Chapter 2
Basics of Structure and Mechanisms of Function of Botulinum Toxin - How Does it Work?

Introduction

Botulinum toxin or botulinum neurotoxin (BoNT) is a protein which is produced by a bacteria named clostridium botulinum. The term clostridium refers to the shape of the bacteria which is spindle/rod shaped and the term botulinum is derived from the Greek word of botulus (sausage) since earlier outbreaks of botulism were related to the consumption of rotten sausage. The history of early botulism outbreaks, discovery of the responsible agent, purification and production of the toxin for medical research as well as early clinical trials which led to discovery of BoNT's effectiveness in treatment of medical disorders are presented in detail in Chap. 1. This chapter focuses on an explanation of how this toxin work.

The results of animal research and early human observations which emerged during 60s and 70's, indicating a significant therapeutic potential for BoNT, encouraged basic scientists to explore the molecular structure of the toxin and its mode of action. These efforts succeeded to decipher the exact molecular structure of BoNT and provide a large amount of knowledge about how the toxin molecule reaches the nerves and exerts its therapeutic action after it is injected into the site of concern.

Botulinum toxin is structurally a protein with perfect machinery to exert its function through a set of well –defined mechanisms. There are 7 distinct types of botulinum toxins (A,B,C,D,E,F,G) that are structurally similar with only minor differences. Types A, B,E and F can cause botulism in human, whereas, types C and D mainly cause botulism in domestic animals [1]. Recently, several subtypes have been discovered (A1, A2,…) [2]. Continued research efforts are underway to define the role of these subtypes. Currently, only types A and B are suitable for clinical use.

Botulinum toxin molecule (type A) is an approximately 900 KiloDalton (KD) complex which consists of a core toxin (150KD) and a complex of surrounding proteins (>700 KD). Dalton the unified atomic mass unit, is a standard unit of mass that quantifies mass on an atomic or molecular scale. The surrounding proteins of the core toxin protect the toxin from being degraded in a hostile environment such

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as acid of the stomach after its ingestion. However, when the BoNT is injected into a muscle, the tissue enzymes (protease) quickly separate the toxin from the surrounding proteins by a process termed "nicking". The core toxin molecule then reaches its target at nerve endings probably via blood or lymphatic system [3].

The point where a nerve connects to a muscle is called neuromuscular junction. The point where the end of a nerve (nerve terminal) contacts a muscle is also called synapse in medical terms. In case of nerve-muscle synapse, the synapse has a membrane on the nerve side and a membrane on the muscle side with a cleft in between (synaptic cleft) (Fig. 2.1). The nerve ending close to the muscle contains many small vesicles that contain a chemical called neurotransmitter. When nerve's electrical signals reach the nerve ending the vesicles rupture and pour their neurottansmitter contents into the synaptic cleft . The neurotransmitter then attaches itself to the muscle membrane and activate (contracts) the muscle. The neurotransmitter in the nerve-muscle junction is a chemical called acetylcholine. Injected Botulinum neurotoxins can relax, weaken or even paralyze the muscle (depending on the dose) by preventing release of acetylcholine from the synaptic vesicles. The mechanism through which BoNT exerts its effect on nerve-muscle junction is complex and requires some knowledge of core toxin's molecular structure.

Each molecule of the toxin consists of two structures, called light chain (50 KD) and heavy chain (100 KD). KD stands for kilodalton. Dalton is the unit of atomic weight. These two chains are connected by a disulfide bond (Fig. 2.2).

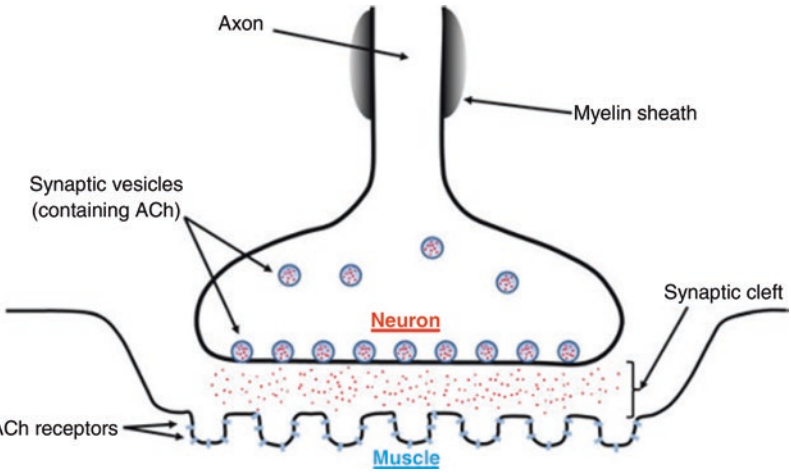

Fig. 2.1 Neuromuscular junction: Nerve and nerve terminal, muscle fiber, and synaptic cleft between. Nerve terminal shows vesicles that contain the neurotransmitter acetylcholine. Nerve signals reaching the nerve terminal at neuro-muscular junction lead to rupture of the vesicles and release of acetylcholine molecules into the synaptic cleft. Acetylcholine molecules attach to muscle receptors on the surface of the muscle and activate the muscle

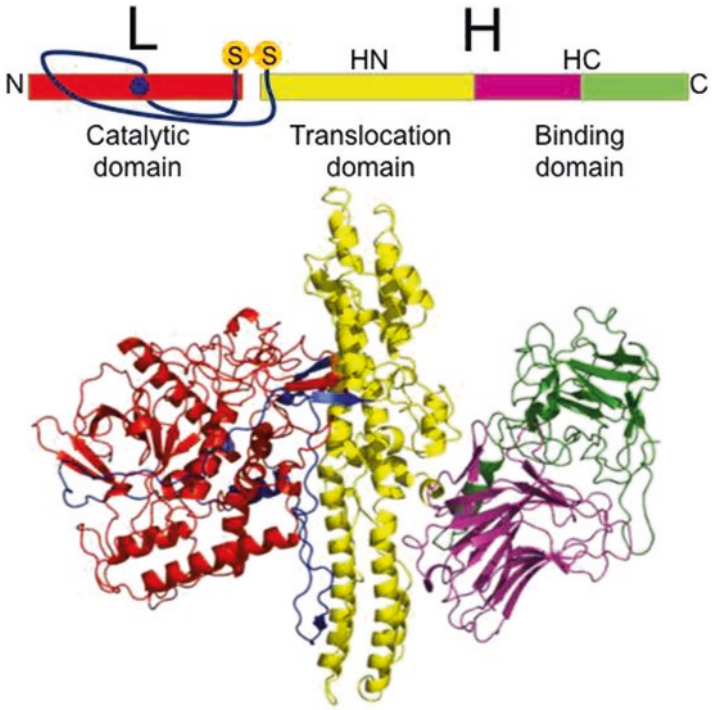

Fig. 2.2 Molecular structure of botulinum toxin. From Rossetto O (2018), in Botulinum toxin Treatment in Clinical medicine (Jabbari B -Editor) . Reproduced by permission from Publisher-Springer

The light chain, the catalytic domain, is the active moiety of the toxin. The heavy chain has two parts called HC and HN domains (Fig. 2.2). The HC domain (binding domain) attaches the toxin to the membrain receptors of the nerve cell. There are specific receptors on the nerve cell membrane that the HC domain of the toxin can attach itself to. The receptor for type A toxin is a protein called SV2. For type B toxin, two receptors have been identified a ganglioside (a form of complex sugar) and a protein called synaptogamin. After the toxin attaches to the receptor, the receptor undergo structural modification and works like a channel letting the toxin go through. The HN domain (translocation domain) of the toxin then moves the whole toxin molecule inside the nerve cell terminal through the chanelled receptor. After entering the nerve terminal, the disulfide bond of BoNT breaks and the two chains of the toxin separate from each other. The light chain (active moiety of the toxin) is now free to exert its effect and prevent the release of acetylcholine from the synaptic vesicles. It does this via attaching itself to specific synapse proteins whose function is to promote the fusion of the vesicle onto the nerve membrane. Vesicle fusion to the synapse membrane leads to its rupture and release of the neurotransmitter, acetylcholine into the synaptic cleft. The synapse proteins that promote vesicular fusion and rupture are called SNARE (Soluble NSF Attachment Protein).

Over the past 30–40 years, a group of cell biologists succeeded to determine the mechanisms of vesicle fusion and synaptic machinery including the function of SNAREs. [4] Most notable among these scientists are J.Rothman, R.Schekman and TC.Sudhof who won the Nobel prize in Medicine & Physiology in 2013 for their work in this area, (Fig. 2.3).

While inside the nerve terminal and detached from the heavy chain, the light chain of the BoNT attaches itself to a specific SNARE that attracts that specific type of the toxin (for instance type A or B). After attachment to the SNARE protein the light chain of the toxin deactivate the SNARE protein via light chain's enzymatic function (a Zinc activated protease). The result is inhibition of release of the neurotransmitter from the vesicle and, in case of nerve-muscle synapse, relaxation, weakness or even paralysis of the muscle depending on the dose of the injected toxin. The SNARE for Type A toxins (Botox, Xeomin, Dysport) was first discovered by a group of Yale investigators and named SNAP 25. [5] It is attached to the membrane of the nerve terminal. For the type B toxin, the SNARE is attached to the vesicle wall itself and is designated as Synaptobrevin. (Table 2.1)

The binding of the BoNTs A and B to the nerve terminal is a long-term binding that in case of nerve- muscle junction lasts for 3–4 months [6]. This long period of binding is medically desirable. For instance in spastic and tense muscles of patients with stroke or children with cerebral palsy, one injection could maintain the muscle relaxation for the entire period of binding. Over time, the nerve ending starts to sprout and the new endings make contact with different muscle fibers. Finally when the binding is over the synapse resumes its full function. This reversibility which is the hallmark of BoNT function is very different from the disease conditions that often destroy the synapse and lead to neurodegeneration and often perment loss of function.

Fig. 2.3 Dr. James Rothman, Yale Cell biologist who won the Nobel prize in physiology and Medicine in 2013 for his work on physiology of the synapse

Table 2.1 Sequence of Botulinum toxin action after injection into the muscle

1.	After injection in to the muscle, protease, an enzyme inside the muscle separates the core toxin from protective proteins around the core toxin
2.	The released toxin molecule reaches nerve muscle junction probably via blood or lymphatic system
3.	Heavy chain of the toxin attaches the toxin molecule to certain receptors on the surface of terminal nerve ending (SV2 for Botox)
4.	Receptors open as a channel and let the toxin molecule enter into the nerve terminal
5.	The disulfide bond of the toxin break inside of the nerve terminal via function of heavy chain
6.	Freed light chain of the toxin (active or catalytic moiety) reaches the SNARE proteins and deactivate them via its enzymatic function
7.	Deactivation of SNARE protein prevents rupture of synaptic vesicles and release of acetylcholine
8.	Muscle deprived from acetylcholine activation relaxes and slightly weakens, an effect that improves muscle spasms, abnormally high muscle tone (spasticity) and involuntary movements.

Excessive Sweating and Drooling

The nerves exciting sweat, tear and salivary glands originate from the sympathetic nervous system. Acetylcholine is also the neurotransmitter for the sympathetic nerve endings that supply nerves to sweat and salivary glands. BoNT injections into and under the skin in the areas where these glands are located (for instance arm pit, hands and feet for sweat glands or face for salivary glands) effectively reduces sweating and drooling (Chap. 13 of this book). The injections can be very helpful in patients with excessive sweating on the hands or feet or at the arm pit. Also patients with excessive drooling may do well when botulinum toxins (type A or B) are injected into the salivary glands. The parotid glans is just under the skin above the angle of the jaw and the submandibular glands are under the jaw at the junction of medial one third and lateral two third. For reasons which are not yet well understood, effects of BoNT over sympathetic nerves controlling salivation and drooling lasts longer than that observed in nerve-muscle junction (usually 6 months, and in some cases as long as a year, after one injection). The molecular mechanism of blockage of sweat, tear and saliva secretion is similar to that provided for the nerve-muscle junction.

Pain

This a relatively new area of BoNT indication. For migraine, the efficacy of BoNT-A (Botox) has been proven by several high quality studies [7] and Botox was approved for use in treatment of chronic migraine by FDA (2010) in the US. Further studies

have shown that BoNTs are effective in a number of other pain syndromes [8] (Chaps. 4 and 5 of the book). In case of pain, the molecules of Botox exert their effect on the sensory nerve fibers through a similar cascade of mechanisms. Animal studies have shown that injection of Botox into the muscle (intramuscular) or under the skin can block the release of several well recognized pain transmitters such as glutamate, substance P and Calicitonin gene-relater peptide (CGRP). These agents accumulate in peripheral nerve endings in reaction to noxious peripheral stimulation and through their action the abnormal sensation invoked in the peripheral nerves is conveyed to the brain and perceived as pain. Blocking the release of pain transmitters from peripheral nerve endings reduced sensitization of peripheral nerve endings and alleviates pain.

More recently, an additional "central" mechanism for the action of botulinum toxin molecules on pain has been elucidated based on animal studies. The support for a central (spinal cord and possibly brain) mechanism comes from several lines of research, two of which are described below:

1- Direct application of BoNT to dura matter (the brain covering) alleviated facial pain and reduced the inflammation caused by experimentally induced pain (ligation of a facial nerve) in laboratory animals. [9]
2- In an animal model of leg pain caused by diabetic neuropathy (nerve damage due to diabetes) injection of BoNT into one leg, not only reduced the pain in that leg but also in the other leg implying an analgesic function through a spinal cord loop with participation of spinal cord nerve cells [10].

These central mechanisms, however, do not seem to exert any deleterious effect on the spinal cord or brain (in doses approved for clinical use) since millions of patient who receive BoNT injections every year do not complain of any untoward side effects related to central nervous system.

Recently, scientists have succeeded in making a toxin molecule consisting of combination of two toxins (chimera- for instance for instance E/A toxins), that can specifically target the sensory nerve cells and hence specifically treat pain [11]. It remains to be seen how effective these chimeric molecules will work in human and in clinical practice.

The details of Botulinum Neurotoxins: Biology, Pharmacology, and Toxicology can be found in a recently published comprehensive review. [12]

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