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Clostridial Neurotoxins: Current Diagnosis and Therapeutic Approach

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Abstract **Review Article**

Clostridium is a Gram-positive, anaerobic, endo spore-forming bacillus, species of Clostridium, notably *C. botulinum* and *C. tetani*, have ability to produce potent neurotoxins. Clostridial neurotoxins include botulinum neurotoxins (BoNTs) and tetanus neurotoxin (TeNT). They are the most potent toxins to mankind. Botulism and tetanus are responsible for severe neurological diseases, respectively. While BoNTs cause flaccid paralysis, TeNT causes spastic paralysis. Eventually, both BoNTs and TeNT will lead to respiratory failure and death. Tetanus is mainly caused by deep penetrating wounds where anaerobic bacterial growth is facilitated. Human botulism, however, can occur in one of three natural forms: foodborne, wound, and intestinal. While inhalational botulism does not occur naturally, it is a concern as a potential bioterror attack. In BoNT the current antitoxin is a heptavalent polyclonal antibody formulation which contains neutralizing antibodies against seven toxin serotypes; A, B, C, D, E, F, and G and has superseded the older trivalent antitoxin which is only able to neutralize serotypes A, B, and E. For TeNT, Wounds from which the infection originated should be surgically debrided and an antibiotic administered. Penicillin has been the worldwide antibiotic of choice. However, the structure of penicillin is similar to γ aminobutyric acid (GABA); it therefore acts as a competitive GABA antagonist, and in high doses may cause central nervous system (CNS) hyperexcitability and convulsions. In tetanus this potential side effect of penicillin may act synergistically with the toxin to block GABA neuronal activity. Metronidazole is therefore considered to be the antibiotic of choice in the treatment of tetanus.

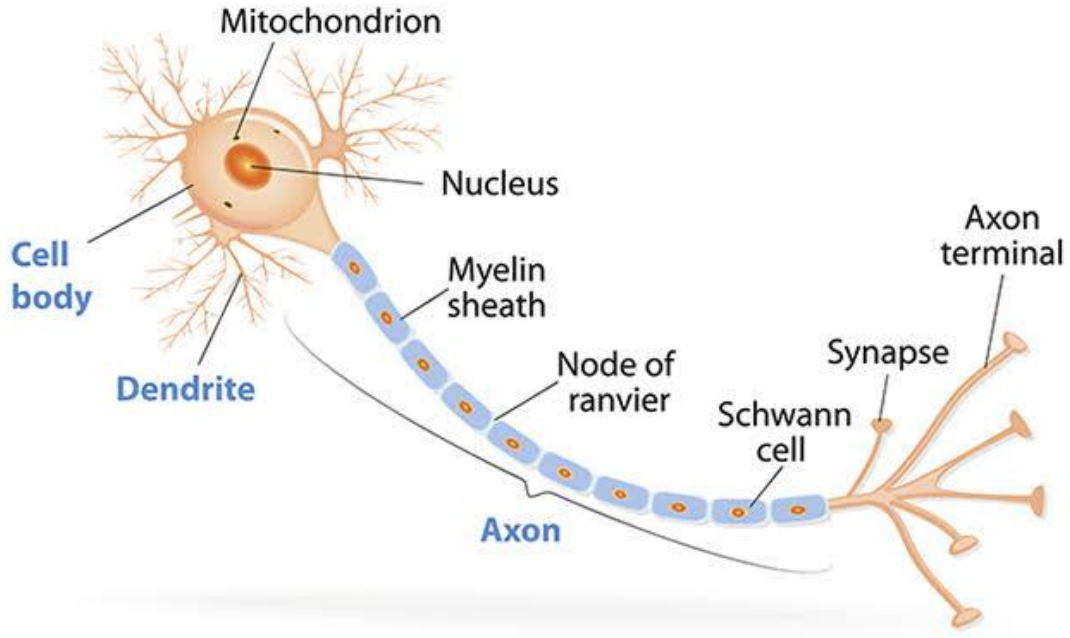
Keywords: Clostridial neurotoxins, botulinum neurotoxin (BoNT), tetanus neurotoxin (TeNT), botulism and tetanus, antitoxin therapy.

Introduction

Clostridium is an anaerobic, Gram-positive, endospore-forming bacillus (Johnson, 2019). Several species of Clostridium, most notably C. botulinum and C. tetani, are capable of producing potent neurotoxins (Popoff & Brüggemann, 2022). Botulinum neurotoxins (BoNTs) exist as seven major serotypes (A–G) with numerous amino acid variants, known as subtypes, identified through nucleotide sequencing (Gregory & Acharya, 2023). Along with tetanus neurotoxin (TeNT), BoNTs form the clostridial neurotoxin (CNT) family of protein exotoxins. Exposure to these neurotoxins can cause life-threatening neuroparalytic diseases, including botulism and tetanus. The active form of CNTs consists of a ~100 kDa heavy chain (HC), which includes translocation (HCT) and receptor-binding (HCR) domains, linked via a single disulfide bond to a ~50 kDa light chain (LC) that contains the catalytic domain (Prajapati et al., 2022). Neuronal intoxication is a multistep process involving receptor-mediated endocytosis, translocation of the LC into the cytosol, and proteolytic cleavage of neuronal SNARE (soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor) proteins. SNARE cleavage blocks synaptic vesicle exocytosis, ultimately leading to paralysis of the affected muscles or glands (Kumar & Singh, 2025). Among BoNT/A serotypes, eight subtypes (A1–A8) have been identified, with amino acid similarities ranging from 98% (A1 vs. A5) to 84% (A1 vs. A3). Functional differences between subtypes have been studied only to a limited extent (Grenda et al., 2023). Comparative analyses of subtypes A1–A5 show that BoNT/A4 is ~1000-fold less active than A1, BoNT/A3 has a shorter neuronal lifespan, and BoNT/A2 enters cells more rapidly than A1. Notably, studies in ex vivo cell cultures, animal models, and humans indicate that BoNT/A2 exhibits greater clinical efficacy and less diffusion to neighboring muscles compared with BoNT/A1 (Kaji, 2023).

Neurons

The fundamental task of neurons is to sense, conduct, receive and transmit signals. Neurons transfer signals from the sense organs inward to the central nervous system (CNS), consisting the brain and spinal cord (Sabina *et al.*, 2024). In the CNS the signals are processed and interpreted by a system of neurons, which later produce a response. The response is sent, by neurons, outward for action to muscle cells and glands. Neurons come in many shapes and sizes, but they all have some common features. A typical neuron has four main components: the cell body (or soma), which contains the nucleus and organelles such as the endoplasmic reticulum and mitochondria; dendrites, which branch out to receive signals from other neurons; an axon, which carries signals away from the cell body; and terminal branches at the end of the axon called nerve terminals or presynaptic terminals (ten Donkelaar et al., 2023). The cell body and axon are surrounded by glial cells, which support neurons and form insulating layers known as myelin sheaths around many large axons. The total number of neurons and glial cells in the human body is estimated to be around 10 (Rahman et al., 2023). Axon length varies widely depending on the type of neuron, ranging from less than 1 mm to about 1 meter, while their diameter ranges from 0.1 µm to 20 µm (Rahman et al., 2023). Dendrites receive small electrical signals from the nerve terminals of other neurons. These impulses travel to a region of the soma called the axon hillock (Wang et al., 2024). When the combined electrical input at the hillock exceeds a certain threshold, it initiates a propagating electrical signal along the plasma membrane known as an action potential. If the plasma membrane functioned like a simple conductor, the electrical impulse would quickly diminish as it traveled along the membrane (Wang et al., 2024).



Source: queensland brain institute

Fig.1: Structure of a Neuron

Clostridial Neurotoxins

Clostridial neurotoxins consist of botulinum neurotoxins (BoNTs) and tetanus neurotoxin (TeNT), which are considered among the most powerful toxins known to affect humans. They cause severe neurological illnesses—botulism and tetanus, respectively. BoNTs lead to flaccid paralysis, whereas TeNT results in spastic paralysis (Swink & Gilsenan, 2022). In severe cases, both toxins can ultimately cause respiratory failure and death. Tetanus typically develops from deep, penetrating wounds that create anaerobic conditions favorable for bacterial growth (Pal et al., 2024). In contrast, botulism occurs naturally in three main forms: foodborne, wound, and intestinal (which includes infant botulism and sporadic adult intestinal botulism) (Rao, 2021). Although inhalational botulism does not occur naturally, it remains a concern due to its potential use in bioterrorism (Taşkın & Akpınar, 2024).With the growing therapeutic and cosmetic use of BoNT—particularly BoNT/A—for neuromuscular disorders and aesthetic procedures, cases of iatrogenic botulism have also emerged as a rare but serious complication (Rashid et al., 2018). Foodborne botulism results from consuming food contaminated with preformed toxin. Wound botulism, though uncommon, has been increasingly reported among individuals who inject drugs, arising from toxin production by *C. botulinum* spores within contaminated wounds. Intestinal botulism occurs when spores colonize the gut and produce toxin in situ. This form is most common in infants, whose intestinal microbiota is not yet fully developed, but it can also affect adults with prior bowel surgery, intestinal abnormalities, or recent antibiotic use that disrupts normal gut flora (Harris et al., 2020). Although BoNT therapy is generally safe and well tolerated, a very rare but serious complication resembling botulism known as iatrogenic botulism can occur (Ibatullin & Magjanov, 2019; Jeffery & Karim, 2021). Among all forms, foodborne botulism and infant intestinal botulism are the most prevalent. Both involve absorption of BoNT through the gastrointestinal tract, an unusual process for a large protein toxin such as BoNT (Kumar & Singh, 2025).

Diversity of Clostridial Neurotoxins

Tetanus Neurotoxin

Tetanus neurotoxin (TeNT) is produced by *Clostridium tetani*, whereas botulinum neurotoxins (BoNTs) are produced by *Clostridium botulinum*. Both *C. tetani* and *C. botulinum* are strictly anaerobic, Gram-positive bacteria. A total of 43 strains of *C. tetani* have been identified, most of which were isolated from human wounds, and their complete genome sequences have been documented (Garrigues et al., 2021; NCBI, 2021). These strains are generally divided into two primary groups: the Harvard strains, which originate from the ancestral Harvard strain, and wild-type strains obtained from clinical cases. Differences between strains are mainly associated with proteins located in prophage regions as well as in CRISPR/Cas loci and their spacer sequences (Cohen et al., 2017). Despite these variations, the genes responsible for toxin production are highly conserved, resulting in the existence of only a single type of tetanus neurotoxin (Garrigues et al., 2021).

Botulinum Neurotoxin

Compared with *Clostridium tetani* and its single tetanus neurotoxin, *Clostridium botulinum* strains and the botulinum neurotoxins (BoNTs) they produce are highly diverse. Using serotype-specific neutralization assays, seven BoNT serotypes (A–G) have been confirmed (Dong et al., 2019). A proposed type H identified in 2013 was later shown to be a hybrid of types A and F and is now referred to as BoNT/FA or BoNT/HA (Smith et al., 2023). More recently, a distinct serotype, BoNT/X, was discovered; it has low sequence similarity to other serotypes and is not neutralized by antisera against types A–G (Masuyer et al., 2018). Beyond serotype variation, each BoNT serotype is subdivided into multiple subtypes based on amino acid sequence differences, designated numerically (e.g., BoNT/A1, A2, A3) (Peck et al., 2017). Advances in DNA sequencing have expanded the number of recognized subtypes. Currently, there are eight subtypes of BoNT/A (A1–A8), eight of BoNT/B (B1–B8), two of BoNT/C (C1 and CD, a C/D chimera), two of BoNT/D (D and DC, a D/C mosaic), twelve of BoNT/E (E1–E12), and eight of BoNT/F (F1–F8). Subtype differences can influence antibody binding, neutralization capacity, and catalytic efficiency toward target substrates (Zhang et al., 2023). *C. botulinum* is classified into six heterogeneous groups (I–VI) that share the ability to produce BoNT (Cai et al., 2021). Group I (proteolytic, mesophilic, highly heat-resistant spores) is a major cause of human botulism and produces toxins A, B, and/or F; strains may carry up to three toxin genes and express multiple serotypes (Meurens et al., 2023). BoNT/X was identified in Group I strain 111, originally isolated from an infant botulism case in Japan. Although this strain lost toxicity after repeated passages—likely due to loss of a neurotoxigenic plasmid—sequence data enabled recombinant production of the toxin, which is not neutralized by existing antisera (Grenda et al., 2023; Ruan & Bourne, 2024). Group II (nonproteolytic, saccharolytic, psychrotrophic, moderately heat-resistant spores) produces a single toxin—type B (B4), E, or F (F6)—and is an important cause of foodborne botulism (Munir et al., 2023). Genome sequencing has revealed remnants of other toxin genes in some strains. Group III (saccharolytic, mesophilic, heat-resistant spores) produces type C, type D, or hybrid C/D or D/C toxins and mainly causes botulism in animals (Ezzati et al., 2025). Group IV, also known as *Clostridium argentinense*, produces BoNT/G, which has not been definitively linked to human or animal disease (Grenda et al., 2024). Additional toxin-producing species expand this diversity: some strains of *Clostridium baratii* produce BoNT/F (F7), and certain strains of *Clostridium butyricum* produce BoNT/E (E4 or E5), both associated with human botulism. These are classified as Groups V and VI, respectively (Cai et al., 2021). Toxin gene clusters across the botulinum family share moderate sequence similarity (~40%) but are located variably on chromosomes, plasmids, or bacteriophages (Benyamini, 2024). In contrast, *C. tetani* is genetically simpler, harboring a single plasmid encoding TetX (the TeNT gene) and TetR, its transcriptional regulator (Shitada et al., 2023). Notably, BoNT-like genes have also been

identified in non-clostridial species, including *Weissella oryzae*, *Enterococcus* sp. 3G1_DIV0629, and *Chryseobacterium piperi*. Recombinant expression of these homologs demonstrated SNARE protein–cleaving activity. For example, BoNT/Wo cleaves VAMP-2, while BoNT/En (formerly eBoNT/J) cleaves multiple SNARE proteins but does not cause toxicity in mice unless combined with a BoNT/A heavy chain, suggesting receptor limitations (Cai et al., 2021; Benyamini, 2024). This remarkable diversity of BoNTs is likely driven by horizontal gene transfer (Brunt et al., 2020). Despite being among the most potent toxins known, BoNTs are also widely used in medicine for treating neuromuscular disorders and in aesthetic applications (Padda & Tadi, 2021). Their extensive variability is therefore significant not only for microbiology and infectious disease research but also as a valuable resource for developing new therapeutic applications (Hafeez et al., 2021).

Mechanism of Action of clostridial neurotoxins

The mechanisms of action of tetanus neurotoxin (TeNT) and botulinum neurotoxins (BoNTs) share several similarities. Both toxins bind strongly to receptors on peripheral nerve terminals (Marinelli, 2025). After binding, the toxin–receptor complex is internalized through receptor-mediated endocytosis in a temperature-dependent process, forming an endocytic vesicle (Kenworthy et al., 2021). As the vesicle becomes acidified by a proton pump, structural changes occur in the toxin, leading to oligomerization and the formation of ion channels (MacDonald et al., 2025). Biophysical evidence suggests that multiple toxin molecules are required to create a functional ion channel (Kenworthy et al., 2021). Subsequently, disulfide bonds linking the heavy (H) and light (L) chains are reduced, allowing the enzymatically active L-chain to translocate into the cytosol, where the pH is neutral. From this stage onward, the actions of TeNT and BoNTs differ. BoNTs remain localized at the presynaptic terminals of peripheral nerves, where they cleave specific proteins essential for synaptic vesicle fusion. Although all BoNTs function as zinc-dependent metalloproteases, each serotype targets distinct SNARE proteins. BoNT/A and BoNT/E cleave SNAP-25; BoNT/C cleaves both syntaxin and SNAP-25; and the other serotypes primarily cleave VAMP (Jonsuu et al., 2023). Disruption of these proteins blocks calcium-dependent docking and fusion of acetylcholine-containing vesicles with the presynaptic membrane. As a result, neurotransmitter release is inhibited, leading to the characteristic descending flaccid paralysis seen in botulism (Kenworthy et al., 2021). TeNT, in contrast, although capable of inhibiting neurotransmitter release at peripheral sites—sometimes producing flaccid paralysis similar to BoNT/B (Marinelli, 2025)—primarily exerts its major effects within the central nervous system (CNS). After uptake by peripheral motor, sensory, or adrenergic neurons, TeNT undergoes retrograde axonal transport to the ventral spinal cord and brainstem (Rissardo et al., 2023). There, it blocks the release of inhibitory neurotransmitters, resulting in central disinhibition and the development of spastic paralysis (Rissardo et al., 2023).

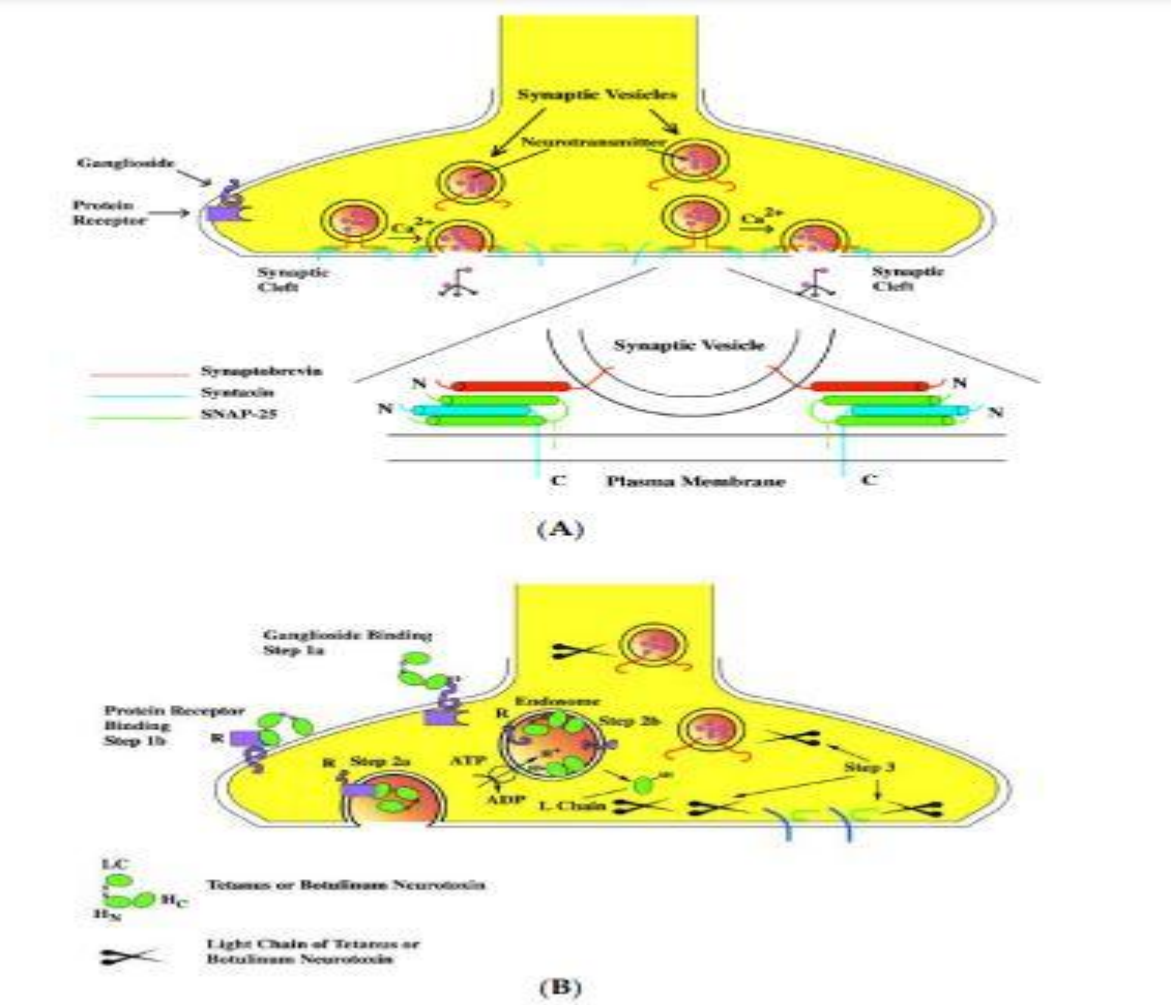


Fig. 2: mechanism of clostridial neurotoxin. source: Singh, 2000

(A) synaptic vesicles containing neurotransmitters dock with the plasma membrane

(B) Botulinum or tetanus toxin binds to the presynaptic membrane through gangliosides and a protein receptor

Pathophysiology of clostridial neurotoxin

BoNT

Botulism is a severe and potentially life-threatening neuroparalytic illness affecting humans and animals, caused exclusively by botulinum neurotoxins (BoNTs) (Rawson et al., 2023). In humans, the disease typically presents as symmetrical, descending muscle weakness. Early symptoms usually involve the cranial nerves and include blurred or double vision, dry mouth, and difficulty speaking. The classic early signs are often remembered using the “four D’s” mnemonic: dysarthria, diplopia, dysphonia, and dysphagia (Rawson et al., 2023). As the toxin spreads, muscle weakness progresses to the torso and upper limbs (Nickson, 2023). Without treatment, paralysis can extend to the respiratory muscles, leading to respiratory failure and potentially death (Rawson et al., 2023). Although BoNT is extremely potent, human botulism has become relatively uncommon. According to data from the Centers for Disease Control and Prevention, an average of about 160 cases are reported annually in the United States (CDC, 2022). Mortality rates have also declined significantly over the past century—from approximately 70% to less than 5% in developed

countries (Franjić, 2024). This improvement is largely attributed to better clinical awareness, timely treatment, and strict food safety and processing standards. In contrast, botulism remains common among wild and domesticated animals, where outbreaks frequently occur through natural environmental transmission cycles (Meurens et al., 2023). In humans, botulism is categorized into three main clinical forms based on the route of exposure: foodborne, wound, and infant botulism. While symptoms are generally similar across these forms, foodborne botulism may initially present with gastrointestinal symptoms—such as diarrhea, nausea, vomiting, and abdominal cramps—before the onset of neurological signs (Buzatu et al., 2024).

Foodborne botulism

Foodborne botulism occurs after consuming food that contains preformed botulinum neurotoxin (BoNT). Even a very small amount of contaminated food can produce severe illness and may be fatal if not treated promptly (Rawson, 2023). Symptoms usually appear within 12–72 hours after ingestion, depending on the quantity of toxin consumed (Yu et al., 2022). Historically, foodborne botulism was the most common form of human botulism. It is typically linked to improperly processed foods, whether commercially produced or home-prepared, especially uncooked or inadequately preserved items (Meurens et al., 2023). High-risk preservation methods include fermentation or pickling followed by canning or bottling without sufficient heat treatment, as these practices create anaerobic conditions that allow spores to germinate and bacteria to grow. The implementation of strict food safety measures—such as the "botulinum cook" (heating at 121 °C for 3 minutes), proper freezing, and refrigeration below 4 °C—has significantly reduced outbreaks in recent decades (Rawson et al., 2023). Growth of vegetative cells and toxin production occur only under specific conditions: anaerobic environments, low salt levels (generally below 5%), high water activity, and non-acidic conditions (pH above 4.6) (An et al., 2024). Therefore, careful product formulation and preservation practices are essential in preventing contamination. Importantly, BoNT itself is heat-sensitive. Any preformed toxin present in food can be destroyed by heating at 85 °C for at least five minutes, making adequate reheating an effective preventive measure (Monash et al., 2025).

Infant botulism

Environmental *Clostridium botulinum* spores commonly pass through the adult human digestive tract without causing disease, as a healthy, mature gut environment does not favor spore germination (Harris et al., 2024). In contrast, infants—particularly those between 1 and 6 months of age, though cases have been reported up to 12 months—have an immature gastrointestinal system that is more susceptible to colonization after ingesting spores (Munnir et al., 2023). This vulnerability is largely due to underdeveloped gut physiology and an incomplete intestinal microbiota. Following spore ingestion and germination in the infant gut, the bacteria produce botulinum neurotoxin in situ. The toxin is then absorbed from the intestinal lumen into the bloodstream, leading to the characteristic presentation of "floppy baby syndrome," marked by poor muscle tone and difficulty sucking or swallowing. Symptoms typically develop 18–36 hours after ingestion (Rawson et al., 2023). Most cases are associated with exposure to untreated natural foods. Honey and corn syrup, for example, have frequently been found to contain high concentrations of *C. botulinum* spores (Salaria et al., 2024). Additional sources include contaminated powdered infant formula and household dust (Hebishy et al., 2024). First recognized in 1976, infant botulism has since become the most common form of human botulism in the United States, according to the Centers for Disease Control and Prevention (Harris et al., 2024).

Wound botulism

Wound botulism, first formally described in 1951, occurs when *Clostridium botulinum* spores contaminate a wound and subsequently produce botulinum neurotoxin (BoNT), which is

absorbed into the bloodstream (Chimienti et al., 2025). The anaerobic conditions within a wound—particularly in abscesses—create an ideal environment for spore germination and bacterial growth (Fachi et al., 2024). Because toxin production takes place locally within infected tissue, the onset of symptoms is generally slower than in other forms of botulism, typically appearing between 4 and 14 days after contamination (Fachi et al., 2024). Historically, wound botulism was rare and most often associated with traumatic injuries. However, its incidence has risen significantly with the increasing prevalence of injection drug use (Kuehn, 2019). A notable outbreak occurred in the late 1980s and 1990s in the state of California, where numerous cases were linked to the injection of black tar heroin (Mars et al., 2024).

TeNT

Tetanus is a severe toxic infection caused by the obligate anaerobe *Clostridium tetani*. When spores enter a wound, they proliferate under anaerobic conditions and produce two toxins: tetanolysin and tetanospasmin (Pal et al., 2024). Tetanolysin damages surrounding tissues and lowers the local redox potential, creating conditions favorable for bacterial growth. Tetanospasmin is responsible for the clinical manifestations of the disease. After being released, tetanospasmin spreads through local tissues and enters the bloodstream, from where it diffuses to nerve terminals throughout the body (Kiron et al., 2024). The circulating toxin does not cross the blood-brain barrier directly, except at the fourth ventricle (Susa et al., 2024). The toxin binds to gangliosides on nerve terminal membranes, is internalized, and transported retrogradely along axons to the neuronal cell body at a rate of 75–250 mm per day (Guo, 2023). This transport to the central nervous system (CNS) typically takes 2–14 days. From the cell body, the toxin spreads trans-synaptically to presynaptic inhibitory neurons (Wang et al., 2023), where it blocks the release of γ-aminobutyric acid (GABA) and glycine, the primary inhibitory neurotransmitters (Kiron et al., 2024). The α-motor neuron interneurons are the first to lose inhibitory control (Yildiz et al., 2025), while preganglionic sympathetic neurons in the lateral horn and parasympathetic centers are affected similarly, leading to autonomic dysfunction that develops days after initial muscle spasms (Roche et al., 2024). At the molecular level, tetanospasmin is synthesized as a 150 kDa single-chain polypeptide with three domains connected by protease-sensitive loops (Kiron et al., 2024). Activation occurs through selective proteolytic cleavage, producing a light (L) chain linked by a disulfide bond to a heavy (H) chain (Wang et al., 2023). The H-chain's amino terminus mediates cell penetration, while the carboxyl terminus directs specific neuronal binding (Mallesh et al., 2024). Once inside inhibitory neurons, the disulfide bond is reduced, releasing the L-chain to act presynaptically by cleaving synaptobrevin, a protein essential for vesicular neurotransmitter release (Overhoff, 2021). Inhibition of glycine and GABA release produces the characteristic symptoms of tetanus (Kiss et al., 2025). Although tetanospasmin also reduces acetylcholine release at the neuromuscular junction—similar to botulinum toxin—this effect is generally masked by predominant α-motor neuron inhibition, resulting in muscle rigidity and spasms rather than weakness, except in cephalic tetanus (Nevsky et al., 2025). Autonomic dysfunction results from the slower transport of toxin in autonomic nerves, leading to sympathetic and parasympathetic overactivity (Banerjee et al., 2023; Roche et al., 2024). This includes a basal increase in sympathetic tone and episodes of intense hyperactivity mediated by both α- and β-receptors, with noradrenaline surges up to ten times normal basal levels, comparable to those seen in pheochromocytoma (Wang et al., 2023). Contributing factors may include increased thyroid hormone release and inhibition of endogenous opiates (Hagberg et al., 2022). Neuronal binding of tetanospasmin is considered irreversible. Recovery relies on the regeneration of new nerve terminals, which explains the prolonged duration of clinical symptoms (Kumar & Singh, 2025). The hallmark clinical features of tetanus are muscle rigidity, muscle spasms, and autonomic dysfunction, with autonomic

symptoms typically appearing days after the onset of spasms (Roche et al., 2024).

Molecular Mechanisms of Clostridial Neurotoxins toward the Targeted Neuronal Cells

After crossing the intestinal barrier, botulinum neurotoxins (BoNTs) enter the extracellular space, with some molecules reaching the general circulation via blood or lymph (Cheng et al., 2019). Only a small fraction of BoNTs ultimately reach their target neurons to induce the clinical symptoms of botulism, and the precise pathways of toxin dissemination remain incompletely understood. Locally, BoNTs interact with the enteric nervous system (ENS), although not all neurons within the ENS are susceptible to BoNTs (Fujinaga & Popoff, 2018). Systemic dissemination through blood and lymph enables BoNT molecules to reach peripheral nerve endings (Poulain & Popoff, 2019). An alternative hypothesis suggests that BoNTs may also spread via axonal transport, similar to tetanus neurotoxin (TeNT) (Poulain & Popoff, 2019). This is supported by the typical descending flaccid paralysis observed in botulism, which begins with cranial nerve involvement. If blood circulation were the primary route, BoNTs would likely distribute more uniformly to all neuromuscular junctions, producing generalized symptoms (Chimienti et al., 2025). Evidence also shows that BoNT/A can undergo retrograde transport to motor neurons and the central nervous system, followed by transcytosis to afferent neurons (Marinelli, 2025). Functionally, BoNTs primarily target motor neuron terminals at neuromuscular junctions, whereas TeNT exclusively uses motor neurons to reach and act on central nervous system neurons via a transcytotic pathway (Marinelli, 2025). Despite these differences in targeting, both toxins share a similar molecular mechanism. In A–B-type toxins, the heavy chain functions as the receptor-binding domain, while the light chain serves as the intracellular enzymatic domain (Jiang et al., 2024). Over two decades ago, a three-step intoxication model was proposed: receptor binding and endocytosis, translocation into the cytosol, and enzymatic cleavage of SNARE proteins, which disrupts neurotransmitter release (Zangh et al., 2024).

Binding to the Neural Cells

The initial step in the action of clostridial neurotoxins involves binding to neural cells. The C-terminal portion of the heavy chain (HC), referred to as HC, serves as the receptor-binding domain. Structural studies from X-ray crystallography divide HC into two distinct folds: the carboxyl-terminal β-trefoil (HCC) and the amino-terminal lectin-like jelly roll (HCN) (Davies et al., 2018). HCC exhibits high sequence variability among tetanus neurotoxin (TeNT) and different botulinum neurotoxin (BoNT) serotypes, whereas HCN is highly conserved (Zangh et al., 2024). Clostridial neurotoxins initially bind to gangliosides on neuronal membranes, preferentially to the b-series (Cai et al., 2021). Specifically, gangliosides GT1b, GD1b, and GD1a have been shown to interact with both TeNT and BoNTs (Cai et al., 2021). The ganglioside-binding sites are located on the HCC domain. Cocrystallization studies have identified a conserved SxWY motif within the WY loop of HCC as the primary ganglioside-binding site (Flood et al., 2024). This SxWY motif is conserved in TeNT and BoNT serotypes A, B, E, F, and G (Masuyer et al., 2025). Although SxWY is not conserved in BoNT/C and D, structural studies suggest analogous loops in these serotypes serve as ganglioside-binding pockets (Cai et al., 2021). Additional research has identified a second ganglioside-binding site, a sialic acid–binding pocket, in TeNT, BoNT/C, and BoNT/D (Liu et al., 2023). Binding between gangliosides and the toxins is relatively weak (Liu et al., 2023), but the widespread distribution of gangliosides on neuronal surfaces may facilitate toxin accumulation, allowing subsequent stronger and more specific interactions with protein receptors (Popoff, 2024). For BoNTs, the protein receptors are synaptotagmin (SYT) and synaptic vesicle glycoprotein 2 (SV2) (Chang et al., 2024). The protein receptor for BoNT/C has not yet been identified, though it may involve other synaptic vesicle structures (Schenck et al., 2025).

Structural studies show that protein receptors also interact with HCC (Chang et al., 2024). A ternary crystal structure of the HC/B–SYT–ganglioside complex revealed that the ganglioside-binding site and the protein receptor-binding site are spatially distinct, supporting the double-receptor hypothesis in which the toxin simultaneously engages both anchor points (Cai et al., 2021). The role of the HCN domain appears to involve membrane interactions. HCN can bind to sphingomyelin-enriched membrane microdomains and may help prime the translocation domain to adopt an insertion-competent orientation within the neuronal membrane (Schenck et al., 2025).

Internalization and Translocation

Clostridial neurotoxins target cytosolic SNARE proteins, so their catalytic domain must reach the cytosol of nerve cells to block neurotransmitter release (Popoff, 2024). These toxins do not penetrate the cell directly through the plasma membrane; instead, they are internalized via endocytosis into acidic intracellular compartments (Popoff, 2024). Electron microscopy studies indicate that, following binding, the toxins enter the lumen of vesicular structures in a temperature- and energy-dependent manner (Wang et al., 2022). In murine spinal cord neurons, the heavy chain (HC) receptor-binding domain alone is sufficient to mediate this internalization process (Viravathana et al., 2024).

Retrograde of Clostridial Neurotoxins to the CNS

Botulinum neurotoxins (BoNTs) primarily act at motor neuron terminals at the neuromuscular junction. In contrast, tetanus neurotoxin (TeNT) can enter both motor and sensory neuron endings and undergo retrograde transport along the axon. Once transported, TeNT is transcytosed and delivered to central inhibitory neurons (Surana et al., 2018). Similar to BoNTs, TeNT binds to gangliosides on the neuronal membrane and also interacts with an as-yet-uncharacterized glycophosphatidylinositol (GPI)-anchored protein as its protein receptor (Tian & Zhou, 2023). Although synaptic vesicle proteins SV2A and SV2B have been reported to facilitate TeNT entry into cultured hippocampal and cortical neurons, direct interactions with TeNT remain unconfirmed. TeNT is internalized through clathrin-mediated endocytosis at both motor and sensory neuron endings (Cai et al., 2021). This internalization relies on a specialized subset of clathrin adaptors that direct the toxin into non-acidified endosomal compartments, preventing premature translocation of the L chain into the cytoplasm (Cai et al., 2021). The heavy chain (HC) of TeNT has been implicated in driving retrograde transport; however, recent evidence indicates that the full-length TeNT protein is required for efficient transport, suggesting that regions outside the HC also contribute to intracellular trafficking (Zhou et al., 2022). Small GTPases Rab5 and Rab7 act sequentially to control TeNT HC retrograde transport in motor neurons (Krzystek et al., 2023), in a pathway shared with neurotrophic factor receptors such as p75^NTR^ and TrkB, and the ligand BDNF, which depends on both Rab5 and Rab7. Retrograde transport occurs via neutral pH endosomes, similar to the mechanism used by neurotrophic signaling endosomes (Tian & Zhou, 2023). Understanding TeNT retrograde transport is essential not only for elucidating its mode of action but also as a model for studying axonal retrograde trafficking of signaling endosomes (Sleigh et al., 2020).

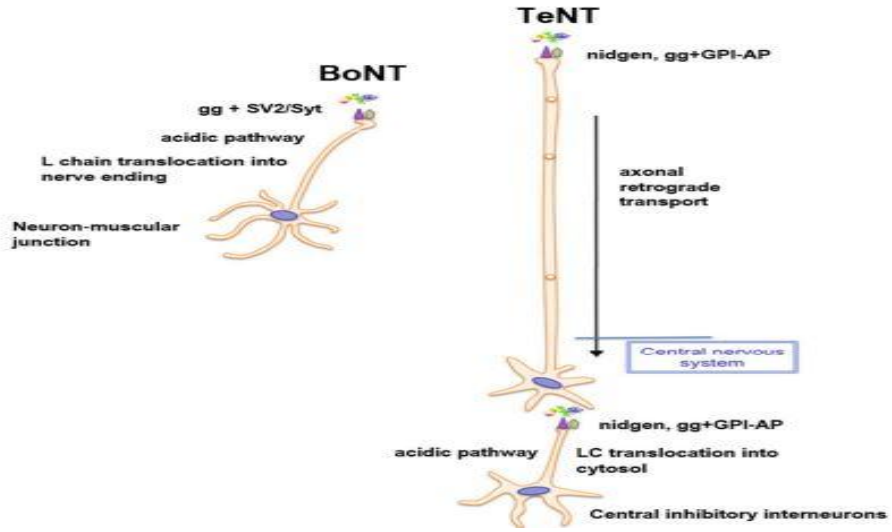


Fig.3: retrograde movement of TeNT. Source: (Popoff 2024).

Enzymatic Activity

Once inside the cytosol of target neurons, the light chain (LC) of clostridial neurotoxins binds to and cleaves SNARE proteins, either on the synaptic vesicle membrane (v-SNARE, e.g., synaptobrevin/VAMP) or on the plasma membrane (t-SNARE, e.g., SNAP-25 and syntaxin). Cleavage of these SNARE proteins prevents formation of the SNARE complex, thereby blocking vesicle docking and inhibiting neurotransmitter release at the synaptic cleft (Kumar & Singh, 2025). The LC of both botulinum neurotoxins (BoNTs) and tetanus neurotoxin (TeNT) contains a highly conserved 20-residue segment, including a HEXXH zinc-binding motif essential for catalytic activity (Cai et al., 2021). Unlike many zinc metalloproteases, clostridial neurotoxins are highly substrate-specific: each serotype targets distinct SNARE proteins or unique cleavage sites on the same substrate. For example, TeNT shares its cleavage site with BoNT/B (Gregory & Acharya, 2023). Specifically, BoNT/A, /E, and /C cleave SNAP-25, while BoNT/C also targets syntaxin. BoNT/B, /D, /F, /G, /H, /X, and /En cleave synaptobrevin/VAMP. The specificity of LC relies on recognition of precise peptide bonds within the SNARE substrate. Although the catalytic efficiency of LC is modest—for instance, BoNT/A LC has a kcat/KM of 10^4–10^6 $s^{-1}M^{-1}$ with SNAP-25 fragments >61 residues (compared to acetylcholinesterase at 1.5×10^8 $s^{-1}M^{-1}$)—the toxin is extremely potent. It is estimated that as few as 1,000 BoNT molecules can completely block neurotransmission in a muscle (Hanig & Lamanna, 2019). The resulting paralysis can last weeks to months, depending on the BoNT serotype. BoNT/A causes the longest-lasting paralysis (3–6 months in humans), with BoNT/C showing a similar duration and potential as an alternative therapeutic agent (Rasetti-Escargueil & Palea, 2024; Ayoub, 2025). BoNT/B induces paralysis for 12–16 weeks, BoNT/E for 2–7 weeks, and BoNT/D and /F have durations comparable to BoNT/B (Pons et al., 2019; Moreau et al., 2025). Importantly, BoNT-induced paralysis is reversible, as the toxin blocks neurotransmitter release without killing neurons (Pons et al., 2019). The unusually long duration of action, despite the typical half-life of intracellular proteins (~1.5–48 hours), is influenced by two factors: the intracellular degradation pathway of the LC and the persistent effect of cleaved SNARE substrates on synaptic vesicle docking and exocytosis (Liu, 2024).

Laboratory Diagnosis

TeNT

Table summarizing the four methods for detecting *Clostridium tetani* or tetanus antibodies**:**

Method	Sample/Specimen	Principle	Key Details	Reference
Culture	Wound swabs	Isolation and growth on selective media	Colonies show characteristic "drumstick" morphology; confirmed by Gram stain, swarming, and biochemical tests (gelatin hydrolysis, nitrate reduction, starch hydrolysis, H_2S, DNase, lipase, lecithinase)	Kumurya, 2019; Bukar et al., 2008
Tetanos Quick Stick (TQS)	Finger-prick blood	Immunochromatographic test for anti-tetanus antibodies	Blood + diluent flows along strip; complexes with toxoid-gold conjugate bind to immobilized toxoid → pink line in "T" window if antibodies present; control line "C" ensures validity; read after 20 min; lab TQS and serum TQS performed within 6 h	Colombette et al., 2023
Molecular (PCR)	Bacterial colonies	Detection of *TetX* gene	DNA extracted by boiling colonies; PCR using Tet-F and Tet-R primers; amplicons visualized on 1% agarose gel	Bruggemann, 2005
Serology (ELISA)	Serum or blood	Quantitative detection of IgG antibodies against tetanus toxoid	Microplate coated with tetanus toxoid; calibrated with WHO standard; performed blind to TQS results	Colombette et al., 2023

BoNT

Table summarizing the main laboratory diagnostic methods for botulism:

Method	Sample/Specimen	Principle	Key Details	Reference
Culture	Implicated food, stool, or wound tissue	Isolation of *Clostridium botulinum*	Confirms presence of BoNT-producing bacteria; requires appropriate selective media and incubation	Kumurya, 2019; Rao et al., 2021
Mouse Bioassay	Food, stool, serum, or wound extracts	Detection of biologically active BoNT	Patient sample injected into mice; development of botulism symptoms in 24–48 h indicates	Solomon et al., 2001; Lindström & Korkeala,

Method	Sample/Specimen	Principle	Key Details	Reference
			toxin presence; antitoxins identify serotype. Highly sensitive (5–10 pg/mL) but costly, time-consuming, and ethically challenging	2006; Taylor, 2019
Molecular (PCR)	Food, stool, serum, or bacterial isolates	Detection of BoNT gene sequences (bont A–G)	Rapid results within hours; confirms presence of toxin gene but cannot detect active neurotoxin	Kakinuma et al., 1997; Chellapandi & Prisilla, 2018

Notes for Sample Handling:

- Collect specimens as early as possible after suspicion.
- Store samples at 2–8 °C to avoid false negatives.
- Serum should be collected before antitoxin therapy; stool can be collected post-antitoxin but preferably before antibiotics.

Therapeutic approach

BoNT

Treatment of Botulism

1. Antitoxin Therapy

- The primary treatment for botulism is **heptavalent botulinum antitoxin (HBAT®),** which contains neutralizing antibodies against all seven BoNT serotypes (A–G).
- HBAT® has replaced the older **trivalent antitoxin** used in the US and UK, which only neutralized serotypes A, B, and E. Some trivalent formulations are still in use in certain European countries.
- HBAT® is derived from **equine serum,** obtained from horses hyperimmunized with toxoid strains of each BoNT serotype. Recombinant toxin subunit fragments have also been proposed to produce safer and equally effective antitoxins.
- HBAT® was **FDA-approved in 2013** in the US and is used off-label in the UK (Yu et al., 2018; Barker et al., 2019; FDA, 2022).

Mechanism of Action

- The antitoxin binds and neutralizes **circulating BoNT in the bloodstream** by preventing the heavy-chain receptor from binding to presynaptic receptors.
- It **cannot reverse toxin already internalized** into neurons.
- Early administration, ideally within **48 hours of symptom onset**, reduces hospital stay and the intensity of care needed (Lonati et al., 2020; Ben et al., 2022).

2. Supportive Care

- Patients with suspected botulism should be managed in an **intensive care unit**, due to the high risk of respiratory muscle involvement.

- **Elective intubation and mechanical ventilation** should be considered in patients at risk of respiratory failure.
- **Gastric decontamination** (lavage or induced emesis) may be used if contaminated food was ingested recently.
- **Wound botulism** requires prompt **surgical debridement** and **antibiotic therapy**.
- Drugs that **block neuromuscular transmission** (e.g., aminoglycosides, magnesium-containing compounds) should be avoided (Cherry, 2023; Root et al., 2024; Huglum et al., 2024).

TeNT

Wound Management and Antibiotic Therapy in Tetanus

- **Surgical Wound Care:**
  - Wounds that serve as the site of *Clostridium tetani* infection should be **surgically debrided** to remove necrotic tissue and reduce bacterial load.
  - **Antibiotic therapy** should be administered alongside surgical care (Boer et al., 2024; Radkowski et al., 2025).

- **Choice of Antibiotic:**
  - **Penicillin** has historically been the most commonly used antibiotic worldwide.
  - However, penicillin is structurally similar to **γ-aminobutyric acid (GABA)** and can act as a **competitive GABA antagonist**, potentially causing CNS hyperexcitability or convulsions at high doses.
  - In tetanus, penicillin's GABA-blocking effect may **synergize with tetanospasmin**, worsening inhibition of inhibitory neurons.
  - **Metronidazole** is therefore preferred, as it avoids these CNS effects and has been shown to be **superior to penicillin** in human studies (Olseen et al., 2024; Huang et al., 2025).

- **Duration and Alternatives:**
  - Antibiotics should generally be given for **7–10 days**.
  - Alternative options include **erythromycin, tetracycline, vancomycin, clindamycin, doxycycline, and chloramphenicol (**Lee et al., 2021; Kiron et al., 2024; Wang et al., 2025**)**.

CONCLUSION

Bacterial toxins function like precise molecular machines, with each domain and folding pattern tailored to a specific role. Clostridial neurotoxins are a prime example of this design. The remarkable diversity of botulinum neurotoxins likely arose through horizontal gene transfer over billions of years of evolution. These neurotoxins are exquisitely structured to recognize receptors, translocate their catalytic light chain (LC) into target cells, and selectively cleave their substrates. At every step, the folding and domain flexibility of the toxin adapts to fulfill its function. Such activation and structural flexibility are common among Gram-positive bacterial toxins, suggesting they share ancient evolutionary origins. Protein folding is critical for biological function and evolves more slowly than genetic sequences, offering valuable insights into natural selection. In clostridial neurotoxins, molten globule–type flexible structures are essential for activity, yet studying these dynamic conformations is challenging. High-resolution techniques like X-ray crystallography may not fully capture their flexibility, necessitating multidimensional approaches in solution to understand their functional dynamics. A deeper understanding of these highly dynamic structures is crucial not

only for designing effective antidotes against bacterial toxins but also for harnessing them as therapeutic agents for various medical conditions.

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