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The Pharmacology of Botulinum Toxin Type A

Anna V. Reznik

Abstract

The aim of this chapter is to structure current information clarifying the most disputable issues of botulinum neurotoxin type A (BoNT/A) pharmacology after systemic (botulism) impact and local medical application. Botulinum neurotoxin (BoNT) pharmacological features evaluated open ways to study factors affecting its biological activity: to extend/shorten its effect duration, to increase/decrease BoNT sensitivity in specific patient populations. The chapter presents unique molecular mechanisms underlying BoNT/A pharmacokinetics and pharmacodynamics: entering the body, distribution, receptor binding, translocation, mediator release suppression, zinc metabolism as well as factors affecting body sensitivity to BoNT at each of those stages. The specific biological effects of BoNT/A, which may underlie its analgesic, anticancer and anti-inflammatory effects, are described. Botulinum neurotoxin pharmacokinetics and pharmacodynamics features discussed herein represent significant clinical relevance since they determine botulinum treatment safety and effectiveness. And also they open ways to develop both BoNT-based therapies and anti-botulinic agents.

Keywords: botulinum, neurotoxin, ganglioside, synaptic vesicle protein, fibroblast growth factor receptor, SNARE proteins, mediator release block, zinc metabolism, thioredoxin reductase - thioredoxin system, botulism, neuromuscular blockade

1. Introduction

Botulinum neurotoxins (BoNTs) are the most potent protein toxins among bacterial, animal, plant and chemical toxic compounds and are the cause of botulism [1]. However, BoNT-based therapeutics are widely used for treatment of various diseases and esthetic disorders. Various features of BoNT pharmacology holding a great promise for development of both BoNT-based therapeutics and anti-botulinic agents are currently studied. Unique molecular mechanisms underlying various stages of botulinum neurotoxin type A pharmacological activity as well as potential factors affecting body sensitivity to BoNT are described herein.

2. Neurotoxin complex and BoNT molecule structure

Botulinum neurotoxin is a protein dimer with molecular weight of 150 kDa and chemical formula C₆₇₆₀H₁₀₄₄₇N₁₇₄₃O₂₀₁₀S₃₂ consisting of two chains: light and

heavy [1]. The light chain represents approximately one third of toxin molecular weight and is bound to the heavy one with a disulfide link [2].

The light chain (L-chain) is a protease blocking synaptic release. It forms the BoNT molecule catalytic domain. The heavy chain (H-chain) consists of two domains: binding domain binds to target cell surface receptors, translocation domain is involved in light chain translocation creating cell membrane channel. The BoNT molecule is a dipole with an electric charge attenuating from the binding domain to the catalytic one [3]. It is of importance when the molecule is directed relative to cell membrane that facilitates receptor binding.

In natural settings BoNTs are synthesized by bacteria as a complex with several proteins: one non-toxic non-hemagglutinin (NTNHA) and several hemagglutinins [1].

NTNHA has a molecular weight of 130 kDa and its amino acid sequences are highly homologous to BoNT but without protease motif as the only difference. "Hand in glove"-type interaction with the BoNT molecule protects the one from aggressive effects of environmental factors including GIT proteolytic enzymes [4].

There are three classes of hemagglutinins with molecular weight of 33–35, 15–18 and 70 kDa [5]. They do not contact with the BoNT molecule directly but with NTNHA working as an adhesin molecule when such toxin complex is absorbed.

Non-toxic hemagglutinin and hemagglutinin proteins can form various multimeric complexes with BoNT called botulinum neurotoxin complexes. Each of them contains only one BoNT molecule released from the complex if medium pH changes [2].

3. BoNT absorption and distribution

BoNTs can enter human body via both injured and intact tissues. Therapeutically botulinum neurotoxin type-A (BoNT/A) based agents are mainly injected as close as possible to their target cells. However, BoNT/A forms to be applied without the need to damage skin are already under development though they are not yet through Phase III clinical studies [6, 7].

In natural settings BoNTs show systemic action causing botulism and enter the body mainly through intact membranes.

Depending on toxin mode of entry botulism forms can be classified as follows: food botulism (ingestion of BoNT-contaminated food), infant (ingestion of food with bacteria spores), inhalation (breathing-in BoNT-containing aerosols), wound (in majority of cases it is related to injectable drug use), iatrogenic [8].

In natural settings botulinum neurotoxin should cross epithelial barriers and reach general circulation to hit its target cells. Such process is called absorption.

BoNT might utilize two modes of penetrating intestinal or pulmonary epithelium: intracellular route and intercellular junction-related one.

In case of transcytosis (penetration through epithelial cell) BoNT binds to ganglioside receptors at epithelial cell surface and undergoes endocytosis (being captured in a vesicle). Transport vesicles transfer toxin through the whole cell and release it into general circulation. Neither toxin structure is altered, nor it is released in cell cytosol during transcytosis, which differentiates BoNT binding with epithelial cell from binding with neuronal ones [9, 10].

Paracellular route (through intercellular junctions) may or may not involve complexing proteins. Complexin hemagglutinins can bind to E-cadherin in epithelial intercellular junctions and disrupts the latter allowing BoNT in general circulation [4]. However, BoNT molecules are able to break epithelial barriers without complexing proteins.

Studies by Maksymowych et al. [11] and Al-Saleem et al. [12] showed that introduction of equimolar amounts of free BoNT/A and BoNT/A complexes resulted in equivalent BoNT titers in general circulation with similar toxicity and effectiveness. However, hemagglutinins are assumed to boost BoNT transportation through epithelium.

When transported through intestinal wall BoNT may bind to cholinergic and serotonergic neurons of enteric (intestinal) nervous system located in intestinal submucosa blocking gut motor and secretory activity. It explains impaired bowel movement (constipation) as one of early signs for alimentary and infant botulism [13].

BoNT penetrating epithelial barrier reaches general circulation and is distributed in all extracellular fluid compartments in the body but the ones of central nervous system.

Eisele et al. [14] had a series of experiments demonstrating that with pH values close to neutral (arterial blood pH of 7.37–7.43 [15, 16]) botulinum neurotoxin complex dissociates on active BoNT and complexing proteins with half-life below 1 minute. Once such toxin complex dissociates complexing proteins are not any more of any significance for the occurrence of the clinical effect of BoNT.

Al-Saleem et al. [17] works proved that toxin reaches general circulation without any evident structural or biological activity changes. General circulation performs as toxin storage compartment until BoNT reaches its target cells. While in general circulation BoNT undergoes slight biotransformation, it is not accumulated in blood cells and mostly remains in its free active form. Such concept of “general circulation—botulinum neurotoxin storage compartment” has been confirmed by many researchers. Fagan et al. [18] described active BoNT/A presence in human blood serum 11 days after contaminated food ingestion; Sheth et al. [19], 25 days after disease onset; Delbrassinne et al. [20], 29 days after contaminated food was taken.

From intravascular fluid compartment botulinum neurotoxin enters extravascular one and then intercellular fluid. Being locally injected with therapeutic purposes botulinum neurotoxin is directly introduced in extravascular compartment (or intravascular one if it is in a blood vessel) next to target cells bypassing absorption stage. From intercellular compartment botulinum neurotoxin should reach its target—peripheral cholinergic nerve endings and bind to receptors there.

To better understand the mechanism of botulinum neurotoxin binding with receptors knowledge of normal neurotransmission in synapses is required.

4. Normal synapse neurotransmission

Neuromediators are synthesized in neuron cytosol and then stored in pre-synaptic nerve endings within synaptic vesicles. Synaptic vesicle membrane contains proton pump (vesicular ATPase), which, when activated, increases intravesicular proton concentration [8]. Electrochemical proton gradient ensures mediator influx from cytosol and its accumulation in such vesicles. The uptake of mediators within the synaptic vesicles is also regulated by receptors on the neuronal membrane, not only by the proton gradient. Mediator-containing vesicles are located in neuron cytoplasm and are bound to specific presynaptic membrane regions (active zones [21]) during so-called docking [22]. Vesicles are docked with cell membrane in active zones only and docking is controlled by a great deal of transport proteins [23].

When a nerve impulse arrives axonal presynaptic membrane is depolarized, calcium channels open and Ca^{2+} ions flow into axon [24]. In response to Ca^{2+} influx the mediator-containing vesicle fuses with presynaptic membrane in active zone. This stage is called priming. It is regulated with two integral membrane synaptic vesicle proteins

(synaptobrevin and synaptotagmin) as well as two presynaptic membrane proteins (SNAP25 and syntaxin) and cytosol proteins including complexin [25–28].

Rapid vesicle conformation changes by regulatory proteins result in full synaptic vesicle fusion with presynaptic membrane and pore formation where through neuro-mediator is released in a synaptic cleft [29].

Neuromediator diffuses from its nerve terminal and binds to post-synaptic receptors that trigger post-synaptic cell signaling. In neuromuscular junctions acetyl choline binds with its receptor on myocyte plasmalemma resulting in muscle cell membrane depolarization. Membrane depolarization kicks off Ca^{2+} influx in myocyte and muscle contraction.

While neuromediator is released synaptic vesicle lumen opens temporarily into a synaptic cleft but later it internalizes in nerve terminal during endocytosis. After endocytosis the vesicle is again filled in with neuromediator and next neurotransmission cycle starts [30].

5. BoNT/A binding with target cells

Active BoNT/A molecules bind with target cells via their receptors on cell surface [31]. To bind with neuronal membrane BoNT/A molecule should interact with a set of high and low affinity receptors [32]. Currently three receptors (polysialoganglioside GT1b, fibroblast growth factor receptor 3, transmembrane vesicular receptor SV2) and several co-receptors have been described with such combination.

Active neurotoxin molecule endocytosis and its further changes are possible only once it binds with entire receptor combination at axonal surface [5]. Binding to one of the receptors without interaction with others does not induce toxin internalization. Such multistage process for BoNT/A binding with receptors makes up for low BoNT/A concentration in circulating fluids, high rate of extracellular flow around cells and small axonal surface area.

5.1 First receptor: Polysialoganglioside

First BoNT/A receptor at neuronal surface is polysialoganglioside GT1b (PSG).

Gangliosides are glycosylated lipids being a part of cell membranes. Though gangliosides are present in all tissues of vertebrates they are more prevalent in neuronal membranes [33] where they are involved in optimal myelin production, axon-myelin interactions, peripheral and central axon stability [32].

PSG density on presynaptic membrane is high. PSGs are grouped as microdomains next to presynaptic membrane active zones [34]. PSG receptor presence in these zones is important for processes of botulinum neurotoxin binding with other receptors.

Oligosaccharide (BoNT-binding part) PSG projects quite far outside membrane surface in a synaptic gap and is negatively charged [8]. BoNT/A molecule is a dipole with positively charged binding domain [3]. Such electric charge difference of BoNT/A binding domain and PSG receptors (and other anion lipids at axonal membrane) makes possible to redirect BoNT/A molecule on its way to cell membrane enhancing receptor binding chances.

Currently polysialoganglioside are considered as initial binding regions drawing toxin from relatively vast 3D extracellular fluid space into 2D membrane surface one [5]. It is required, in turn, for toxin binding to following receptors. On one hand,

binding to PSG is irreversible since BoNT/A is extracted from ground substance and is fixed on axonal membrane. On the other, at that stage toxin can still be affected and neutralizing antibodies can still reach it.

However, polysialogangliosides are membrane receptors for both botulinum neurotoxin and human neuropathy-associated antiganglioside autoantibodies. Anti-PSG autoantibody production in neuropathy patients may induce diminished botulinum neurotoxin sensitivity and resistance development [35].

5.2 Second receptor: Fibroblast growth factor receptor 3

HC subdomain structure of botulinum neurotoxin type A is homologous to basic fibroblast growth factor (FGF) [36]. That similarity enables BoNT/A high-affinity binding with protein fibroblast growth factor receptor 3 (FGFR3b) on neuronal surface [37].

However, FGFR3b receptors are affine not only to BoNT/A but also to multiple fibroblast growth factors. Moreover, this receptor affinity to growth factors exceeds the one to botulinum neurotoxin. Native FGFR3 ligands—growth factors FGF1, FGF2 and FGF9—compete for binding with FGFR3 and occupying receptors are able to jam BoNT/A absorption by cells [8].

Besides, FGFR3b receptor activity is regulated by several low-affinity cofactors including heparansulfate, neuropilin-1, anosmin, etc. [38]. Non-specificity and competitive binding of FGFR3 receptors with BoNT/A and fibroblast growth factors, cofactor impact on receptor activity may explain fragility of the said receptor mechanism and, therefore, variable sensitivity to botulinum neurotoxin. Moreover, some FGFR3 mutation-related conditions (skeletal dysplasias, epidermal nevus, seborrheic keratosis, hyperinsulinemia) might demonstrate defective FGFR3 expression [39–43]. FGFR3 mutation influence on botulinum neurotoxin sensitivity is yet to be studied.

5.3 Third receptor: Transmembrane vesicular receptor SV2

SV2 is a protein receptor located on vesicular membrane [44] of all peripheral and central neurons as well as on secretory granule membrane of endocrine cells [45]. SV2 is expressed on vesicular membranes in cells accumulating not only acetyl choline but also GABA, dopamine, glutamate, substance P and several other mediators [46].

Unlike polysialoganglioside receptors expressed into a synaptic gap the SV2-receptor BoNT/A-binding site is projected into synaptic vesicle lumen and is not approachable for neurotoxin while such vesicle is in axonal cytosol [47]. SV2 becomes reachable for BoNT/A at the time of vesicle fusion with presynaptic membrane and acetyl choline exocytosis [48].

Thus, BoNT/A binding with entire receptor combination happens in active zones only after synaptic vesicle fusion with presynaptic membrane and opening of vesicular lumen into synaptic cleft facilitating further BoNT/A endocytosis. After binding with receptor combination and endocytosis botulinum neurotoxin cannot be reached by neutralizing antibodies anymore.

6. Endocytosis

BoNT/A molecule binding with receptors results in receptor-mediated endocytosis of both receptors and toxin [49].

Immediately after endocytosis vesicular lumen has neutral pH. Vesicular ATPase proton pump controls mediator re-uptake [50] and injects protons into synaptic vesicle, therefore, gradually decreasing vesicular lumen pH [51].

7. Light chain translocation

Vesicular medium acidification results in irreversible conformation changes of both heavy and light BoNT/A chains. With these changes the heavy chain being linked via receptors with vesicular membrane forms transmembrane H-channel there [52, 53]. Through the channel the conformation-altered light chain leaves the vesicle for cytosol [54] and then chain-binding disulfide link breaks up.

L-chain translocation occurs with pH between 4.5 and 6 [55]. pH decrease results in protonation of carboxylated amino acid residues present in BoNT/A heavy and light chains. Carboxylated residues are located at one side of toxin molecule and their protonation results in significant changes of molecular shape [55]. BoNT/A molecule with its positively charged surface interacts with anion vesicular membrane surface forming protein and lipid complex [56]. L-chain is assumed to turn into “molten protein globule” gaining hydrophobic features [8]. On one hand, L-chain hydrophobicity ensures its translocation via the H-chain-formed membrane channel. On the other, with lower pH molecular surface where the disulfide bond is located becomes more hydrophobic. It ensures disulfide bond integrity until complete L-chain translocation.

To cross vesicular membrane L-chain should have disulfide bond with H-chain throughout entire translocation sequence [55]. Premature disulfide bond breakage at any stage until it exits into cytosol interrupts L-chain translocation [57].

At the end of translocation process the disulfide bond is destroyed by thioredoxin reductase-thioredoxin system releasing light chain to express its catalytic activity in cytosol [58].

Thioredoxin reductase (TrxR)—thioredoxin (Trx) system is a main cellular redox system. TrxR and Trx are cytosol side proteins of vesicular membrane and their inhibition may block BoNT/A action on stages when neurotoxin cannot be reached by neutralizing antibodies [59]. In vitro experiments of Zanetti et al. [60] showed that inhibitors for TrxR-Trx enzymatic couple hampers L-chain protease activity for all known botulinum neurotoxin serotypes in cultured neurons. While in vivo they prevent toxin-induced paralysis in mice irrespective of botulinum neurotoxin serotype.

In terms of life cycle model disulfide bond reduction is the end of intracellular existence of intact active BoNT/A molecule (holotoxin). Even if a light or heavy chain was exported out of cell none of them on its own should be able to disrupt cell functioning. Only holotoxin can undergo through multiple stages ended up with conduction block [61]. On the other side, conformation changes related to pH-induced L-chain translocation in cytosol create “a trap” making impossible both retrotranslocation into endosome and active toxin molecule return in extracellular environment [5].

8. L-chain cleaves transport proteins

Modified L-chain enters neuron cytosol through H-channel where it behaves as a metalloprotease. It catalytically cleaves nine amino acids from C-terminal of soluble

N-ethylmaleimide-sensitive factor-attachment protein receptor (SNARE) for SNAP25 protein (SNAP25206) forming SNAP25197 [62, 63]. Intact SNAP25 is required for mediator-containing vesicle attachment with further neurotransmitter release and it is also involved directly in Ca-channels activity regulation in presynaptic membrane [64]. SNAP25 cleavage impairs mediator exocytosis causing nerve impulse conduction block and muscle paralysis [65].

Synaptic activity is highly sensitive to cleavage of minimal SNAP25 amounts. It was hypothesized that SNAP25 in neuron cytosol exists as various pools and that only small amounts of SNAP25 are actively involved in exocytosis and reachable for L-protease effects [66]. It was confirmed experimentally showing that cleavage of 10–15% of total intracellular SNAP25 pool is sufficient for complete neuromediator release block [67–69]. L-protease cleavage of as little as 2–3% of SNAP25 pool results in block of miniature post-synaptic cell potentials (weak depolarization of post-synaptic membranes at neuromuscular rest) [70].

Along with that SNAP25 proteolysis product, SNAP25197 protein, on its own inhibits exocytosis [71]. Meunier et al [72]. described that SNAP25197 is able to persist for a long while in cytosol as a component of the non-productive SNARE complex prolongating BoNT/A effects. While removal of several amino acids from SNAP25197 results in rapid exocytosis restoration.

9. Zinc metabolism and translocation

Zinc is necessary for light chain catalytic activity. One botulinum holotoxin molecule contains 1 zinc atom retained by L-chain zinc-binding amino acid sequence and such binding is reversible [73].

Vesicle acidification causes protonation of zinc-binding sections in the BoNT molecule. Translocation causes light chain denaturation obliterating chelate site integrity. As a result bound zinc dissociates and adds up to cytosol zinc pool.

Simpson et al [74]. in their in vitro studies demonstrated that zinc removal from active botulinum neurotoxin molecule caused L-chain catalytic activity loss in cell-free samples. Though activity in intact neuromuscular junctions retained since internalized toxin bound cytosol zinc. Thus, zinc retained by holotoxin (intact active molecule) is not the same zinc that is bound with catalytically active light chain. Light chain binds cytosol zinc.

10. Mediator release block

Main BoNT/A target is peripheral neurons where botulinum neurotoxin inhibits acetylcholine release [75].

Many of cell-based studies showed that BoNT/A not only blocks acetylcholine release but also prevents release of multiple other neuromediators if they are accumulated and stored in vesicles [32]. These neuromediators are as follows: epinephrine, norepinephrine, dopamine [76, 77], glutamate [78], glycine [79], serotonin [80], substance P [81], etc. Therefore, botulinum neurotoxin is to be considered not as specific acetylcholine release inhibitor but rather as an exocytosis blocker for various mediators that offers tremendous promise for treatment and prevention of various disorders.

11. Specific biological effects

In addition, BoNT/A can affect cells not only as a blocker of exocytosis mediators, but also by binding to various receptors on the cell membrane, cause specific biological effects. Including influencing the expression of genes by the cell. Grando and Zachary [82] described that many cells are capable of expressing one or more BoNT/A receptors and binding BoNT/A: epidermal keratinocytes, mesenchymal stem cells from subcutaneous adipose tissue, nasal mucosa cells, urothelium, intestinal epithelial cells, prostate epithelial cells, alveolar epithelial cells, neutrophils, macrophages, etc. In addition to SNAP25, BoNT/A can also cleave SNAP-23, which is expressed in various human tissues.

Kim et al. [83] experimentally proved that BoNT/A is able to bind to TLR2 receptors on macrophages, changing the expression of genes responsible for signal transduction, protein metabolism and modification, nucleic acid metabolism, apoptosis, proliferation, cell differentiation. Which may explain the anti-inflammatory effect of BoNT/A.

12. Cytotoxicity

Cytotoxicity for BoNT/A has not been established either in cell-based studies [84] or in electrophysiological studies in healthy humans [85]. In addition, the experience of therapeutic use of BoNT/A for various indications indicates the absence of any signs of neuronal damage even with long-term regular use [66, 86, 87].

13. Conclusion

Further studies of unique pharmacological mechanisms for botulinum neurotoxin are quite promising for the search of the ways to influence its effects: to extend/shorten its action duration, to increase/decrease BoNT sensitivity in specific patient populations. Also it will help to develop protocols for optimal combinations of botulinum neurotoxin with esthetic medicine procedures of all kinds. Better insights on multiple aspects of not only BoNT neuronal selectivity but also BoNT/A interaction with non-neuronal cells will show ways to find new therapeutic applications of botulinum neurotoxin-based agents in various areas of medicine.

One of the promising areas of botulinum therapy and bioengineering of botulinum toxin is the treatment of pain syndromes. BoNT/A is effective in the treatment of various neuropathic pain syndromes, including chronic migraine, postherpetic neuralgia, trigeminal neuralgia and peripheral neuralgia [32].

Hybrid preparations of botulinum toxin are being developed to suppress secretion in various cell populations, including the secretion of growth hormone [88]. Various effects of BoNT/A on the enhancement and suppression of gene expression in neuronal and non-neuronal cells are described [89]. This implies a fundamentally different response of neuronal and epithelial cells to the action of botulinum toxin and is of great importance in the development of anti-cancer treatments based on BoNT/A.

Thus, BoNT/A should not be considered as a specific blocker of acetylcholine release by motor neurons, but mainly as a blocker of exocytosis of various mediators by various cells, neuronal and non-neuronal. Moreover, the biological effect of BoNT/A can be realized not only through the blockade of exocytosis. And it can be absolutely different in different types of cells, which has great prospects in the treatment and prevention of many diseases.

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Author details

Anna V. Reznik
ARclinic Medical Center, Saint-Petersburg, Russia

*Address all correspondence to: ksho@yandex.ru

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