aesthetic medicine

Review

Current understanding of the pharmacology of botulinum toxin type A: penetration, distribution, triple receptor binding, the mechanism of action and factors affecting it

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Abstract

The aim of this review 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. The evaluation of botulinum neurotoxin (BoNT) pharmacological features requires the study of factors affecting its biological activity to extend/shorten the duration of its effect and to increase/decrease BoNT sensitivity in specific patient populations.

This review presents unique molecular mechanisms underlying BoNT/A pharmacokinetics and pharmacodynamics: entry into 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.

Botulinum neurotoxin pharmacokinetics and pharmacodynamics features discussed herein are of significant clinical relevance since they determine botulinum treatment safety and effectiveness. They also open the way for developing both BoNT-based therapies and anti-botulinic agents.

Keywords

Botulinum neurotoxin, Ganglioside, Synaptic vesicle protein, Fibroblast Growth Factor Receptor, SNARE proteins, translocation

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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]. BoNT-based therapeutics are widely used in the treatment of various diseases and esthetic disorders. Various highly promising features of BoNT pharmacology in the development of both BoNT-based therapeutics and anti-botulinic agents are currently under study. 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 in this paper.

Neurotoxin complex and BoNT molecule structure

Botulinum neurotoxin is a protein dimer with a molecular weight of 150 kDa and the chemical formula C6760H10447N1743O2010S32, consisting of two chains: light and heavy. The light chain constitutes 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 2 domains: the binding domain bounds to target cell surface receptors; the translocation domain is involved in light chain translocation, creating the 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 to cell membranes as it facilitates receptor binding.
In natural settings BoNTs are synthesized by bacteria as a complex with several proteins: one non-toxin non-hemagglutin (NTNHA) and several hemagglutinins[1].
NTNHA has a molecular weight of 130 kDa and its amino acid sequences are extremely similar to BoNT but without protease motif. "Hand in glove"-type interaction with the BoNT molecule protects from the 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 directly come into contact with the BoNT molecule, but rather with NTNHA, working as an adhesin molecule when said toxin complex is absorbed.
Non-toxic hemagglutinin and hemagglutinin proteins can form various multimeric complexes with BoNT called botulinum neurotoxin complexes. Each contains only one BoNT molecule, which is released from the complex if medium pH changes[2].

BoNT absorption and distribution

BoNTs can enter the 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 that can be applied without damaging skin are already under development, yet to reach 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 the mode of toxin 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 related to injectable drug use), iatrogenic[8].
In natural settings, the botulinum neurotoxin must cross epithelial barriers and reach general circulation in order to hit its target cells. This process is called absorption.
There are two modes of BoNT intestinal or pulmonary epithelium penetration: intracellular route and intercellular junction-related.
In the case of transcytosis (penetration through epithelial cells), BoNT binds to ganglioside receptors at the epithelial cell surface and undergoes endocytosis (captured in a vesicle). Transport vesicles transfer the toxin to the entire cell and release it into general circulation. Toxin structure is not altered, nor is it released in cell cytosol during transcytosis, which differentiates BoNT binding with epithelial cell from binding with neuronal ones[9,10].
The paracellular route (through intercellular junctions) may or may not involve complexing proteins. Complexin hemagglutinins can bind to E-cadherin in epithelial intercellular junctions and disrupt the latter, releasing BoNT into general circulation[4]. BoNT molecules are also able to break epithelial barriers without complexing proteins. Studies by Maksymowych et al., 1999; Al-Saleem et al., 2012[11,12] showed that the 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 the epithelium.
When transported through the intestinal wall, BoNT may bind to cholinergic and serotoninergic neurons of the enteral (intestinal) nervous system located in the intestinal submucosa, blocking gut motor and secretory activity. This explains impaired bowel movement (constipation) as one of the early signs of alimentary and infant botulism[13].
BoNT penetrates the epithelial barrier into general circulation and is distributed in all extracellular fluid compartments in the body except for in the central nervous system.
Eisele et al. (2011) conducted a series of experiments[14] 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 a half-life of under one minute. Once the toxin complex dissociates, complexing proteins are no longer of any significance in the occurrence of the clinical effect of BoNT. Studies by Al-Saleem et al. (2008)[17] proved that the toxin reaches general circulation without any evident structural or biological activity changes. General circulation acts as a 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. This concept of "general circulation - botulinum neurotoxin storage compartment" has been confirmed by many researchers. Fagan et al., 2009[18] described active BoNT/A presence in human blood serum 11 days after contaminated food ingestion, Sheth et al.,2008[19] - 25 days after disease onset, Delbrassinne

et al., 2018[20] - 29 days after the consumption of contaminated food. From the intravascular fluid compartment, the botulinum neurotoxin enters the extravascular compartment and then intercellular fluid.
When locally injected for therapeutic purposes, the botulinum neurotoxin is directly introduced into the extravascular compartment (or intravascular one if in a blood vessel) adjacent to target cells, thus bypassing the absorption stage. From the intercellular compartment, the botulinum neurotoxin should reach its target, peripheral cholinergic nerve endings, and bind to receptors there.
In order to better understand the binding mechanism of botulinum neurotoxin with receptors, a knowledge of normal neurotransmission in synapses is required.

Normal synapse neurotransmission

Neuromediators are synthesized in neuron cytosol and then stored in pre-synaptic nerve endings in synaptic vesicles. The Synaptic vesicle membrane contains a proton pump (vesicular ATPase), which increases intravesicular proton concentration when activated[8]. The 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 membranes in active zones only and docking is controlled by numerous transport proteins[23].
When a nerve impulse arrives, the axonal presynaptic membrane is depolarized, calcium channels open and Ca^{2+} ions flow into the axon[24]. In response to the Ca^{2+} influx, the mediator- containing vesicle fuses with the presynaptic membrane in the active zone. This stage is called priming. It is regulated by 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 caused by regulatory proteins result in full synaptic vesicle fusion with presynaptic membrane and pore formation, enabling neuromediator release into a synaptic cleft[29].
The 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.
During neuromediator release the synaptic vesicle lumen opens temporarily into a synaptic cleft; later it internalizes in the nerve terminal during endocytosis. After endocytosis, the vesicle is filled once more with the neuromediator; this is followed by the start of the neurotransmission cycle[30].

BoNT/A binding with target cells

Active BoNT/A molecules bind with target cells via their receptors on cell surface[31]. In order to bind with the neuronal membrane, BoNT/A molecule must interact with a set of high and low affinity receptors[32]. Currently 3 receptors (polysyaloganglioside GT1b, fibroblast growth factor receptor 3, transmembrane vesicular receptor SV2) and several co-receptors have been described in such combination. Active neurotoxin molecule endocytosis and further changes are possible only when it binds with the entire receptor combination on the axonal surface[5]. Binding to one of the receptors without interaction with others does not induce toxin internalization.
This multistage process for BoNT/A binding with receptors accounts for a low BoNT/A concentration in circulating fluids, a high rate of extracellular flow around cells and a small axonal surface area.

First receptor - polysyaloganglioside

The first BoNT/A receptor on the neuronal surface is polysyaloganglioside GT1b (PSG). Gangliosides are glycosylated lipids contained in 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]. The presence of PSG receptors in these zones is important for binding processes of botulinum neurotoxin with other receptors.
Oligosaccharide (BoNT-binding part) PSG projects quite far outside the membrane surface into a synaptic gap and is negatively charged[8]. The BoNT/A molecule is a dipole with a positively charged binding domain[3].
Such a difference in electric charge of the BoNT/A binding domain and PSG receptors (and other anion lipids on the axonal membrane) makes it possible to redirect BoNT/A molecule to the cell membrane, thus enhancing chances of receptor binding. Currently polysyaloganglioside are considered as initial binding regions, drawing the toxin from a relatively vast 3D extracellular fluid space into a 2D membrane surface one[5]. Therefore toxin binding must follow receptors. On the one hand, binding to PSG is irreversible since BoNT/A is extracted from ground substance and is fixed onto the axonal membrane. On the other hand, at this stage the toxin can still be affected and neutralizing antibodies can still reach it.
However, polysyalogangliosides are membrane receptors for both botulinum neurotoxin and human neuropathy-associated antiganglioside autoantibodies. Anti-PSG autoantibody production in neuropathic patients may induce diminished botulinum neurotoxin sensitivity and resistance development[35].

Second receptor - fibroblast growth factor receptor 3

The HC subdomain structure of botulinum neurotoxin type A is similar to basic fibroblast growth factor (FGF)[36]. This similarity enables BoNT/A high-affinity binding with protein fibroblast growth factor receptor 3 (FGFR3b) on a neuronal surface[37].
However, FGFR3b receptors bear an affinity not only to BoNT/A but also to multiple fibroblast growth factors. Moreover, this receptor affinity to growth factors exceeds the one to the 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].
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, which may explain the fragility of this 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.

Third receptor - transmembrane vesicular receptor SV2

SV2 is a protein receptor located on the vesicular membrane[44] of all peripheral and central neurons as well as on the 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 polysyaloganglioside receptors expressed into a synaptic gap, the SV2-receptor BoNT/A- binding site is projected into synaptic vesicle lumen and is not approachable for the 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 the entire receptor combination occurs in active zones only after synaptic vesicle fusion with the presynaptic membrane and the opening of vesicular lumen into the synaptic cleft, facilitating further BoNT/A endocytosis. After binding with the receptor combination and endocytosis, the botulinum neurotoxin can no longer be reached by neutralizing antibodies.

Endocytosis

BoNT/A molecule binding with receptors results in receptor-mediated endocytosis of both receptors and toxin[49]. The vesicular lumen has a neutral pH immediately after endocytosis. The vesicular ATPase proton pump controls mediator re-uptake[50] and injects protons into the synaptic vesicle, thus gradually decreasing vesicular lumen pH[51].

Light chain translocation

Vesicular medium acidification results in irreversible conformation changes to both heavy and light BoNT/A chains. With these changes, via receptors the heavy chain links to the vesicular membrane to form a transmembrane H-channel[52,53]. The conformation-altered light chain leaves the vesicle for cytosol[54] through the channel, resulting in the break-up of the chain-binding disulfide link. L-chain translocation occurs with a pH of between 4.5 and 6[55]. A pH decrease results in the protonation of carboxylated amino acid residues present in BoNT/A heavy and light chains. Carboxylated residues are located on one side of the toxin molecule and their protonation results in significant changes in molecular shape[55]. With its positively charged surface, the BoNT/A molecule interacts with the anion vesicular membrane surface to form a protein and lipid complex[56]. The L-chain is assumed to turn into a “molten protein globule”, thus assuming hydrophobic features[8]. On the one hand, L-chain hydrophobicity ensures its translocation via the H-chain-formed membrane channel. On the other, a lower pH molecular surface where the disulfide bond is located results in increased hydrophobicity. This ensures disulfide bond integrity until complete L-chain translocation. In order to cross the vesicular membrane, L-chain should maintain a disulfide bond with the H-chain throughout the entire translocation sequence[55]. Premature disulfide bond breakage at any stage before exit into the cytosol interrupts L-chain translocation[57].

At the end of translocation process, the disulfide bond is destroyed by the thioredoxin reductase- thioredoxin system, releasing a light chain to express its catalytic activity in cytosol[58]. The Thioredoxin reductase (TrxR) - thioredoxin (Trx) system is a main cellular redox system. TrxR and Trx are cytosol side proteins of the vesicular membrane and their inhibition may block BoNT/A action in stages in which the neurotoxin is beyond the reach of neutralizing antibodies[59]. In vitro experiments by Zanetti et al., 2015 showed that inhibitors for the TrxR-Trx enzymatic couple hamper L-chain protease activity for all known botulinum neurotoxin serotypes in cultured neurons. In vivo, they prevent toxin-induced paralysis in mice irrespective of botulinum neurotoxin serotype[60].

In life cycle model terms, disulfide bond reduction is the end of intracellular existence for the intact active BoNT/A molecule (holotoxin). Even if a light or heavy chain were to be exported beyond the cell, neither would be able to disrupt cell functioning. Only holotoxin can undergo multiple stages that result in conduction block[61]. On the other hand, conformation changes related to pH-induced L-chain translocation in cytosol create “a trap”, preventing both retrotranslocation into the endosome and active toxin molecule return in the extracellular environment[5].

L-chain cleaves transport proteins

The modified L-chain enters the neuron cytosol through the H-channel, where it behaves as a metalloprotease. It catalytically cleaves nine amino acids from the 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 the presynaptic membrane[64]. SNAP25 cleavage impairs mediator exocytosis, causing nerve impulse conduction block and muscle paralysis[65].

Synaptic activity is highly sensitive to the 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 by showing that a cleavage of 10-15% of the 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 the blockage of miniature post-synaptic cell potentials (weak depolarization of post-synaptic membranes at neuromuscular rest)[70]. Along with the SNAP25 proteolysis product, the SNAP25197 protein inhibits exocytosis on its own[71]. Meunier et al.[72] reported how SNAP25197 is able to persist for a long time while

in cytosol, as a component of the non-productive SNARE complex, thus prolongating BoNT/A effects. Conversely, the removal of several amino acids from SNAP25197 results in rapid exocytosis restoration.

Zinc metabolism and translocation

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

Vesicle acidification causes the 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 enters the cytosol zinc pool.

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

Mediator release block

The main BoNT/A target is peripheral neurons, where the botulinum neurotoxin inhibits acetylcholine release[75].

Many cell-based studies have shown that BoNT/A not only blocks acetylcholine release but also prevents the release of numerous 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, the botulinum neurotoxin is to be considered not as a specific acetylcholine release inhibitor, but rather as an exocytosis blocker for various mediators that offers tremendous promise for the treatment and prevention of various disorders.

Conclusion

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

Conflict of interest

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REFERENCES

1. Poulain B, Popoff M. Why Are Botulinum Neurotoxin-Producing Bacteria So Diverse and Botulinum Neurotoxins So Toxic?. *Toxins (Basel).* 2019; 11(1):34.
2. Berry M. Botulinum Neurotoxin: Basic Facts, Physiology and Pharmacology. *Atlas of Surgical Therapy for Migraine and Tension-Type Headache.* 2019; 45-50.
3. Fogolari F, Tosatto S, Muraro L, Montecucco C. Electric dipole reorientation in the interaction of botulinum neurotoxins with neuronal membranes. *FEBS Lett.* 2009; 583(14):2321-2325.
4. Gu S, Rumpel S, Zhou J et al. Botulinum Neurotoxin Is Shielded by NTNHA in an Interlocked Complex. *Science.* 2012; 335(6071):977-981.
5. Simpson L. The life history of a botulinum toxin molecule. *Toxicon.* 2013; 68:40-59.
6. Zhu Z, Stone H, Thach T, Garcia L, Ruegg C. A Novel Botulinum Neurotoxin Topical Gel: Treatment of Allergic Rhinitis in Rats and Comparative Safety Profile. *Am J Rhinol Allergy.* 2012; 26(6):450-454.
7. Collins A, Nasir A. Topical Botulinum Toxin. *J Clin Aesthet Dermatol.* 2010; 3(3):35-39.
8. Rossetto O, Pirazzini M, Montecucco C. Botulinum neurotoxins: genetic, structural and mechanistic insights. *Nat Rev Microbiol.* 2014; 12(8):535-549.
9. Couesnon A, Shimizu T, Popoff M. Differential entry of botulinum neurotoxin A into neuronal and intestinal cells. *Cell Microbiol.* 2009; 11(2):289-308.
10. Elias M, al-Saleem F, Ancharski D et al. Evidence That Botulinum Toxin Receptors on Epithelial Cells and Neuronal Cells Are Not Identical: Implications for Development of a Non-Neurotropic Vaccine. *J Pharmacol Exp Ther.* 2010; 336(3):605-612.
11. Maksymowych A, Reinhard M, Malizio C, Goodnough M, Johnson E, Simpson L. Pure Botulinum Neurotoxin Is Absorbed from the Stomach and Small Intestine and Produces Peripheral Neuromuscular Blockade. *Infect Immun.* 1999; 67(9):4708-4712.
12. Al-Saleem F, Ancharski D, Joshi S et al. Analysis of the Mechanisms That Underlie Absorption of Botulinum Toxin by the Inhalation Route. *Infect Immun.* 2012; 80(12):4133-4142.
13. Rosow L, Strober J. Infant Botulism: Review and Clinical Update. *Pediatr Neurol.* 2015; 52(5):487-492.
14. Eisele K, Fink K, Vey M, Taylor H. Studies on the dissociation of botulinum neurotoxin type A complexes. *Toxicon.* 2011; 57(4):555-565.
15. Boyle J, Weitzman J, Berne C. Indications for measurement of arterial blood pH. *Am J Surg.* 1960; 100(2):346-353.
16. Kaplan L, Kellum J. Fluids, pH, ions and electrolytes. *Curr Opin Crit Care.* 2010; 16(4):323-331.
17. Al-Saleem F, Ancharski D, Ravichandran E et al. The Role of Systemic Handling in the Pathophysiologic Actions of Botulinum Toxin. *J Pharmacol Exp Ther.* 2008; 326(3):856-863.
18. Fagan R, McLaughlin J, Middaugh J. Persistence of Botulinum Toxin in Patients' Serum: Alaska, 1959–2007. *J Infect Dis.* 2009; 199(7):1029-1031.
19. Sheth A, Wiersma P, Atrubin D et al. International Outbreak of Severe Botulism with Prolonged Toxemia Caused by Commercial Carrot Juice. *Clin Infect Dis.* 2008; 47(10):1245-1251.
20. Delbrassinne L, Laisnez V, De Weweire M, Vanderpas J, Dierick K, Denayer S. Very Long Persistence of Botulinum Toxin B in a Patient's Serum. *Open Infect Dis J.* 2018; 10(1):187-191.
21. Zhai R, Bellen H. The Architecture of the Active Zone in the Presynaptic Nerve Terminal. *Physiology.* 2004; 19(5):262-270.
22. Heuser J, Reese T. Evidence for recycling of synaptic vesicle membrane during transmitter release at the frog neuromuscular junction. *J Cell Biol.* 1973; 57(2):315-344.
23. Ahmari S, Buchanan J, Smith S. Assembly of presynaptic active zones from cytoplasmic transport packets. *Nat Neurosci.* 2000; 3(5):445-451.
24. Stanley E. The calcium channel and the organization of the presynaptic transmitter release face. *Trends Neurosci.* 1997; 20(9):404-409.
25. CATTERALL W. Interactions of Presynaptic Ca2+ Channels and Snare Proteins in Neurotransmitter Release. *Ann N Y Acad Sci.* 1999; 868(1):144-159.
26. Hilfiker S, Pieribone V, Czernik A, Kao H, Augustine G, Greengard P. Synapsins as regulators of neurotransmitter release. *Philos Trans R Soc Lond B Biol Sci.* 1999; 354(1381):269-279.
27. Hallam S, Goncharov A, McEwen J, Baran R, Jin Y. SYD-1, a presynaptic protein with PDZ, C2 and rhoGAP-like domains, specifies axon identity in C. elegans. *Nat Neurosci.* 2002; 5(11):1137-1146.
28. Jarvis S, Barr W, Feng Z, Hamid J, Zamponi G. Molecular Determinants of Syntaxin 1 Modulation of N-type Calcium Channels. *J Biol Chem.* 2002; 277(46):44399-44407.
29. Taverna E, Saba E, Rowe J, Francolini M, Clementi F, Rosa P. Role of Lipid Microdomains in P/Q-type Calcium Channel (Cav2.1) Clustering and Function in Presynaptic Membranes. *J Biol Chem.* 2004; 279(7):5127-5134.
30. Burns M, Augustine G. Synaptic structure and function: Dynamic organization yields architectural precision. *Cell.* 1995; 83(2):187-194.
31. Simpson LL. Molecular pharmacology of botulinum toxin and tetanus toxin. *Annu Rev Pharmacol Toxicol.* 1986; 26(1):427-453.
32. Poulain B, Lemichez E, Popoff M. Neuronal selectivity of botulinum neurotoxins. *Toxicon.* 2020; 178:20-32.
33. Schnaar RL. Gangliosides of the Vertebrate Nervous System. *J Mol Biol.* 2016; 428(16):3325-3336.
34. Prinetti A, Loberto N, Chigorno V, Sonnino S. Glycosphingolipid behaviour in complex membranes. *Biochim Biophys Acta.* 2009; 1788(1):184-193.
35. Bullens R, O'Hanlon G, Wagner E et al. Complex Gangliosides at the Neuromuscular Junction Are Membrane Receptors for Autoantibodies and Botulinum Neurotoxin But Redundant for Normal Synaptic Function. *J Neurosci.* 2002; 22(16):6876-6884.
36. Jacky B, Garay P, Dupuy J et al. Identification of Fibroblast Growth Factor Receptor 3 (FGFR3) as a Protein Receptor for Botulinum Neurotoxin Serotype A (BoNT/A). *PLoS Pathog.* 2013; 9(5):e1003369.
37. James N, Malik S, Sanstrum B et al. Characterization of clostridium botulinum neurotoxin serotype A (BoNT/A) and fibroblast growth factor receptor interactions using a novel receptor dimerization assay. 2019.
38. Rummel A. Double Receptor Anchorage of Botulinum Neurotoxins Accounts for their Exquisite Neurospecificity. *Curr Top Microbiol Immunol.* 2013; 364:61-90.
39. Hernández S, Toll A, Baselga E, et al. Fibroblast Growth Factor Receptor 3 Mutations in Epidermal Nevi and Associated Low Grade Bladder Tumors. *J Invest Dermatol.* 2007; 127(7):1664-1666.
40. Hafner C, Hartmann A, Vogt T. FGFR3 Mutations in Epidermal Nevi and Seborrheic Keratoses: Lessons from Urothelium and Skin. *J Invest Dermatol.* 2007; 127(7):1572-1573.
41. Chen J, Liu J, Zhou Y et al. Molecular therapeutic strategies for FGFR3 gene-related skeletal dysplasia. *J Mol Med (Berl).* 2017; 95(12):1303-1313.
42. Mustafa M, Moghrabi N, Bin-Abbas B. Hypochondroplasia, Acanthosis Nigricans, and Insulin Resistance in a Child with FGFR3 Mutation: Is It Just an Association?. *Case Rep Endocrinol.* 2014; 2014:1-6.
43. Blomberg M, Jeppesen E, Skovby F, Benfeldt E. FGFR3 Mutations and the Skin: Report of a Patient with a FGFR3 Gene Mutation, Acanthosis Nigricans, Hypochondroplasia and Hyperinsulinemia and Review of the Literature. *Dermatology.* 2010; 220(4):297-305.
44. Dong M, Yeh F, Tepp WH, et al. SV2 Is the Protein Receptor for Botulinum Neurotoxin A. *Science.* 2006; 312(5773):592-596.

45. Bartholome O, Van den Ackerveken P, Sánchez Gil J, et al. Puzzling Out Synaptic Vesicle 2 Family Members Functions. *Front Mol Neurosci.* 2017; 10:148.

46. Dunn A, Hoffman C, Stout K, Ozawa M, Dhamsania R, Miller G. Immunochemical analysis of the expression of SV2C in mouse, macaque and human brain. *Brain Res.* 2019; 1702:85-95.

47. Strotmeier J, Mahrhold S, Krez N, et al. Identification of the synaptic vesicle glycoprotein 2 receptor binding site in botulinum neurotoxin A. *FEBS Lett.* 2014; 588(7):1087-1093.

48. Yao G, Zhang S, Mahrhold S, et al. N-linked glycosylation of SV2 is required for binding and uptake of botulinum neurotoxin A. *Nat Struct Mol Biol.* 2016; 23(7):656-662.

49. Colasante C, Rossetto O, Morbiato L, Pirazzini M, Molgó J, Montecucco C. Botulinum Neurotoxin Type A is Internalized and Translocated from Small Synaptic Vesicles at the Neuromuscular Junction. *Mol Neurobiol.* 2013; 48(1):120-127.

50. Takamori S, Holt M, Stenius K, et al. Molecular Anatomy of a Trafficking Organelle. *Cell.* 2006; 127(4):831-846.

51. Saheki Y, De Camilli P. Synaptic Vesicle Endocytosis. *Cold Spring Harb Perspect Biol.* 2012; 4(9):a005645-a005645.

52. Montecucco C, Schiavo G. Structure and function of tetanus and botulinum neurotoxins. *Q Rev Biophys.* 1995; 28(4):423-472.

53. Montal M. Translocation of botulinum neurotoxin light chain protease by the heavy chain protein-conducting channel. *Toxicon.* 2009; 54(5):565-569.

54. Brunger A, Breidenbach M, Jin R, Fischer A, Santos J, Montal M. Botulinum Neurotoxin Heavy Chain Belt as an Intramolecular Chaperone for the Light Chain. *PLoS Pathog.* 2007; 3(9):e113.

55. Pirazzini M, Rossetto O, Bolognese P, Shone C, Montecucco C. Double anchorage to the membrane and intact inter-chain disulfide bond are required for the low pH induced entry of tetanus and botulinum neurotoxins into neurons. *Cell Microbiol.* 2011; 13(11):1731-1743.

56. Galloux M, Vitrac H, Montagner C, et al. Membrane Interaction of Botulinum Neurotoxin A Translocation (T) Domain. *J Biol Chem.* 2008; 283(41):27668-27676.

57. Fischer A, Montal M. Crucial Role of the Disulfide Bridge between Botulinum Neurotoxin Light and Heavy Chains in Protease Translocation across Membranes. *J Biol Chem.* 2007; 282(40):29604-29611.

58. Pirazzini M, Azarnia Tehran D, Zanetti G, Rossetto O, Montecucco C. Hsp90 and Thioredoxin-Thioredoxin Reductase enable the catalytic activity of Clostridial neurotoxins inside nerve terminals. *Toxicon.* 2018; 147:32-37.

59. Pirazzini M, Azarnia Tehran D, Zanetti G, et al. Thioredoxin and Its Reductase Are Present on Synaptic Vesicles, and Their Inhibition Prevents the Paralysis Induced by Botulinum Neurotoxins. *Cell Rep.* 2014; 8(6):1870-1878.

60. Zanetti G, Azarnia Tehran D, Pirazzini, M et al. Inhibition of botulinum neurotoxins interchain disulfide bond reduction prevents the peripheral neuroparalysis of botulism. *Biochem Pharmacol.* 2015; 98(3):522-530.

61. Fischer A, Montal M. Single molecule detection of intermediates during botulinum neurotoxin translocation across membranes. *Proc Natl Acad Sci U S A.* 2007; 104(25):10447-10452.

62. Blasi J, Chapman E, Link E, et al. Botulinum neurotoxin A selectively cleaves the synaptic protein SNAP-25. *Nature.* 1993; 365(6442):160-163.

63. Fernandez-Salas E, Steward L, Ho H, et al. Plasma membrane localization signals in the light chain of botulinum neurotoxin. *Proc Natl Acad Sci U S A.* 2004; 101(9):3208-3213.

64. Pozzi D, Corradini I, Matteoli M. The Control of Neuronal Calcium Homeostasis by SNAP-25 and its Impact on Neurotransmitter Release. *Neuroscience.* 2019; 420:72-78.

65. Kasai H, Takahashi N, Tokumaru H. Distinct Initial SNARE Configurations Underlying the Diversity of Exocytosis. *Physiol Rev.* 2012; 92(4):1915-1964.

66. Pirazzini M, Rossetto O, Eleopra R, Montecucco C. Botulinum Neurotoxins: Biology, Pharmacology, and Toxicology. *Pharmacol Rev.* 2017; 69(2):200-235.

67. Raciborska D, Trimble W, Charlton M. Presynaptic protein interactionsin vivo: evidence from botulinum A, C, D and E action at frog neuromuscular junction. *Eur J Neurosci.* 1998; 10(8):2617-2628.

68. Keller J, Cai F, Neale E. Uptake of Botulinum Neurotoxin into Cultured Neurons. *Biochemistry.* 2004; 43(2):526-532.

69. Montecucco C, Schiavo G, Pantano S. SNARE complexes and neuroexocytosis: how many, how close?. *Trends Biochem Sci.* 2005; 30(7):367-372.

70. Beske P, Scheeler S, Adler M, McNutt P. Accelerated intoxication of GABAergic synapses by botulinum neurotoxin A disinhibits stem cell-derived neuron networks prior to network silencing. *Front Cell Neurosci.* 2015; 9:159.

71. Huang X, Wheeler MB, Kang YH, et al. Truncated SNAP-25 (1-197), Like Botulinum Neurotoxin A, Can Inhibit Insulin Secretion from HIT-T15 Insulinoma Cells. *Mol Endocrinol.* 1998; 12(7):1060-1070.

72. Meunier F, Lisk G, Sesardic D, Dolly J. Dynamics of motor nerve terminal remodeling unveiled using SNARE-cleaving botulinum toxins: the extent and duration are dictated by the sites of SNAP-25 truncation. *Mol Cell Neurosci.* 2003; 22(4):454-466.

73. Schiavo G, Rossetto O, Benfenati F, Poulain B, Montecucco C. Tetanus and Botulinum Neurotoxins Are Zinc Proteases Specific for Components of the Neuroexocytosis Apparatus. *Ann N Y Acad Sci.* 1994; 710(1 Toxins and Exocytosis):65-75.

74. Simpson L, Maksymowych A, Hao S. The Role of Zinc Binding in the Biological Activity of Botulinum Toxin. *J Biol Chem.* 2001; 276(29):27034-27041.

75. Pantano S, Montecucco C. The blockade of the neurotransmitter release apparatus by botulinum neurotoxins. *Cell Mol Life Sci.* 2013; 71(5):793-811.

76. Ashton A, Dolly J. Characterization of the Inhibitory Action of Botulinum Neurotoxin Type A on the Release of Several Transmitters from Rat Cerebrocortical Synaptosomes. *J Neurochem.* 1988; 50(6):1808-1816.

77. Maisey E, Wadsworth J, Poulain B, et al. Involvement of the constituent chains of botulinum neurotoxins A and B in the blockade of neurotransmitter release. *Eur J Biochem.* 1988; 177(3):683-691.

78. Foran P, Mohammed N, Lisk G, et al. Evaluation of the Therapeutic Usefulness of Botulinum Neurotoxin B, C1, E, and F Compared with the Long Lasting Type A. *J Biol Chem.* 2003; 278(2):1363-1371.

79. Neale E, Bowers L, Jia M, Bateman K, Williamson L. Botulinum Neurotoxin a Blocks Synaptic Vesicle Exocytosis but Not Endocytosis at the Nerve Terminal. *J Cell Biol.* 1999; 147(6):1249-1260.

80. Najib A, Pelliccioni P, Gil C, Aguilera J. Clostridium Neurotoxins Influence Serotonin Uptake and Release Differently in Rat Brain Synaptosomes. *J Neurochem.* 1999; 72(5):1991-1998.

81. Lucioni A, Bales G, Lotan T, McGehee D, Cook S, Rapp D. Botulinum toxin type A inhibits sensory neuropeptide release in rat bladder models of acute injury and chronic inflammation. *BJU Int.* 2008; 101(3):366-370.