain docked in terminals treated with the clostridial toxins that cleave VAMP or syntaxin (30). Synaptotagmin could provide a toxin-resistant tether between the vesicle and the membrane by binding to SNAP-25. Injection of synaptotagmin peptides into squid nerve has been shown to cause accumulation of synaptic vesicles at active zones (31).

Localization of calcium influx as close as possible to the synaptic vesicle release apparatus is also necessary to promote a rapid response to calcium influx. Both of the presynaptic membrane-associated proteins, syntaxin and SNAP-25 (32, 33), can associate with the N-type calcium channel, which would position calcium influx near the release apparatus. Injection of peptides into presynaptic neurons, which dissociates the calcium channel from the SNARE complex, has been shown to cause attenuation of transmitter release (13).

Future of Clostridial Toxins in Basic Research

The important insights into synaptic vesicle exocytosis that derive from the use of clostridial toxins all rely upon activities of only the short chain of the molecule. The other remarkable properties of these toxins, including their highly specific binding, endocytosis, transport, and release from appropriate neurons, derive from activities provided by the long chain fragment. Future studies using this portion of the molecule may yield new insights into endocytosis, and protein trafficking.

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Fig. 1. Schematic diagram summarizing inter-relationships among synaptic proteins, critical binding domains for the formation of a ternary complex, and sites of cleavage by the clostridial neurotoxins (see refs. 1, 20, and 22 for details).

Table 1.

Clostridial neurotoxins and their synaptic protein substrates (see references cited and refs. 2 and 20 for details).

Clostridial neurotoxin			
Toxin	Substrate	Inhibition of Binding	Reference
TeNT	VAMP (synaptobrevin) Gln 76-Phe 77	syntaxin, SNAP-25	4
BoNT/A	SNAP-25 Gln 197-Arg 198	syntaxin (no effect on VAMP)	5
BoNT/B	VAMP (synaptobrevin) Gln 76-Phe 77	syntaxin, SNAP-25	4
BoNT/C	Syntaxin Carboxyl-terminal	SNAP-25 (no effect on VAMP)	6, 7
BoNT/D	VAMP (synaptobrevin) Lys 59-Leu 60	syntaxin SNAP-25	5
BoNT/E	SNAP-25 Arg 180-Ile 181	syntaxin (no effect on VAMP)	5
BoNT/F	VAMP (synaptobrevin) Gln 58-Lys 59	syntaxin SNAP-25	8
BoNT/G	VAMP (synaptobrevin) Ala 81-Ala 82	syntaxin SNAP-25	8
Synaptic Proteins			
Protein	Localization	Comments	Reference
NSF (N-ethylmaleimide-sensitive fusion protein)	Soluble	APase	9
SNAP (soluble NSF attachment protein)	Soluble	adaptor molecule, recruits NSF to membrane	10
SNAP-25 (synaptosome-associated protein of 25 kDa)	Presynaptic membrane	binds syntaxin	11
SNARE (SNAP receptor)	Bridges synaptic vesicle and membrane	docking and fusion particle, recruits NSF-SNAP complex	5
Synaptobrevin (see VAMP)	Synaptic vesicle	see VAMP	11
Synaptotagmin	Synaptic vesicle	calcium sensor and binds β -SNAP and SNAP-25. Not a substrate for TeNT or any BoNT	11, 12, 13
Syntaxin	Presynaptic membrane	binds SNAP-25 and N-type calcium channel	14
t-SNARE (target-localized SNARE)	Presynaptic membrane	(syntaxin + SNAP-25)	10, 15
v-SNARE (vesicle-localized SNARE)	Synaptic vesicle	(VAMP + synaptotagmin)	10, 15
VAMP (synaptobrevin) (vesicle-associated membrane protein)	Synaptic vesicle	Binds SNAP-25 and syntaxin	11, 12, 13