Botulinum neurotoxins: genetic, structural and mechanistic insights

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Abstract | Botulinum neurotoxins (BoNTs) are produced by anaerobic bacteria of the genus *Clostridium* and cause a persistent paralysis of peripheral nerve terminals, which is known as botulism. Neurotoxigenic clostridia belong to six phylogenetically distinct groups and produce more than 40 different BoNT types, which inactivate neurotransmitter release owing to their metalloprotease activity. In this Review, we discuss recent studies that have improved our understanding of the genetics and structure of BoNT complexes. We also describe recent insights into the mechanisms of BoNT entry into the general circulation, neuronal binding, membrane translocation and neuroparalysis.

Neurotransmitter
An endogenous chemical that transmits signals across a synapse from a neuron to a postsynaptic cell.

Metalloprotease
A proteolytic enzyme that is defined by the presence of an essential active-site metal ion, which is most often zinc.

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Clostridium is a genus of sporulating and anaerobic Gram-positive, rod-shaped bacteria that includes more than 150 species. These bacteria are widely distributed in the environment and in anaerobic regions of the intestines of several animals, where they are typically found as spores, which are resistant to physical and chemical stresses and can persist for long periods of time until favourable conditions enable germination[1,2]. Under appropriate environmental conditions (such as humidity, nutrients and the absence of oxygen), spores germinate into vegetative cells; conversely, exposure to oxygen, as well as water and nutrient deprivation, trigger sporulation. Several clostridia, including *Clostridium difficile*, *Clostridium perfringens* and *Clostridium sordelli*, are pathogenic, owing to the release of protein toxins, but only a few species are neurotoxigenic. For example, *Clostridium tetani* produces tetanus neurotoxin, which blocks neurotransmitter release in spinal cord interneurons and causes the spastic paralysis of tetanus[3]. In addition, six phylogenetically distinct clostridia produce more than 40 different botulinum neurotoxins (BoNTs) (BOX 1). BoNTs consist of three primary domains: two of these domains enable binding to nerve terminals and translocation of the toxin into the neuronal cytosol, and the third domain comprises a metalloprotease that inhibits the release of neurotransmitter by peripheral nerve terminals (BOX 2), which causes the flaccid paralysis and autonomic dysfunctions that are typical of botulism[2,4]. The neurospecificity and toxic potency of BoNTs make them the most powerful known toxins, and they are potential bioterrorism weapons[5,6]. By contrast, their absolute neurospecificity has enabled BoNTs to be used as effective therapeutic agents for human diseases that are characterized by hyperfunctioning nerve terminals, as the local injection of minute amounts of these toxins counteracts hyperactivity of the nerve terminal[7].

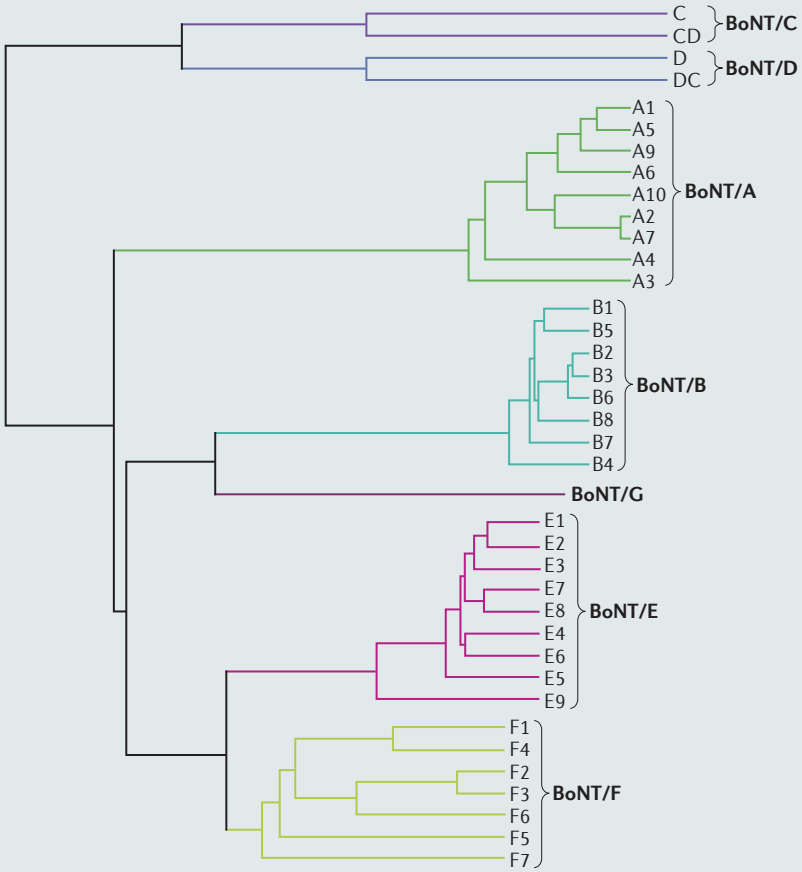
In the past few years, major advances in our understanding of the structures and mechanism of action of BoNTs have been made. In this Review, we summarize the life cycle of *Clostridium botulinum* in humans and animals and discuss the recent structural and mechanistic studies that have advanced our understanding of BoNT entry into neurons, trafficking in nerve cells and intoxication.

Botulism

Botulism mostly affects wild and domesticated animals, and outbreaks of animal botulism can spread rapidly, leading to the intoxication of hundreds of thousands of animals in just a few days[8]. Outbreaks typically occur in environments that contain *C. botulinum* spores, which can germinate in decomposing organic material under anaerobiosis. Environmental conditions that favour botulism outbreaks include warm temperatures, shallow alkaline waters that contain abundant invertebrate populations, and decomposing vertebrate carcasses[8]. Toxigenic clostridial strains are responsible for outbreaks, but horizontal gene transfer to non-toxigenic strains can occur, including the transfer of toxin-encoding loci, which causes non-toxigenic strains to become toxigenic[9]. The life cycle of toxigenic clostridia in wildlife begins with the growth of vegetative cells in decomposing organic material and the release of BoNTs via autolysis. The infected organic material is ingested by BoNT-insensitive invertebrates such as worms, mussels and larvae. These invertebrates are consumed by

Box 1 | **Serotypes and subtypes of BoNTs produced by different classes of neurotoxigenic *Clostridium* spp.**

Six phylogenetically distinct clostridia (*Clostridium botulinum* groups I-IV and some strains of *Clostridium butyricum* and *Clostridium baratii*) produce seven serotypically distinct botulinum neurotoxins (BoNTs) (serotypes A–G; see the table). Recent data suggest that there is an eighth serotype (known as BoNT/H), but this requires experimental validation[16]. Each toxin serotype is categorized into various subtypes on the basis of their amino acid sequences (see the table). BoNT serotypes C and D are closely related to each other, as are serotypes B and G, and E and F (see the figure). Although most strains of *C. botulinum* express a single toxin serotype, some isolates produce more than one serotype; for example, some proteolytic *C. botulinum* group I isolates produce a mixture of Ab, Af, Ba and Bf subtypes[9,133–135], and several *C. botulinum* strains that cause food-borne and infant botulism, and produce type A toxins, have been found to also encode a silent *bontb* gene and are denoted A(B). This finding suggests that the toxin types in these strains are actively evolving. Furthermore, a *C. botulinum* strain that expresses three *bont* genes (chromosomally encoded *bonta2* and *bontf4* genes and a plasmid-borne *bontf5* gene) has recently been described[136]. BoNT/DC and BoNT/CD are mosaic toxins; BoNT/DC comprises the L chain and HN domain of serotype D and the HC domain of serotype C, whereas BoNT/CD consists of the L chain and HN domain of serotype C and the HC domain of serotype D.



The number of subtypes has grown in recent years, owing to the increased use of whole-genome sequencing and mass spectrometry, as well as the availability of high-affinity monoclonal antibodies. This has revealed the striking variety of distinct BoNT subtypes that are produced by clostridial species (see the table), in contrast to the production of only one tetanus neurotoxin by *Clostridium tetani* (although a comparable genomic effort has not been made for this species). Notably, *C. botulinum* strains from groups I and II produce the widest range of neurotoxins, whereas group III and the other three clostridial species produce substantially fewer toxin types. We propose that this difference might result from differences in the usual physiological state of bacteria in the environment. Specifically, bacteria that produce only one or a few toxin types might be present in the environment mostly as spores (in this case, *C. tetani*, *C. botulinum* strains from group III and the three other clostridial species). As spores are in a non-replicative state, these bacteria have less opportunity to evolve compared with strains that are mostly in a vegetative state (such as those from *C. botulinum* groups I and II). That is, the number of replicating cells and the ratio of spores/vegetative cells are key factors that could possibly determine the 'evolvability' of *bont* genes. However, this hypothesis is speculative and requires experimental validation. Oxygen tension is one of the main parameters that control the ratio of spores/vegetative cells. The hypothesis would then predict that *C. tetani*, *C. botulinum* group III and *Clostridium argentinense* are strict anaerobes, whereas *C. botulinum* group I and II strains are capable of growth in micro-aerophilic conditions. Given the general interest of explaining the basis of the evolvability of *bont* genes, it will be interesting to test this hypothesis. The data on BoNT subtypes that is presented in the table include the BoNTs whose sequences are available.

Clostridial species	Proteolytic *C. botulinum* group I	Non-proteolytic *C. botulinum* group II	*C. botulinum* group III	*C. argentinense* (group IV)	*C. butyricum*	*C. baratii*
Type	A; B; F; (H)*	B; E; F	C; D	G	E	F
Subtype	A1; A2; A3; A4; A5; A6; A7; A8; A9; A10; B1; B2; B3; B5 (Ba); B6; B7; A(B); Ab; Af; Af84; Bf; F1; F2; F3; F4; F5	B4; E1; E2; E3; E6; E7; E8; E9; E10; E11; F6	C; D; CD; DC		E4; E5	F7

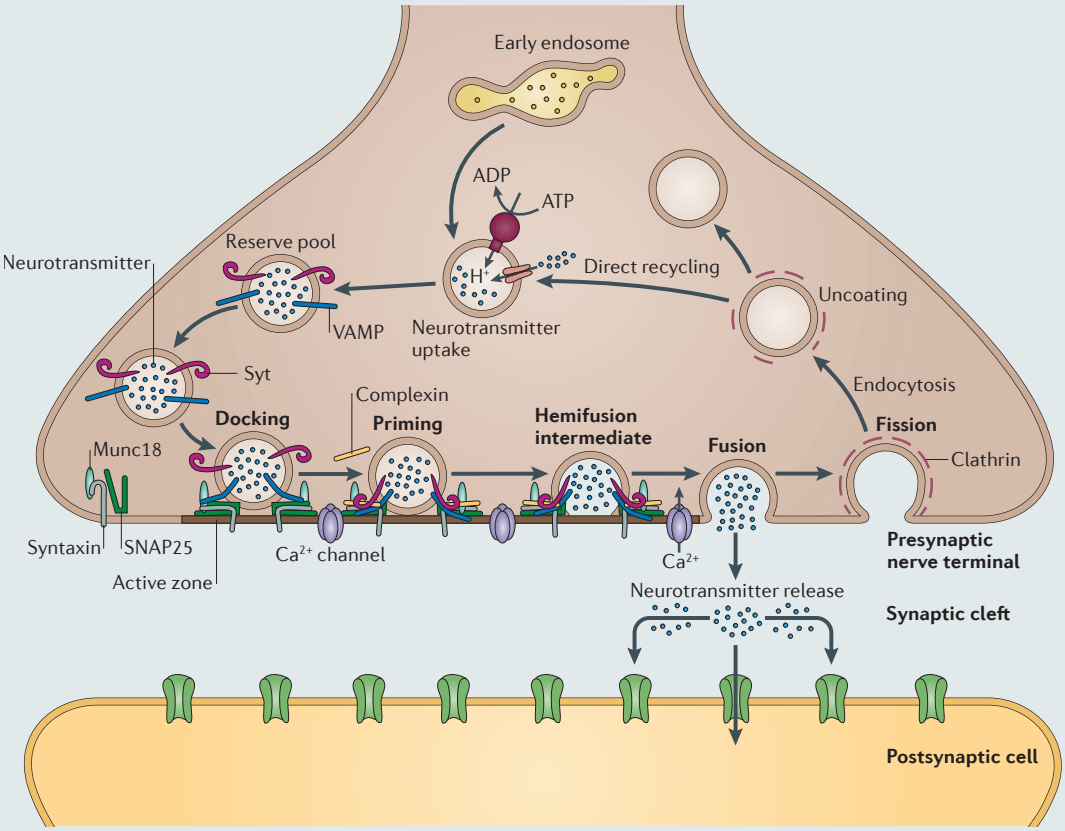
*Serotype H has been proposed as a novel serotype, but this remains to be experimentally verified.

Mouse lethal dose
Corresponds to the toxin dose that is required to kill 50% of exposed mice; it is usually expressed as the median lethal dose (LD_{50}). The mouse LD_{50} of BoNTs is 0.1–1 ng per kg for subtype 1 of the seven serotypes.

fish, birds and other terrestrial or aquatic vertebrate animals that are sensitive to the toxin and become paralysed, eventually resulting in death[8,10]. Higher temperatures and the presence of vertebrate carcasses favour the deposition of insect eggs on the cadavers, and a bird–maggot or fish–maggot cycle for the propagation of botulism is established (FIG. 1a). Vertebrate animals feed on the BoNT-containing maggots that are present on intoxicated cadavers and become paralysed. Thus, a new cycle of transmission begins, and such cycles are amplified by the death of an increasing number of animals. Importantly, the ingestion of only a small amount of toxin (that is, much less than the mouse lethal dose that has been determined in the laboratory; see below) can

Box 2 | **Neurotransmission at synapses**

Signalling at chemical synapses is mediated by neurotransmitters, which are released from the presynaptic nerve terminal and bind to receptors that are located on the postsynaptic cell (such as muscle or exocrine cells; see the figure). Neurotransmitters are synthesized in the neuronal cytosol and are stored in the presynaptic nerve terminal inside small synaptic vesicles[137]. The accumulation of neurotransmitters in the lumen of synaptic vesicles is mainly driven by the electrochemical proton gradient that is generated by the vesicular ATPase proton pump, which is located in the synaptic vesicle membrane and pumps protons into the synaptic vesicle using the energy that is released by ATP hydrolysis. The synaptic vesicles form a reserve pool of neurotransmitters within the nerve terminal or bind to specialized sites of the presynaptic membrane that are known as active zones[138,139], in a process known as docking[115,140]. The large set of proteins that regulate synaptic vesicle docking[115,137,140] are not depicted in the figure for simplicity. Two synaptic vesicle integral membrane proteins, VAMP (also known as synaptobrevin) and synaptotagmin (Syt); two proteins in the presynaptic membrane, SNAP25 and syntaxin; and cytosolic proteins, including complexin and Munc18, are involved in the subsequent step, which is known as priming and enables the synaptic vesicle to fuse rapidly with the presynaptic membrane in response to Ca^{2+} influx (see the figure). Syt interacts with presynaptic membrane inositol phospholipids, whereas VAMP forms a coiled-coil complex with SNAP25 and syntaxin, which is known as the SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) complex, in a process that is regulated by Munc18 and other proteins. After docking, fusion is prevented by complexin, which functions as a brake and, together with Munc18, also promotes the assembly of several SNARE complexes to form a radial super-SNARE complex[116,120]. This is the core of the nanomachine that mediates neurotransmitter release. The carboxy terminus of SNAP25 has an essential role in protein–protein interactions between the SNARE complexes within the super-SNARE complex[116,120]. It is likely that the synaptic vesicle and presynaptic membrane are hemifused in the primed state[141], which would account for the ultrafast (milliseconds or less) release of neurotransmitter at the neuromuscular junction (NMJ)[140]. Depolarization of the nerve terminal results in the opening of the Ca^{2+} channels and the influx of Ca^{2+} ions that induce release of the primed synaptic vesicle following binding to Syt. Such binding triggers a rapid conformational change that leads to complete synaptic vesicle–presynaptic membrane fusion and the formation of a pore through which neurotransmitter is released into the synaptic cleft (see the figure). Neurotransmitter diffuses out of the nerve terminal and binds to a postsynaptic receptor, which triggers signalling in the postsynaptic cell. In the case of the NMJ, acetylcholine is released and binds to the acetylcholine receptor, which results in depolarization of the muscle plasma membrane, leading to Ca^{2+} entry and muscle contraction. During neurotransmitter release, the lumen of the synaptic vesicle is transiently opened to the outside, but it is later internalized into the nerve terminal by endocytosis[70,137]. The exocytosis and endocytosis of neurotransmitter are strictly coupled: inhibition of one process leads to inhibition of the other[70]. Most endocytosis of synaptic vesicles at the NMJ is mediated by a clathrin coat. After internalization and uncoating, the synaptic vesicle is refilled with neurotransmitter and the next cycle of neurotransmission begins.



lead to physiological dysfunctions that make the intoxicated animal unfit; it therefore becomes either prey or a cadaver in the wild. For example, only a minimal amount of BoNT is required to cause visual impairment, which reduces survival in the wild but not in the laboratory. Furthermore, several vertebrates can carry neurotoxigenic *C. botulinum* as part of their intestinal microbiota, which can invade the cadaver post-mortem. Thus, the consumption of contaminated carcasses is particularly relevant on farms, as it facilitates the spread of botulism among livestock[8].

Human botulism is much rarer than animal botulism and is mostly caused by BoNT type A (BoNT/A), BoNT/B, BoNT/E, and rarely by BoNT/F[2,8] (BOX 1). There are five different forms of the disease, which are classified according to the route of entry of the toxin (FIG. 1b). Food-borne botulism occurs after the ingestion of BoNT-contaminated food (typically canned food that contains the pre-formed toxin)[2], and the toxin must survive the proteolytic environment of the gastrointestinal tract to reach the intestines, where it is absorbed. Similarly, infant botulism is typically caused by the consumption of food that is contaminated with neurotoxigenic spores that germinate in the intestine[11,12]. The colonization of infants is facilitated by a lack of competition from the resident microbiota, as infants tend to have a less robust bowel microbiota compared with adults[2,12]. In this case, BoNTs are produced and released in the intestines for prolonged periods of time unless the infant is treated with antibiotics. Although rare, an adult form of infant botulism has been documented in individuals that have anatomical or functional bowel abnormalities, or following antibiotic therapy, both of which might protect clostridial species from being outcompeted by the intestinal microbiota[2,4]. In food-borne and infant botulism, BoNTs cross the intestinal mucus layer and the polarized intestinal epithelial monolayer from the apical to the basolateral side to reach the general circulation[2,13]. BoNTs then reach peripheral cholinergic nerve terminals and paralyse the nerve terminals, which causes botulism (FIG. 1b). Wound botulism results from tissue contamination with spores and is almost exclusively associated with injection drug users[14]. Iatrogenic botulism occurs as a result of excessive exposure to BoNTs for cosmetic or therapeutic purposes[15]. In wound and iatrogenic botulism, BoNTs bypass intestinal absorption and directly enter the general circulation. It should be noted that BoNTs are much more toxic when injected (the LD_{50} ranges from 0.1 ng per kg to 1 ng per kg in laboratory mice) than when administered orally (which is >100–1000 times less toxic)[8]. Finally, in inhalational botulism, the toxin enters via the respiratory tract; however, delivery via aerosols is inefficient[2,6]. Food-borne and infant botulism are the predominant forms of the disease in humans, and the other forms are rarely encountered.

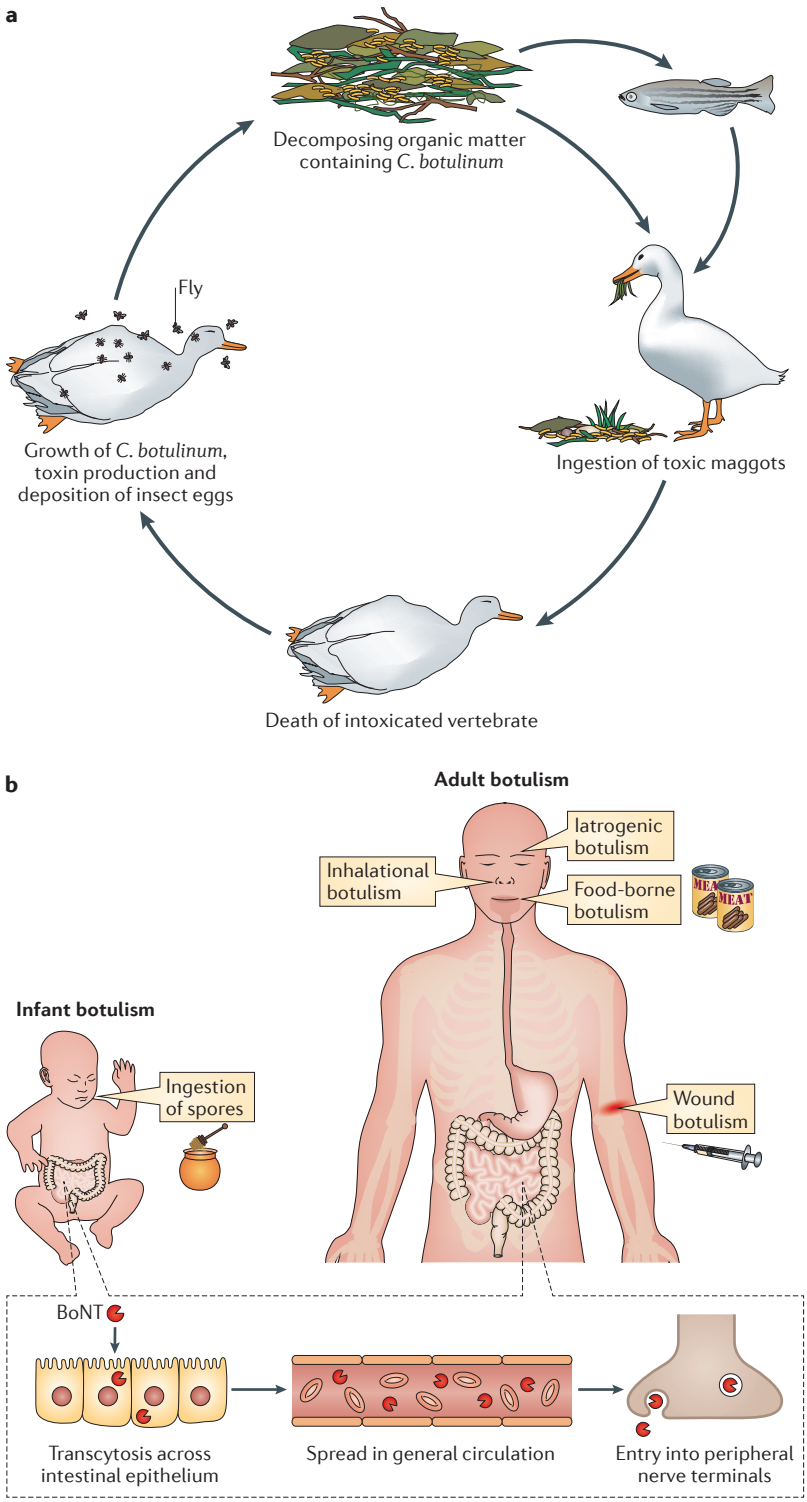
Figure 1 | **Animal and human botulism. a** | Botulism mainly affects wild and domesticated animals and begins with the growth of toxigenic clostridia in decaying anaerobic material, followed by release of the toxin. This infected material is consumed by botulinum neurotoxin (BoNT)-insensitive invertebrates (such as maggots), which disseminate the bacterium and the toxin to vertebrates. The cadavers of intoxicated animals provide an anaerobic environment that enables the bacterium to proliferate and release the toxin. The deposition of insect eggs (for example, from flies) leads to the growth of many intoxicated larvae, which are eaten by birds (or fish), generating a self-amplifying cycle that may rapidly involve many birds and/or fish. **b** | There are five forms of human botulism. The two most common forms are food-borne botulism (which occurs following the ingestion of BoNT-containing foods — typically canned foods) and infant botulism, which is caused by the ingestion of food contaminated with spores that germinate into neurotoxigenic clostridia in the gastrointestinal tract. In the infant gut, the bacterium has the potential to proliferate, owing to a lack of competition from the resident microbiota, which tends to be less robust in infants. The other three forms of human botulism are much rarer and include inhalational botulism (owing to inhalation of BoNT-containing aerosols), iatrogenic botulism (which is caused by the injection of excessive clinical doses of BoNT) and wound botulism (which is almost exclusively associated with drug injection). Following transcytosis across the intestinal epithelium and subsequent entry into the general circulation, the toxin eventually enters peripheral cholinergic nerve terminals, which causes the flaccid paralysis of botulism.

Diversity and structures of BoNTs

As summarized in BOX 1, six phylogenetically distinct clostridial groups (*C. botulinum* groups I–III, *Clostridium argentinense* and some strains of *Clostridium baratii* and *Clostridium butyricum*) produce seven serotypically distinct BoNTs (which are denoted BoNT/A–BoNT/G)[1,9]. An additional serotype (known as BoNT/H) has been proposed, but its confirmation as a novel toxin serotype requires further experimental validation[16]. BoNT serotypes are divided into subtypes on the basis of their amino acid sequences (BOX 1). The *bont* genes are encoded by mobile genetic elements that enable horizontal transfer among different isolates, which is thought to contribute to evolution of the *bont* loci and thereby to the large number of distinct BoNTs that are currently known[1,9] (BOX 1).

BoNT proteins are initially synthesized as single polypeptide chains of ~150 kDa, which are cleaved by proteases at a loop that is formed by a disulphide bond to yield the mature toxin, which consists of a light chain (L chain; which is 50 kDa) and a heavy chain (H chain; which is 100 kDa). The L chain and H chain are held together by a long peptide belt, non-covalent interactions and a single inter-chain disulphide bond (FIG. 2a). The crystallographic structures of the entire BoNT/A1, BoNT/B1 and BoNT/E1 are available[17–19], in addition to some individual domains and L chain–substrate complexes. Similarly to all bacterial exotoxins that have intracellular targets, BoNTs consist of multiple domains that fulfil different functions during the intoxication process: the L chain encodes the toxic moiety, which is a metalloprotease domain that specifically cleaves the SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) proteins that are necessary for neurotransmitter exocytosis; the HN domain (the N terminus

Cholinergic nerve terminals
Axonal terminals that use acetylcholine for neurotransmission.



of the H chain) is required for translocation of the L chain across the membrane of endocytic vesicles into the neuronal cytosol; and the HC domain (the C terminus of the H chain) is responsible for presynaptic binding and endocytosis and consists of two subdomains that have different folding and binding properties (FIG. 2a). A unique feature of BoNTs is the presence of a belt that encircles the L chain and attaches it tightly to the HN domain. The level of amino acid sequence similarity and the available single-domain structures suggest that BoNT/C, BoNT/D and BoNT/G are similar to BoNT/A and BoNT/B, whereas BoNT/F is more similar to BoNT/E (BOX 1).

The *bont* gene is located next to the non-toxic non-haemagglutinin gene (*ntnha*), which encodes a protein that forms a heterodimer with BoNT; for example, BoNT/A1 and NTNHA/A1 adopt a similar fold and bind to each other as interlocking hands[20] (FIG. 2b). This arrangement, which results in extensive protein–protein contacts, effectively decreases the exposure of the BoNT to external damaging agents. It also suggests that the two genes resulted from a duplication event and that the *ntnha* gene evolved to have a protective function[1,9,20]. Considering that the toxin is mostly produced in decaying biological material, where it remains active for months to years[8], we propose that the primary role of NTNHA is to protect BoNT from pH denaturation and the many proteases and protein-modifying agents that are present in this material[8], rather than the previously suggested primary role of protection in the gastrointestinal tract[21,22]. As passage through the gastrointestinal tract is relatively rapid (it occurs in minutes to hours), protection against the hostile environment of this *in vivo* compartment is likely to be a secondary role of NTNHA.

The *bont* and *ntnha* genes are in close proximity to genes that encode either haemagglutinin or OrfX proteins; these proteins associate with the BoNT–NTNHA heterodimer and are also thought to have a protective role. The *orfX* locus is present in genomes that encode BoNT subtypes A1 (strain NCTC2916), A2, A3, A4, E1–E11 and F1–F6 (REF. 9). The haemagglutinin operon is present in strains that produce BoNT/A1 (strain Hall), A5, B1–B7, C, D and G (REF. 9). The protein products of the haemagglutinin operon (which are HA17, HA33 and HA70) form large complexes (known as haemagglutinin complexes) that interact with the NTNHA–BoNT/A heterodimer, generating large oligomers that are known as progenitor toxin complexes (PTCs)[23,24] (FIG. 2c). The corresponding PTCs of BoNT/B and BoNT/E have also been structurally characterized[24]. The overall structure of the PTC resembles that of a λ phage, and the haemagglutinin proteins show only little protein–protein contact with NTNHA and no contact with BoNT/A1 (FIG. 2c). The haemagglutinin proteins of PTCs provide nine potential carbohydrate-binding sites[23], and these structural features, as well as recent experiments[25–27], suggest that the main role of the haemagglutinin complex is to facilitate trans-epithelial absorption of the toxin[23] (rather than the previously suggested role in protection[21,22]). Thus, it is possible that haemagglutinins function as adhesins and attach to the mucus layer, epithelial cells or other cells in the intestinal layer, such as M cells and neuroendocrine crypt cells[27]. A complex entry route has been suggested, in which the PTC is proposed to cross the epithelial barrier, followed by its release on the basolateral side. The haemagglutinin complex then dissociates from PTC and disrupts the epithelial barrier by loosening E-cadherin-mediated cell–cell adhesion, which opens the paracellular route to the toxin[25,26].

By contrast, other studies indicate that BoNT alone is capable of crossing epithelial cells[13,28], although with lower efficiency. Additional studies are clearly required to determine the role of the PTC complex *in vivo*. Future research should investigate the role of the range of accessory proteins that are associated with different BoNTs and should use representative models of the intestine that are derived from different animal species and that include the mucus layer.

Entry into the circulation

After breaching the intestinal epithelial barrier, BoNTs disperse in extracellular fluids and enter the lymphatic system and then the blood circulation[13]. The mechanism that is involved in crossing lymphatic and blood vessels is unknown, but BoNTs are unable to cross the blood–brain barrier and therefore cannot enter the central nervous system (CNS) using this route[29]. BoNTs are robust and can remain in the circulation for many days in humans[30,31] and rodents[13].

The specificity of BoNTs is surprising as, among the hundreds of different cell types that are present in the body of vertebrates, BoNTs only bind to peripheral nerve terminals, particularly those of skeletal and autonomic cholinergic nerves[32], the surfaces of which are only a small proportion of the total cell surface area that is exposed to extracellular fluids. This is even more remarkable considering that the mouse lethal dose corresponds to a BoNT concentration of ~10–15 M in circulating fluids.

Dual receptor binding

To selectively target the presynaptic membrane of peripheral nerve terminals, BoNTs have evolved a unique binding mode that is based on the use of two independent receptors: a polysialoganglioside (PSG) and a protein receptor in the lumen of synaptic vesicles[33–37] (FIG. 3). It is also likely that additional, low affinity but selective interactions contribute to neurospecificity[38–40]. This unique binding mode may have evolved to simultaneously overcome several physiological obstacles, such as the low BoNT concentration in circulating fluids, the high velocity of movement of extracellular fluids around cells and the reduced surface area of peripheral nerve terminals compared with that of other cells that are exposed to extracellular fluids.

Initial binding. The 'evolutionary choice' of PSG as the first presynaptic receptor that BoNT contacts on the nerve terminal[41,42] seems to be ideal, as PSG molecules are present at a high density on the presynaptic membrane, are organized in microdomains that also include glycoproteins, and their oligosaccharide portion (which is the BoNT-binding moiety) is flexible and projects far beyond the membrane surface[43,44]. In addition, PSGs form a large family of glycolipids with chemically complex oligosaccharides that can generate very specific interactions with target proteins. PSGs also influence transmembrane signalling, endocytosis and vesicle trafficking[43,44]. Thus, PSGs are perfectly equipped to function as 'antennae' that capture BoNTs as they pass in close proximity and thereby concentrate them on the nerve terminal surface. Indeed, BoNTs bind to the most distal part of the PSG sugar head via a PSG-binding site that is located in the HC domain of the BoNT molecule (FIG. 3). That PSG alone functions as the first and major presynaptic receptor of BoNTs is also supported by the fact that some autoimmune PSG-specific antibodies bind to PSG and recruit complement on the presynaptic membrane, causing entry of Ca^{2+} ions[45,46].

The binding of BoNT to the negatively charged PSG molecule is probably rapid, as it is likely to be controlled only by the rate of diffusion. In fact, BoNTs are dipoles, with their positively charged end located close to the binding site on PSG (FIG. 2a). Thus, PSG and other anionic lipids might be involved in reorienting the BoNT dipole as it approaches the membrane, which would make almost any PSG-binding attempt productive[47]. This effect may contribute to the rapid binding of BoNTs to the nerve terminal *in vivo*[13]. In terms of binding density, studies of the rat NMJ have shown that hundreds of BoNT/A or BoNT/B molecules can bind per square micrometre of the presynaptic membrane[48].

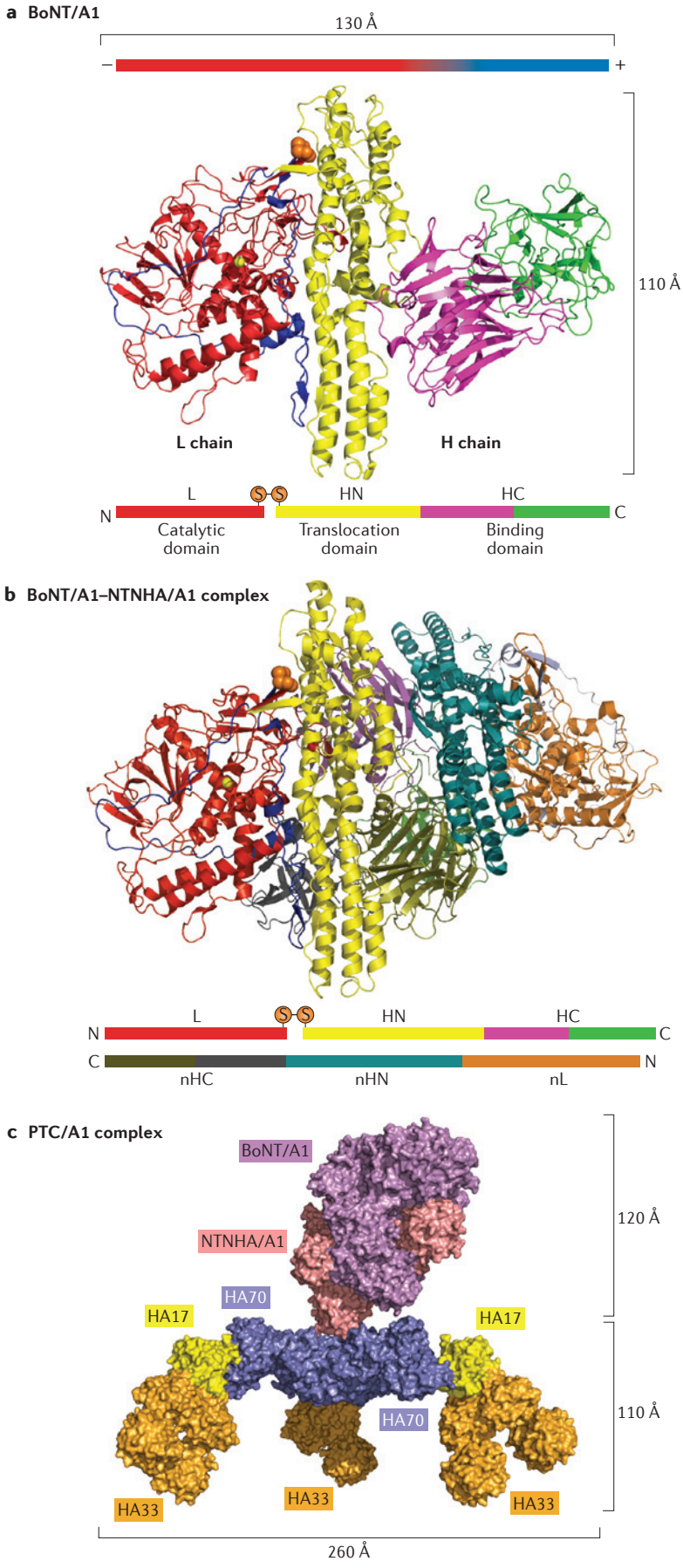
Figure 2 | **Structure of isolated BoNT molecules and BoNT complexes.** **a** | Crystal structure of botulinum neurotoxin A1 (BoNT/A1)[17], showing its associated electrical dipole and the organization of individual toxin domains, each of which has a specific function in cell intoxication: the HC domain binds specifically to nerve terminals; the HN domain translocates the L chain into the nerve terminal cytosol; and the L chain is a metalloprotease that cleaves and inactivates specific SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) proteins that are involved in neurotransmitter release, thereby causing nerve paralysis. A peptide belt (shown in dark blue), which surrounds the L domain and the inter-chain disulphide bond (orange), links the L chain to the HN domain. This unique feature is also present in BoNT/B1 and BoNT/E1 (Protein Data Bank (PDB) accessions 3BTA, 1EPW and 3FFZ). **b** | Crystal structure of BoNT/A1 in complex with the NTNHA/A1 protein[20] (PDB accession 3VOC). NTNHA/A1 has the same domain organization as BoNT/A1, and the two proteins form an interlocking complex, which suggests that NTNHA/A1 protects BoNT/A1 from proteases and other damaging agents that the toxin encounters in the *ex vivo* environment and in the gastrointestinal tract. The lower bar shows the schematic organization of the two proteins. **c** | Structure of the precursor toxin complex (PTC), which contains the NTNHA/A1–BoNT/A1 heterodimer complexed to PTC/A1 (REF. 23), in which the NTNHA/A1–BoNT/A1 heterodimer occupies the central position and the haemagglutinin proteins (HA17, HA33 and HA70) of the PTC are shown underneath. There are six HA33 proteins, three HA17 proteins and three HA70 proteins in each NTNHA/A1–BoNT/A1 complex. PTC/A1 forms three spider-like legs that have little protein–protein contact with NTNHA/A1 and no contact with the toxin. This structure suggests that the haemagluttinin proteins function in binding to the intestinal epithelium to facilitate absorption of the toxin, rather than in the protection of BoNT from protease attack. Structure in part **c** courtesy of R. Jin, University of California, Irvine, USA.

M cells
Specialized epithelial cells of the follicle-associated epithelium of the gastrointestinal tract that are involved in the rapid uptake and presentation of particular antigens and microorganisms to immune cells of the lymphoid follicle, thereby inducing an effective immune response.

Neuroendocrine crypt cells
Cells that are distributed throughout the intestinal epithelium and that secrete peptide hormones in an endocrine or paracrine manner from dense core or neurosecretory granules.

Synaptic vesicles
Neuronal vesicles that store and release neurotransmitters or neuropeptides at the synapse.

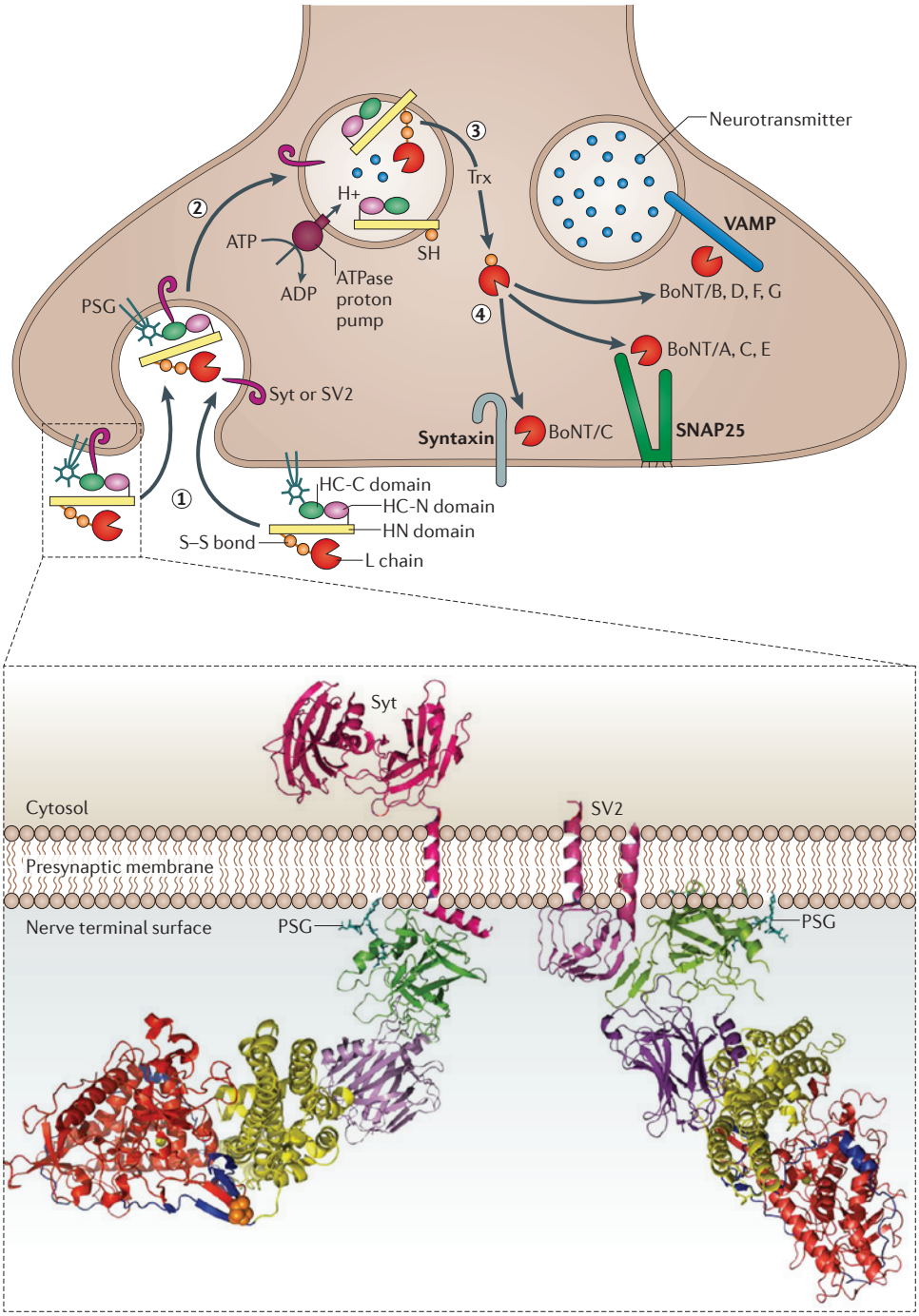
Presynaptic receptor
A receptor that is localized on the surface of the presynaptic membrane; it is either protein or lipid in nature.



The PSG-binding site is located on the surface of the carboxy-terminal subdomain of the HC domain (HC-C domain) (FIG. 2a) and has been characterized in detail[34]. In BoNT/A, BoNT/B, BoNT/E, BoNT/F and BoNT/G, the PSG-binding site is defined by the motif E(or D or Q)---H(or K or G)---SXWY---G (where X is any amino acid and --- denotes a variable number of residues). The PSG-binding site of BoNT/C, BoNT/DC and BoNT/D is located in a similar position, but the binding residues are different. This group of BoNTs also has a second PSG-binding site within the HC domain, which includes the W-Y(or F) residues. These residues are known to be involved in binding to carbohydrate receptors[49–51], and this PSG-binding site is involved in binding to neurons in culture[35,49–54]. In addition, there is evidence that fibroblast growth factor receptor 3 (FGFR3) might be involved in the binding of BoNT/A1 to nerve cells[55]. Such binding could account for the high specificity and affinity of BoNT/A1 for peripheral nerve terminals.

Functional binding. Following attachment to PSG, BoNT/B1, BoNT/DC and BoNT/G bind to segment 40–60 of the synaptic vesicle luminal domain of synaptotagmin (Syt) via a binding site in the HC-C domain that is close to the PSG-binding site[56–59] (FIG. 3). However, the two binding sites are structurally separated, and binding interactions with PSG and Syt are independent of each other[35–37,60]. By contrast, BoNT/A1 and BoNT/E1 bind specifically to two different segments of the fourth luminal loop of the synaptic vesicle transmembrane protein SV2 (REFS 61–64). Although isoform SV2C seems to be the main receptor that is involved in BoNT/A1 binding *in vitro*[63,65] — via an interaction with the N-terminal and C- terminal subdomains of the HC domain[65] (FIG. 3) — both SV2A and SV2B can also mediate BoNT/A1 entry, and all three isoforms are expressed on motor nerve terminals[61,62]. Glycosylated residues are present in the toxin-binding site of SV2 (REF. 65) and are potentially clinically relevant, but this requires further investigation. In fact, a different pattern of glycosylation among individuals would provide a simple explanation for the variable sensitivity of different patients to BoNT/A1 injection, which is often observed in clinical settings. Clearly, this variability might also be applicable to different vertebrate species.

Syt and SV2 are integral proteins of the synaptic vesicle membrane and expose their BoNT-binding sites to the synaptic vesicle lumen (FIG. 3). Therefore, unlike PSG, these protein receptors are not exposed on the nerve terminal surface and are not accessible to BoNT. However, they become available following the fusion of the synaptic vesicle with the presynaptic membrane, which exposes the synaptic vesicle lumen to the extracellular environment (BOX 2). Accordingly, BoNT binding to protein receptors occurs only after fusion of the synaptic vesicle to the presynaptic membrane, and this seems to facilitate the subsequent step of intoxication, which requires the endocytosis of BoNT (FIG. 3). However, it is possible that some Syt molecules might be present on the presynaptic membrane following complete merging of the synaptic vesicle with the plasma membrane[66] (FIG. 3).



Synaptotagmin
(Syt). A protein that spans the membrane of synaptic vesicles and binds to Ca^{2+} to trigger the fusion of synaptic vesicles with the plasma membrane of the neuron.

SV2
A protein that spans the membrane of synaptic vesicles and has an unknown function. Following fusion of the synaptic vesicle to the plasma membrane, the luminal domain of SV2 becomes exposed to the extracellular medium and functions as a receptor for botulinum neurotoxins.

Figure 3 | **Binding and trafficking of botulinum neurotoxins inside nerve terminals.** The first step in intoxication involves the binding of the carboxy-terminal end of the HC domain (the HC-C domain) to a polysialoganglioside (PSG) receptor that is present on the presynaptic membrane, followed by binding to a protein receptor (either synaptotagmin (Syt) or SV2) that is located either inside the exocytosed synaptic vesicle or on the presynaptic membrane (step 1). The crystal structure of botulinum neurotoxin B (BoNT/B) bound to Syt and PSG is shown on the lower left-hand side and the crystal structure of BoNT/A bound to PSG and to SV2 is shown on the lower right-hand side. The BoNT is then endocytosed inside synaptic vesicles (step 2) as it exploits the vesicular ATPase proton pump, which drives the re-uptake of neurotransmitter. Owing to the acidification of the vesicle, the BoNT becomes protonated, which results in translocation of the L chain across the synaptic vesicle membrane (step 3) into the cytosol. Translocation can also occur across the endosomal membrane following the fusion of a synaptic vesicle with an endosome (which seems to occur in cultured neurons[68]). The L chain is released from the HN domain, owing to the action of the thioredoxin reductase–thioredoxin system (TrxR–Trx), which cleaves the inter-chain disulphide bond (S–S; shown in orange). The L-chain metalloproteases of BoNT/B, BoNT/D, BoNT/F and BoNT/G cleave VAMP, the L-chain metalloproteases of BoNT/A and BoNT/E cleave SNAP25 and the L-chain metalloprotease of BoNT/C cleaves both SNAP25 and syntaxin (step 4), all of which result in the inhibition of neurotransmitter release and consequent neuroparalysis.

The protein receptors of other BoNTs have not been characterized in comparable detail so far, and conflicting results have been reported, which indicates that further characterization is needed.

Entry into nerve terminals

The second step of nerve terminal intoxication involves BoNT internalization (FIG. 3). The dual binding interaction with PSG and synaptic vesicle receptors (Syt or SV2, depending on the toxin serotype, as discussed above) increases the strength of BoNT interactions with the membrane, which is the product of the two binding affinities[33].

In both cultured neurons and *in vivo*, BoNT/A1 rapidly enters the synaptic vesicle lumen[67,68], and the number of toxin molecules (either one or two[67,68]) correlates with the number of SV2 molecules in the synaptic vesicle membrane[69]. The rate of entry for BoNT/A1 correlates with the rate of synaptic vesicle endocytosis[70] and with the rate of paralysis of the mouse phrenic nerve hemidiaphragm, which is the standard NMJ that is used to test the potency of BoNTs[71,72]. The mechanism of internalization of other BoNTs remains to be established, but their ability to rapidly paralyse the mouse phrenic nerve hemidiaphragm suggests that they all use the synaptic vesicle as a 'Trojan horse' to enter motor neuron terminals *in vivo*. By contrast, in cultured CNS neurons, other vesicles and trafficking routes might contribute to entry[68], particularly at the very high toxin concentrations that are frequently used in the laboratory[73].

Release into the cytosol

In order to reach their target SNARE proteins in the cytosol of nerve cells, the catalytically active L chain must be translocated from the synaptic vesicle lumen into the cytosol. The main driving force for L-chain translocation is the transmembrane pH gradient that is generated by the vesicular ATPase proton pump, which drives the re-entry of neurotransmitter into the synaptic vesicle (along with H^+ ions) after exocytosis[74] (BOX 2; FIG. 3). This is supported by the observation that specific ATPase inhibitors completely block nerve terminal intoxication by all BoNTs[52,67,73,75–77]. Thus, BoNTs of neurotoxigenic clostridia have evolved to exploit two major physiological events that occur at nerve terminals: synaptic vesicle endocytosis (to enter nerve terminals) and neurotransmitter refilling of the synaptic vesicle (to deliver the L chain metalloprotease into the cytosol). The molecular aspects of BoNT translocation across the synaptic vesicle membrane into the cytosol have been only partially elucidated, but studies that have been carried out in the past decade have provided considerable insights and have led to the proposal of a molecular model for this process[78,79].

Translocation across the synaptic vesicle membrane. It has long been known that BoNTs form ion channels of low conductance in planar lipid bilayers at low pH[80–82], and this process is associated with translocation of the L chain and the cleavage of its target SNARE proteins[83]. A major advance in understanding the mechanisms that are involved was made using the patch clamp technique in Neuro2A cells[78,79,84,85] and PC12 cells[77,86]. This experimental approach mimics *in vivo* conditions and enables events that occur at the single-molecule level to be resolved[79,85]. Collectively, these studies suggest that lowering the pH at the *cis* side of the membrane (that is, the side that faces the synaptic vesicle lumen) induces the L chains of BoNT/A1 and BoNT/E1 to cross the membrane through a channel that is 15–20 Å in diameter[87] (FIG. 4a). These channel dimensions enable the passage of α-helices but not of tertiary structural elements, which suggests that the L chain must unfold to pass through the channel. Stabilization of the L chain tertiary structure with antibodies prevents channel formation[88], which highlights the importance of unfolding for translocation and also suggests that this unfolding is linked to channel formation. This conclusion is also supported by the finding that cargo molecules, which are capable of unfolding at low pH, are transported into the neuronal cytosol when they are attached to the N terminus of BoNT[89]. Further studies have suggested that the HN domain alone is sufficient to form the transmembrane channel and that the peptide belt that links the L chain and the H chain regulates the formation of the HN channel[78,79,90,91]. Residues that are present in all three BoNT domains are responsible for the pH sensitivity of translocation[52,92]. The release of the L chain on the *trans* side (that is, the cytosolic side) of the membrane requires the inter-chain disulphide bond to be reduced[84]. The crucial role of cytosolic disulphide bond reduction is highlighted by the fact that BoNTs that have a reduced inter-chain disulphide bond do not form channels[84]. These data are consistent with the finding that only reduced BoNTs can hydrolyse their substrates[93] and also explain why this disulphide bridge is essential for neurotoxicity[52,94,95]. On the basis of these data, a model for translocation has been proposed (FIG. 4a). This model posits that the low pH of the synaptic vesicle lumen induces a conformational change in the HN domain, which then inserts into the membrane and forms a translocation channel that chaperones the passage of the partially unfolded L chain from the luminal side to the cytosolic side of the synaptic vesicle membrane. The L chain remains attached to the synaptic vesicle until the inter-chain disulphide bond is reduced, which occurs at the end of this process[78,84].

More recent data (discussed below) have clarified the molecular events that are involved in the interaction between BoNT and the membrane (which are induced at low pH) and in the reduction of the inter-chain disulphide bond (FIG. 4b). BoNT/B1, and the L chain and HN domains of BoNT/A1, do not change conformation at low pH in solution[90,96], whereas they do change conformation in the presence of PSG or PSG-containing membranes[77,90,97,98]. Using a protocol that bypasses the synaptic vesicle internalization step and enables the L chain to be translocated from the cell surface into the cytosol, it was found that BoNTs must be anchored to the membrane by two receptors[52] and that translocation occurs within minutes at 37 °C in the pH range 4.5–6

Phrenic nerve hemidiaphragm
An *ex vivo* preparation that includes a portion of the diaphragm, as well as the axon and nerve terminal of the phrenic nerve. This nerve contains motor, as well as sensory and sympathetic, fibres and controls the contraction of the diaphragm muscle via the release of acetylcholine. Its inhibition by botulinum neurotoxins blocks respiration, which causes death.

Patch clamp technique
An electrophysiological technique that is based on microelectrodes that are sealed on the plasma membrane of a cell, which enables the measurement of electrical activity and the properties of ion channels.

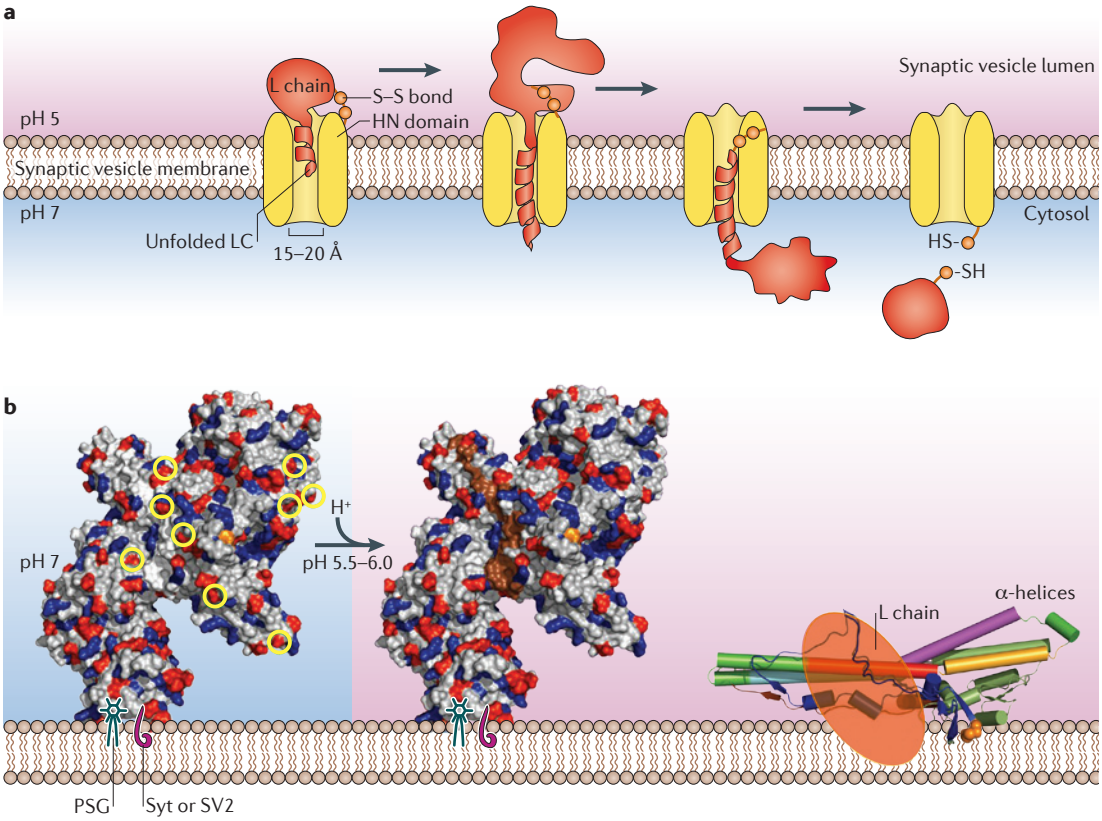


Figure 4 | **Model for the molecular events that occur during L-chain translocation across the synaptic vesicle membrane.** **a** | On the basis of a series of biophysical studies, a model for membrane translocation of the L chain has been proposed[78]. Owing to the action of the vesicular ATPase proton pump, the pH in the lumen of synaptic vesicles becomes acidic, which causes a conformational change in the HN domain, enabling it to penetrate the lipid bilayer. This leads to the formation of a channel that chaperones the partially unfolded L chain across the membrane. The inter-chain disulphide bond (S–S bond) is proposed to cross the membrane at a late stage during translocation, and its reduction on the cytosolic side of the synaptic vesicle membrane releases the L chain into the cytosol. **b** | Proposed steps that are suggested to occur before membrane insertion of botulinum neurotoxins (BoNTs) (BoNT/B, Protein Data Bank accession 1EPW, is shown). The process begins with the binding of BoNT to its two receptors (polysialoganglioside (PSG) and Syt or SV2), which is a prerequisite for subsequent steps (left-hand structure). Cationic and anionic residues in BoNT/B are shown in blue and red, respectively. The acidification of the synaptic vesicle lumen (owing to the action of the ATPase proton pump) causes protonation of some conserved glutamic acid and aspartic acid residues that have high pKa values (circled in yellow in the left-hand structure), which are clustered on one face of the toxin. This face of the toxin also contains the inter-chain disulphide bond[52] (orange) and a segment that is predicted to have high membrane insertion propensity[96] (brown; centre structure). The partially protonated, positively charged face of BoNT is attracted by the anionic membrane surface, which is rich in negatively charged lipids, and the toxin eventually collapses onto the membrane. The ensuing events are unclear, but it is likely that the long α-helices of the HN domain break into shorter helices (shown in right-hand panel with different colours), which then insert into the lipid bilayer to form the ion channel. The L chain is predicted to become a molten globule, which is a protein conformation that is capable of inserting into the lipid bilayer of the membrane.

(REFS 77,99), which is consistent with the pH inside the synaptic vesicle[100,101]. It was also noted that one face of the BoNT contains several conserved high pKa carboxylates, the inter-chain disulphide bond and a segment that has a high propensity for membrane insertion[52,97]. Replacement of three of the carboxylate residues with the corresponding amides in BoNT/B1 eliminates the requirement for their protonation and causes the L chain to enter the cytosol more rapidly, thus increasing toxicity[92]. These data suggest that there is no single pH sensor in BoNTs, but instead, several carboxylates that have high pKa values have a role in the low pH-driven release of the L chain into the cytosol.

pH sensor
In the context of this Review; amino acid residues that change protonation state according to variations in pH. A change in protein structure may consequently occur, owing to altered hydrogen bonding and electrostatic interactions.

Updated model of BoNT translocation. By considering the findings that are described above, an updated model for BoNT translocation can now be presented. It should be noted that this model requires further experimental studies to determine whether the steps that are outlined below are indeed correct. BoNT initially binds to its two receptors (PSG and SV2 or Syt) inside the synaptic vesicle lumen, which has a neutral pH, immediately after endocytosis (FIG. 4b). The vesicular ATPase then pumps protons into the synaptic vesicle and the luminal pH becomes progressively more acidic. Notably, protons and other cations are attracted to the anionic membrane surface of the synaptic vesicle and their local

concentration reduces the pH near the membrane to 1–1.5 units below that of the lumen[100–103] (FIG. 4b).

The amino acids histidine, glutamate and aspartate become protonated within the pH range (4.5–6) and are predicted to be involved in L-chain translocation. However, the actual pKa values of these residues depend on their molecular surroundings. BoNTs lack conserved histidine residues, except those that are in the active site, but they do contain conserved carboxylate residues that are predicted to have high pKa values[52]. Assuming that the residues that are important for the low-pH-driven process are conserved, seven conserved carboxylates that have high pKa values are located in the HN domain, three are located in the L chain and one is located in the HC domain[52] (FIG. 4b). The spatial distribution of these residues reinforces the suggestion that BoNTs contain more than a single pH sensor[92]. The model posits that these carboxylates become protonated — partially or entirely — in a sequential manner (depending on their pKa values) as the pH of the synaptic vesicle lumen decreases (FIG. 4b). Even a partially protonated BoNT has a net positive charge that favours its interaction with the anionic membrane surface[52,103–105]. The BoNT surface that is involved in membrane interactions is suggested to be the surface that contains the inter-chain disulphide bond and the membrane-inserting segment (residues 637–688) (FIG. 4b); the opposite side of the BoNT molecule lacks carboxylates of appropriate pKa values[52].

The predicted collapse of BoNT onto the membrane surface is not prevented by receptor interactions, as either binding is weakened by the low pH[65] or the two receptors are flexible[35,36]. BoNT is suggested to undergo a gross structural change that involves both the L chain and the HN domain and is facilitated by simultaneous changes in the conformation and organization of membrane lipids (FIG. 4). Such changes are caused by the acidic pH of the lumen, but other factors that might contribute include ionic strength, the high Ca^{2+} concentration and the high negative curvature of the luminal synaptic vesicle membrane. The ensuing molecular events are currently unknown, but, on the basis of previous studies, we suggest that the L chain becomes a 'molten globule', which is a protein variant that retains native secondary structure but has increased hydrophobicity, to enable membrane insertion[98,106–109]. The α-helices of the HN domain contain amphipathic segments and residues that have a low propensity to form a helical structure, which suggests that the long α-helices of the HN domain might break into shorter protein segments that insert into the membrane and thereby form an ion channel. However, whether this actually occurs is currently unknown, and clearly, more studies are needed to clarify this essential step of the BoNT intoxication process.

Importantly, the reduction of the inter-chain disulphide bond at any stage before its exposure to the cytosol prevents L-chain translocation, so this domain must emerge on the cytosolic side before reduction takes place[84]. The reduction of protein disulphide bonds is catalysed in the cell cytosol by different enzymatic systems, including glutaredoxins, thioredoxins and other systems[110–112]. Using a discriminating pharmacological approach, the redox system NADPH–thioredoxin reductase (TrxR)–thioredoxin (Trx) was found to have a major role in release of the L chain into the neuronal cytosol[113]. Following Trx-mediated reduction of the disulphide bond, L-chain translocation is irreversible and the toxin is now free to interact with its target proteins (FIG. 3). The Trx tertiary fold is similar to that of ancestral chaperonins, so it is also possible that Trx functions as a chaperonin for L-chain translocation[112,114].

Mechanism of BoNT-induced neuroparalysis

The L chains of all known BoNTs are metalloproteases that are specific for one of the SNARE proteins: VAMP (vesicle-associated membrane protein; also known as synaptobrevin), SNAP25 (synaptosomal-associated protein of 25 kDa) or syntaxin (FIG. 3). BoNT/C cleaves both SNAP25 and syntaxin, BoNT/B, BoNT/D, BoNT/F and BoNT/G only target VAMP and BoNT/A and BoNT/E cleave SNAP25. The fact that inactivation of any one of these three proteins inhibits neurotransmitter release is the strongest evidence that these three proteins form the core of the neuroexocytosis nanomachine[115,116] (BOX 2). The SNARE family of proteins includes many isoforms of VAMP, SNAP25 and syntaxin, which are differentially expressed in many non-neuronal cells and tissues. Although several of these isoforms can be cleaved by BoNTs, these substrates are not accessible *in vivo*, as non-neuronal cells lack appropriate receptors for the toxin[3,117].

The molecular basis of the neuroparalytic activity of BoNTs has recently been reviewed in depth[116,117], and only the more recent findings are discussed here. With the exception of BoNT/A and BoNT/C, all BoNTs cleave isolated SNARE proteins by removing large cytosolic segments, which prevents the formation of the SNARE complex[118,119] (BOX 2). BoNT/A and BoNT/C remove only a few residues from the C terminus of SNAP25 (REFS 13,116,117), and this truncated form of SNAP25 can form a stable SNARE complex[118]; thus, the molecular mechanism of BoNT/A- and BoNT/C-induced neuroparalysis remains to be elucidated. It is possible that the core of the nanomachine is comprised of a SNARE supercomplex that is formed by several SNARE complexes and that the C terminus of SNAP25 is involved in protein–protein interactions among the individual SNARE complexes[116,120]. An alternative explanation is that BoNT/A cleaves another protein (or proteins) that is (or are) essential for neurotransmitter release. However, such protein substrates have not yet been found, despite extensive searches, and they are unlikely to exist, owing to the unique mode of recognition of VAMP, SNAP25 and syntaxin by the L-chain metalloprotease[116,117]. In fact, the SNARE-binding site of the metalloprotease is a long channel that is occupied by the peptide belt in the intact protein (FIG. 2a); however, when the L chain is released, this channel is vacated and the substrate can then insert into the channel. The L chain interacts extensively with the substrate and contacts several exosites of the protein in addition to the cleavage site[116,117].

Neuroexocytosis nanomachine
A molecular machine of nanometre dimensions that is used for the release of neurotransmitters.

Box 3 | **Vaccines, antibodies and chemical inhibitors**

There is currently no approved pharmacological treatment for botulinum neurotoxin (BoNT) intoxication, but the growing concern for the potential use of BoNTs as biological weapons and the need to prevent botulism outbreaks has stimulated research aimed at developing a range of agents to prevent and/or treat botulism.

Vaccines

Early attempts to create botulism-specific vaccines involved the treatment of partially purified BoNTs with formalin (to inactivate the BoNT) and the addition of aluminium hydroxide as an adjuvant[142]. However, after it was shown that injection of a recombinant version of the HC domain of tetanus neurotoxin was sufficient to induce a protective immune response against tetanus[143], various BoNT HC domains were expressed in *Pichia pastoris* and were shown to induce protective antibodies in animals[144,145]. A recombinant vaccine composed of the HC domains from BoNT/A1 and BoNT/B1 has shown promising results in clinical trials, and vaccines for other serotypes are now under development. Other BoNT domains have been tested in animals[146], but there is currently no licensed vaccine available for human use. However, several animal vaccines are on the market, which are used to prevent botulism outbreaks.

Antibodies

Specific antitoxin antibodies can be used to prevent and treat botulism by eliminating circulating BoNTs. Antibodies are also often used in research laboratories to identify BoNT serotypes. However, BoNT-specific antibodies have difficulty entering neurons so their use for the treatment of overt disease is limited. BoNT-specific antibodies have traditionally been produced in animals — mainly horses[142] — however, although these polyclonal antibodies are efficient at BoNT neutralization, they are usually rapidly eliminated from the human body and can also lead to serum sickness. To overcome these obstacles, BoNT/A-specific polyclonal human antibodies have been isolated from the sera of human volunteers that have been immunized with BoNT toxoids and are used to treat infant botulism[147]. However, sophisticated biotechnologies to produce high-affinity humanized monoclonal antibodies are now available and have been used to produce BoNT/A-, BoNT/B-, BoNT/E- and BoNT/F-specific antibodies[148–150], and work to extend this approach to all serotypes is ongoing. Another promising approach is to generate single-chain toxin-binding camelid-like antibodies, which have the potential for intracellular use[151,152].

Small-molecule inhibitors

Therapeutic inhibitors for post-intoxication treatment must block L-chain metalloprotease activity inside nerve terminals. Such inhibitors must be non-toxic and capable of crossing the plasma membrane of nerve cells. However, the development of such agents is complicated owing to the complex mode by which the L chain binds to its substrate, which involves several interaction sites[116,117]. Thus, despite intensive efforts in several laboratories, including the screening of large chemical and natural compound libraries, structure-based molecular design and several chemical synthesis approaches, few molecules have passed the stage of inhibition of the toxins in cultured neurons and have therefore not yet reached the level of testing in animals[153].

The substrates of only a few of the BoNT subtypes have so far been determined. In addition, the rate of substrate turnover for the metalloproteases inside nerve terminals is unknown, and this parameter has a profound effect on the onset of paralysis. Although it seems unlikely that novel BoNT substrates will be found, it is probable that novel cleavage sites in the SNARE proteins will be revealed, as indicated by the recent report that BoNT/F5 cleaves VAMP[121] at a different peptide bond compared with BoNT/F1 (REF. 122). The available evidence suggests that BoNT/A subtypes have different enzymatic rates[123,124], and it is therefore possible that individual subtypes are highly variable in their potency, onset and duration of action. Understanding the enzyme kinetics of BoNTs *in vivo* is also essential for the design of novel inhibitors of BoNTs (BOX 3).

Duration of action. One special feature of BoNTs is the reversibility of their action *in vivo*. Patients with botulism fully recover if death by respiratory paralysis is prevented by mechanical ventilation[2,4]. This is probably because BoNTs paralyse the nerve terminal but do not kill the neuron, the cell body of which is frequently located a substantial distance away from the paralysed nerve terminal. However, although BoNTs do not cause axonal degeneration at the doses that cause botulism, reversible muscle atrophy is evident.

There is a remarkable diversity in the duration of BoNT-induced neuroparalysis. The lifetime of the metalloprotease within the nerve terminal cytosol is the predominant, but not the only, factor that contributes to the duration of paralysis[116,125,126]. In general, the duration of paralysis in mice is in the following order (with the longest duration of action first): BoNT/A1, BoNT/C1, BoNT/B1, BoNT/D, BoNT/F1, BoNT/G, BoNT/E1. However, the duration of paralysis also varies with the vertebrate species, the activity of the affected muscle and the toxin dose. Moreover, the paralysis of autonomic human cholinergic nerve terminals lasts 3–4 times longer than that of the NMJ[127]. However, as data for less than one-third of the BoNTs subtypes are available, it seems likely that paralytic activity may vary considerably among different subtypes.

Duration of action is the main factor that contributes to the biological action of BoNTs, as it determines the severity of human botulism (type A toxins cause more severe botulism than type B toxins, which cause much more severe botulism than type E toxins)[2–4]. Knowing the duration of action should also provide information about the mechanisms of SNARE protein inactivation and turnover inside nerve terminals[125,128] as well as the assembly of the SNARE complex[116]. In addition, the persistence of BoNT activity is important for their therapeutic application, as a toxin that has a longer duration of action requires fewer injections of lower doses. Nevertheless, for certain conditions, such as facial lacerations or disjointed bone fractures, a toxin that has a short duration of action might be more useful in ameliorating the course and outcome of the illness. There is a growing area of research that aims at changing the binding specificity, affinity and the duration of BoNT action in order to obtain tailor-made therapeutic agents and more sophisticated tools to be used in cell biology studies[129–132].

Conclusions and future perspectives

The toxic potency of BoNTs is the result of targeting a physiological function that is essential for life in all vertebrates. The discovery of many novel BoNTs, which vary in potency and duration of action, raises questions regarding the evolutionary advantage that is associated with the production of such a large number of diverse neurotoxins that have the potential to kill the host. However, this seems to be an obligatory survival strategy for an anaerobic organism that can multiply only within a non-oxygenated medium, such as a cadaver. In turn, this strategy must be coupled to an alternative lifestyle, such as sporulation, which enables the anaerobic organism to survive the complete consumption of nutrients and

Serum sickness
An illness of humans that is caused by a hypersensitive reaction to proteins in antiserum derived from a non-human source; it usually occurs 4–10 days after exposure.

Camelid-like antibodies
Single-domain antibodies that are derived from the heavy-chain antibodies of camelids; they are a new generation of therapeutic agents and immunoreagents.

the ensuing exposure to oxygen. Thus, toxin production and sporulation are essential aspects of the life cycle of toxigenic clostridia.

Recent research has unravelled the molecular basis of BoNT action, including neurospecific binding and the mechanisms that are involved in the catalytic cleavage of the core proteins of the neuroexocytosis nanomachine. However, several outstanding questions remain, particularly regarding the mechanistic details of toxin endocytosis into synaptic vesicles and the process of L-chain translocation across the synaptic vesicle membrane and its subsequent release into the cytosol. Another major challenge is to establish methods for the reliable comparison of the more than 40 distinct BoNTs that have been identified so far, and of those that are yet to be identified, which might reveal novel therapeutic BoNTs that have increased potencies and durations of action. We expect that the number of novel BoNTs will increase in the coming years, following the analysis of more clinical and soil isolates. It will then be important to determine the evolutionary processes that are responsible for the generation of such diversity. From a practical perspective, it is essential to develop stocks of defined, highly specific antibodies and effective small-molecule inhibitors to be used in the prevention and treatment of botulism.

Note added in proof

A recent paper[154] provides robust molecular evidence that the BoNT/A complex trancytoses polarized epithelial cells, disrupts E-cadherin cell–cell adhesion at adherens junctions and so opens the paracellular route of toxin entry into the body previously found for the BoNT/B complex[25].

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Competing interests statement
The authors declare no competing interests.

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