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How does Recent Understanding of Molecular Mechanisms in Botulinum Toxin Impact Therapy?

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Abstract

Botulinum toxin, one of the most lethal toxins identified, is also used as a therapeutic agent for a variety of human conditions. The history of the discovery of botulinum toxin, an understanding of its function at the molecular level and its development into a therapeutic reagent are instructive and allow the reader to build a conceptual framework around which to understand its current therapeutic uses and consider potential further uses of botulinum toxin.

Keywords: botulinum toxin, development, biochemistry, molecular biology, therapeutics

1. History of botulinum toxin

In 1817, Justinus Kerner published for the first time a report on a lethal case of food poisoning from eating spoiled sausages. He proposed that a biological poison was the culprit [1]. Subsequently, Dr. Kerner published two monographs of his observations on “sausage poisoning.” In 1820, he described 76 cases in great detail [2]. Two years later, he described another 155 cases and also provided the first accurate and detailed description of the neuromuscular symptoms of botulism [3]. Dr. Kerner also conducted experiments in animals: cats, rabbits and birds that were given “spoiled sausage extracts.” Dr. Kerner observed that motor neurons were affected in the treated animals, resulting in paralysis, while brain and sensory neurons were largely unchanged. Dr. Kerner went so far as to ingest botulinum neurotoxin (BoNT) himself and record the effects. He experienced difficulty in controlling his eyes, constipation and dryness in both

the palms of his hands and soles of his feet [1]. From his clinical observations and animal experiments, Dr. Kerner concluded that this biological toxin could be used for medical purposes in those with disease resulting from an overly excited nervous system, such as St. Vitus's dance (chorea), excessive mucous production, or excessive sweating.

In 1897, Emile Pierre van Ermengem identified and cultured the bacteria that produced botulinum toxin. After an outbreak of botulism that resulted from the ingestion of contaminated ham, van Ermengem associated the growth of anaerobic bacteria on the meat with botulism [4, 5]. He isolated and cultured the anaerobic bacteria and then prepared a toxin extract. He performed and described many animal experiments and correctly deduced that the bacteria themselves do not cause food-borne botulism, but rather produce a toxin that after ingestion causes the illness. Subsequently, the toxin was named botulinum neurotoxin (BoNT) and the bacteria that produce it were named *Clostridium botulinum*. In 1949, Burgen and colleagues demonstrated that BoNT acts by blocking the release of acetylcholine at the neuromuscular junction [6].

2. Molecular biology and biochemistry

To date, seven different serotypes of BoNT have been identified, designated A–G. Four of the BoNT serotypes (A, B, E and F) cause human botulism, a neuroparalytic disease that may be fatal without proper diagnosis and treatment [7]. Using the tools of molecular biology, the serotypes have been studied in greater detail in order to understand their potential lethality and identify those serotypes with greater therapeutic and less lethal effect.

The genes encoding the BoNT serotypes have been isolated from multiple strains of *C. botulinum*. Six of the serotypes have several subtypes that differ significantly at the amino acid level [8–11]. Sequence comparison has identified eight different subtypes of BoNT/A, designated A1–A8 that vary in amino acid sequence. Similarly, sequence comparison has identified seven different subtypes of BoNT/B [12]. While these data were obtained to help with future identification of the source of botulism outbreaks, the sequence differences may be exploited to develop safer therapeutic forms of BoNT.

Each BoNT isoform is synthesized as a single, inactive polypeptide with a molecular mass of approximately 150 kDa. The precursor protein is cleaved by proteases into a 50-kDa light chain (LC) and a 100-kDa heavy chain (HC) that are linked by a disulfide bridge. The toxicity of BoNT is mediated by four distinct steps: binding to the presynaptic neuron, internalization in the neuron, translocation of the LC into the cytosol and proteolytic cleavage of a target protein. The initial binding sites of BoNT on nerve cells are polysialogangliosides [13–15]. While the exact mechanism is unknown, current models suggest that the polysialogangliosides serve to bring the BoNT out of the fluid phase and into the plane of the cell membrane. The carboxyl terminal of the HC chain of the BoNT then mediates binding to the exterior cell surface, presumably via an as-yet unidentified protein receptor [16]. Endocytosis, a temperature- and energy-dependent process, results in internalized BoNT that resides in a vesicle within the

cytoplasm. Translocation then begins with the acidification of the vesicle lumen. Acidification results in conformational changes in the BoNT protein that facilitate translocation across the vesicle membrane and into the cytosol. During translocation, the disulfide bond between the heavy and light chains is broken and the two chains separate [11, 17]. The unbound LC is then able to express its catalytic activity in the cytosol [17, 18].

The LC portion of the BoNT peptide cleaves specific target proteins of the SNARE complex. BoNT/A and BoNT/E cleave the plasma membrane-associated protein SNAP-25 (synaptosome-associated protein of 25 kDa), whereas BoNT types B, D, F and G cleave synaptobrevin, a vesicle-associated membrane protein, also known as VAMP. The SNARE complex is composed of multiple members of a large protein super family that plays an essential role in the fusion of vesicles to their target membrane. In neurons, the SNARE complex is critical in mediating the docking of synaptic vesicles with the presynaptic membrane [19–21]. Thus, cleavage of a critical protein in the SNARE complex results in decreased vesicle fusion with the presynaptic membrane. The result is reduced neurotransmission. When BoNT is injected into the skeletal muscle, in the vicinity of the neuromuscular junction, the presynaptic neuron is unable to release acetylcholine. The result is a decreased stimulus indicating that the muscle should contract and muscle relaxation ensues.

3. Therapeutic development

It was not until the mid-twentieth century that scientists developed standardized methods for the production and stabilization of BoNT serotype A (BoNT/A) [22–28]. Thanks to these efforts, Alan Scott was then able to demonstrate that BoNT/A could treat strabismus in monkeys by injecting toxin into their extra ocular muscles [29]. This was followed by successful trials in humans with strabismus [30]. Since the 1980s, BoNT/A has been used to treat neuromuscular hyperactivity disorders, as first conceived by Justinus Kerner in the early nineteenth century. The original batch of BoNT/A, purified for human use, was registered under the name Oculinum. In 1991, the company and 125 mg of the originally purified BoNT/A were sold to Allergan (Irvine, CA). The drug was given the trade name of Botox and the initial 125 mg of toxin was the exclusive source of drug until 1998, when a continuous manufacturing process was put into production [31].

Currently, BoNT serotypes A1 and B are available for therapeutic use. The toxin is injected into the immediate vicinity of nerve endings for a host of therapeutic purposes, including the alleviation of focal dystonia, spasticity, hyperhidrosis, prophylactic migraine treatment and reduction of glabellar lines. In all of these conditions, the interruption of cholinergic neurotransmission results in the desired therapeutic effect. For the treatment of dystonia or spasticity, for example, BoNT/A has been shown to have effects on both extrafusal fibers, which compose skeletal muscle and are innervated by acetylcholine released at the neuromuscular junction and intrafusal fibers, which are innervated by acetylcholine released by gamma motor neurons at contractile ends and thus serve as a sensory proprioceptor [32]. Extrafusal fibers are integral to muscle contraction and thus are affected in focal dystonia. Intrafusal fibers monitor the

velocity of a muscle stretch and thus play a role in mediating skeletal muscle spasticity. By reducing cholinergic transmission to extrafusal and intrafusal fibers, both dystonia and spasticity can be reduced. For the treatment of hyperhidrosis, intradermal injection of BoNT/A in the axilla results in reduced cholinergic neurotransmission from the sympathetic fibers that innervate the eccrine glands. For the prophylactic treatment of chronic migraine, the mechanism by which the intramuscular injection of BoNT/A, following a fixed pattern of injection sites, results in reduced headache frequency is unknown.

Three different brands of BoNT/A1 are available in the United States: onabotulinum toxinA (Botox), incobotulinum toxinA (Xeomin) and Abobotulinum toxinA (Dysport). In addition, one form of BoNT/B is available, rimabotulinum toxinB (Myobloc). There are a limited number of comparative studies between the different brands. However, two distinct studies, comparing the effectiveness of onabotulinum toxinA to rimabotulinum toxinB in cervical dystonia subjects, indicate that both serotypes are equally efficacious with a possibly slightly longer duration of action in those treated with onabotulinum toxinA [33, 34]. Thus, in the treatment of cervical dystonia, differences between serotypes and subtypes of BoNT may not produce a significant difference in therapeutic effect. For the treatment of axillary hyperhidrosis, a recent study found that both onabotulinum toxinA and rimabotulinum toxinB were equal in the onset of action, the duration of action and therapeutic efficacy [35].

4. Future applications

With our more detailed knowledge of the mechanism of action of BoNT and better understanding of the mechanisms of various disease states, it is possible that the specificity and proteolytic activity of the various BoNT serotypes may be used for a wide variety of medical purposes. A recent publication demonstrated that modified BoNT may be targeted to inflammatory immune cells, disrupting the SNARE complex in those cells and resulting in the blockade of the release of inflammatory cytokine tumor necrosis factor [36]. In the future, the specificity of the HC portion of BoNT may be used to target a variety of agents to presynaptic cholinergic neurons. In addition, the HC portion of BoNT may be modified, to change its cellular target, to allow for the delivery of small molecules to other cell types.

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