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Строение и механизм действия нейротоксинов ботулизма и столбняка: научный обзор

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АННОТАЦИЯ

Ботулинические нейротоксины и столбнячный нейротоксин являются самыми сильными из известных токсинов и вызывают развитие нейропаралитических синдромов при ботулизме и столбняке. Данная статья систематизирует научные данные о строении и механизме действия ботулинических и столбнячного нейротоксинов. Установлено, что нейротоксины ботулизма и столбняка представляют собой белки, содержащие функциональные домены, которые отвечают за связывание с рецептором, трансмембранную транслокацию и протеолитическое расщепление белков, необходимых для экзоцитоза синаптических везикул и высвобождения нейромедиаторов в синаптическую щель. Описаны основные этапы действия ботулинических и столбнячного нейротоксинов: связывание с пресинаптической мембраной, интернализация связанного токсина в цитозоль посредством эндоцитоза, транслокация L-цепи в цитозоль с помощью домена HN, разрушение межцепочечной дисульфидной связи с высвобождением L-цепи для экспрессии её каталитической активности (как металлопротеазы) в цитозоле и избирательное расщепление одного или более белков комплекса SNARE (soluble N-ethylmaleimide sensitive factor attachment receptor; рецептор связывания растворимых N-этилмалеимид-чувствительных белков) с последующей блокадой высвобождения нейромедиатора.

Ключевые слова: столбняк; ботулизм; нейротоксины; клостридии.

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Structure and mechanism of action of botulinum and tetanus neurotoxins: A review

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ABSTRACT

Botulinum neurotoxins and tetanus neurotoxins are the strongest known toxins that cause neuroparalytic syndromes in botulism and tetanus. This review aimed to systematize scientific data on the structures and mechanism of actions of botulinum and tetanus neurotoxins. Botulinum and tetanus neurotoxins are proteins containing functional domains responsible for receptor binding, transmembrane translocation, and proteolytic cleavage of proteins required for exocytosis of synaptic vesicles and release of neurotransmitters into the synaptic cleft. The main stages of the botulinum neurotoxins and tetanus neurotoxin action include binding to the presynaptic membrane, internalization of bound toxin into the cytosol via endocytosis, translocation of the L-chain into the cytosol via the HN domain, disruption of the interchain disulfide bond with the release of the L-chain to express its catalytic activity (as a metalloprotease) in the cytosol, and selective cleavage of one or more soluble N-ethylmaleimide-sensitive factor attachment receptor complex proteins with subsequent blockade of neurotransmitter release.

Keywords: botulism; Clostridium; neurotoxins; tetanus.

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INTRODUCTION

The history of the study of bacterial toxins began with the isolation of diphtheria toxin in 1888 by bacteriologists Emile Roux and Alexander Jersen [1]. It is now known that bacterial toxins target and disrupt certain physiological functions in animals and humans. Many of the toxins reach the cytosol of target cells and alter specific cellular components [2]. These toxins, acting intracellularly, have evolved as multidomain proteins with different mechanisms of action and diverse cellular targets. The isolation and characterization of bacterial toxins led to the development of anatoxins, one of the major medical triumphs of the twentieth century. [3].

The most potent toxins affect the nervous system and are called neurotoxins. This literature review deals with botulinum neurotoxins and tetanus neurotoxin, which top the list of the most potent toxins known to date [4]. Botulinum neurotoxin, in particular, has been shown to be about 100 billion times more toxic than cyanide [5]. Tetanus and botulism are diseases whose pathogenesis is due to exposure to proteinaceous neurotoxins produced by spore-forming bacteria of the genus *Clostridium.* Clostridia exist in the anaerobic environment and in the intestines of animals, mainly in the form of spores. Under favourable conditions, the spores germinate and the bacteria can produce neurotoxins [6]. Several isoforms of tetanus neurotoxin and many isoforms of botulinum neurotoxin are known, which are grouped into one tetanus neurotoxin serotype and seven botulinum neurotoxin serotypes (designated A, B, C, D, E, F and G) [7]. The serotypes include many isotypes that differ from each other in the amino acid sequence that affects their neurotoxicity [8]. Isotypes are denoted by Arabic numerals followed by the capital letter of the serotype. In humans, three botulinum neurotoxin serotypes, A, B and E, predominantly cause the disease. *C. botulinum* phenotypic group III bacteria produce, in addition to neurotoxins C and D, C/D and D/C, which are mosaic toxins thought to result from genetic recombination between the C and D toxin synthesis genes. Mosaic toxins also include the recently described neurotoxin H, which is a hybridoid structure containing domains of the two toxins, A1 and F5 [9]. Immunity after illness is typospecific, so that reinfection is possible in humans [10].

The several isoforms of tetanus neurotoxin and the many isoforms of botulinum neurotoxin currently known are very similar in terms of amino acid sequence, three-dimensional structure and biochemical mechanism of action in neurons. However, tetanus and botulinum neurotoxins cause two different forms of neuroparalysis (spastic and flaccid, respectively) because they affect different types of neurons: tetanus neurotoxin paralyses central inhibitory interneurons in the spinal cord, while botulinum neurotoxin paralyses peripheral and affects central cholinergic neurons [11].

Tetanus has been described since Hippocrates, who first identified the main symptoms of spastic paralysis in this disease [12]. Symptoms of botulism begin with paralysis of the cranial nerves, ptosis and diplopia, followed by impaired swallowing. The paralysis then gradually spreads to the skeletal and respiratory muscles, including the diaphragm, leading to respiratory depression and death (10). Respiratory and skeletal muscle paralysis is accompanied by damage to the cholinergic neurons of the autonomic nervous system, with associated symptoms [13]. Exposure to botulinum and tetanus neurotoxins is not accompanied by anatomical damage and neuronal necrosis, making their recovery possible upon toxin neutralization [10]. In fact, neurotoxins, once inside neurons, have a limited life cycle, which depends on a number of factors, including neuronal sensitivity and the susceptibility of neurotoxins to intracellular protein degradation mechanisms [14].

STRUCTURE AND MECHANISM OF ACTION OF TETANUS AND BOTULINUM NEUROTOXINS

Tetanus and botulinum neurotoxins are proteins consisting of a light chain (L, molecular weight 50 kDa) and a heavy chain (H, molecular weight 100 kDa) connected by a disulfide bridge and folded into four domains, each playing a specific role in affecting nerve endings [15]. The light chain is folded into N-terminal domain and represents a zinc-dependent endopeptidase specific to one or three SNARE complex component proteins (VAMP, SNAP-25, syntaxin). Membrane proteins of SNARE mediate neuroexocytosis and release of neurotransmitters into synaptic cleft [3, 8].

The L-chain is surrounded by a peptide belt formed by the HN domain (N-terminal part of the heavy chain, 50 kDa). This domain is characterised by two long α-helices and additional shorter helices arranged around the inter-chain disulfide bond [11]. The HN domain, which facilitates L-chain translocation into the cytosol, is linked to the carboxyl end of the 50 kDa heavy chain H, which in turn consists of two domains called HC-N (molecular weight 25 kDa) and HC-C (molecular weight 25 kDa). HC-N mediates neurotoxicity, although its exact role is not fully understood and may differ between different StN and BNT serotypes [16]. There is evidence that HC-N is involved in toxin binding through interaction with negatively charged lipid microdomains [17]. In turn, HC-C is responsible for the neuroselectivity of tetanus and botulinum neurotoxins and for their intraneuronal transport, which determines the peripheral activity of botulinum neurotoxin and the central activity of tetanus neurotoxin after its peripheral capture and retrograde axonal transport into the spinal cord [14, 18, 19].

The different transport pathways of tetanus and botulinum neurotoxin within neurons are not mutually exclusive, as tetanus neurotoxin can cause local peripheral paralysis

and botulinum neurotoxin can migrate retrogradely within neurons and be released into the CNS at different levels [14, 20]. Both tetanus toxin and botulinum neurotoxin affect their specific targets, presynaptic nerve endings, through a similar mechanism related to their modular structure and consisting of five main steps binding to the presynaptic membrane, internalisation of bound toxin into the cytosol via endocytosis, translocation of the L-chain into the cytosol via the HN domain, disruption of the inter-chain disulfide bond with release of the L-chain to express its catalytic activity (as a metalloprotease) in the cytosol and selective cleavage of one or more SNARE complex proteins with subsequent blockade of neurotransmitter release.

Let's take a closer look at each of the five steps listed.

Step 1: Neuroselective binding to peripheral nerve endings

Tetanus neurotoxin and botulinum neurotoxin bind with high levels of selectivity and affinity to the presynaptic plasma membrane through interaction with polysialogangliosides, which are rich in nerve endings and protein receptors [18, 21]. Polysialogangliosides contain an oligosaccharide moiety including several negatively charged sialic acid residues that protrude over the unmyelinated surfaces of the presynaptic membrane of neurons, which facilitates the binding of large proteins such as neurotoxins or oligosaccharide-specific immunoglobulins [7, 22]. This is facilitated by the fact that polysialogangliosides are negatively charged and embedded in membrane regions containing anionic lipids [23, 24], whereas tetanus and botulinum neurotoxin proteins are dipoles in which the binding domain is positively charged. This allows the botulinum neurotoxin molecule to be reoriented as it moves towards the membrane, increasing the probability of binding to the receptor [22, 25].

Step 2: Internalisation of bound toxin

The binding of tetanus and botulinum neurotoxins to polysialogangliosides allows the toxins to diffuse across the lipid presynaptic membrane, which greatly increases the probability of their binding to a second receptor. Available evidence suggests that botulinum neurotoxins bind to the luminal domain of an integrin protein present on the membrane of synaptic vesicles (11). This protein is either synaptotagmin-1/2 for botulinum neurotoxin types B, D/C and G or synaptic vesicular glycoprotein 2 (SV2) for botulinum neurotoxins types A and E as well as tetanus neurotoxin [26]. Neurotoxins subsequently penetrate the lumen of synaptic vesicles [11].

Synaptotagmin is represented by 13 protein isotypes (*Syt1–Syt13*). The synaptotagmin molecule includes an N-terminal transmembrane element-binding fragment and two C2 domains (C2A and C2B) that bind calcium. The C2A domain binds three calcium ions, while C2B binds two ions. Synaptotagmin is a calcium sensor that participates in the last stages of neurotransmitter release into the synaptic cleft. It binds to neurexin and SNAP-25, carrying out secretory vesicle retention at the presynaptic membrane, and is involved in neurotransmitter release through regulation of SNARE complex proteins when calcium is increased [27]. Calcium does not bind directly and does not modify the SNARE complex; however, calcium-binding proteins, which are known to be located near the active sites, act as mediators. In neurons, synaptotagmin isoforms 1 and 2 are major calcium-sensitive proteins that regulate SNARE nucleus formation and vesicle docking, priming and fusion. C2 domains are also known to bind phospholipids, such as phosphoserine and phosphoinositol phosphates, and facilitate vesicle and plasma membrane fusion. Phosphatidylinositol-4,5-diphosphate (PIP2) is an important plasma membrane component required for SNARE-mediated membrane fusion. Synaptotagmin-1 has been shown to interact directly with PIP2, facilitating calcium uptake by the C2 domain [28].

SV2 are transmembrane proteins present on every secretory vesicle (including synaptic vesicles) and are crucial for neurotransmission. The results of the structural and functional studies conducted suggest that SV2 proteins may play several roles in ensuring proper vesicular function. Among these roles are their ability to stabilize mediator content in vesicles, to maintain and orient the released vesicle pool, and to regulate vesicle sensitivity to calcium to ensure efficient and coordinated mediator release [29].

In mammals, SV2 comprises three isoforms (SV2A, SV2B and SV2C). The SV2A isoform is selectively expressed in a subpopulation of motor nerve terminals of slow muscle fibres, whereas the SV2B and SV2C isoforms are expressed in motor nerve terminals [30]. SV2 contains 12 transmembrane helices. Its N- and C-termini are on the cytosolic side. Botulinum neurotoxin type A recognises the fourth luminal domain of SV2 (SV2-L4) and can use all three homologues as its receptors [26]. Studies have established that botulinum neurotoxin type E uses SV2 as its functional receptor, as it has been shown that the binding and penetration of botulinum neurotoxin type E into neurons lacking all SV2 is blocked [31]. Studies on cultured hippocampal and cortical neurons have shown that tetanus neurotoxin uses SV2 as its functional receptor [32]. To date, it is not known whether botulinum neurotoxins types F and C have their own protein receptors [26].

Although tetanus and botulinus neurotoxins are very similar in structure, tetanus-induced tetanus neurotoxin is markedly different from botulism and is characterized by spastic paralysis. Tetanus neurotoxin is produced by *C. tetani*, whose toxin-producing ability is encoded in a plasmid. Characteristically, the tetanus pathogen only becomes pathogenic when it comes into contact with oxygen-deprived tissues of the living organism.

Both tetanus and botulinum neurotoxins target the peripheral motor nerve terminals into which they enter, but

the important difference between them is that the L-chain of botulinum neurotoxin is released into the cytosol of motor neurons, whereas tetanus neurotoxins are preferentially transported retrograde along the axon of the motor neuron to the soma [18]. The tetanus neurotoxins are then released from the motor neurons and re-enter the connective inhibitory neurons, where the L-chain neurotoxin is finally released into the cytosol and blocks neurotransmitter release. Loss of inhibitory input leads to hyperactivity of motor neurons, which in turn causes spastic paralysis [26].

The protein receptor responsible for the retrograde axonal transport of tetanus neurotoxins remained unknown for a long time. Early studies described the penetration of tetanus neurotoxins into a pool of endocytic vesicles called signalling endosomes. Clathrin-dependent endocytosis with sequential activation of guanosine triphosphate hydrolases Rab5 and Rab7 to transmit neurotrophic signals from the periphery to the soma of peripheral neurons was cited as the key mechanism [33]. Recent experimental data have shown that the proteins nidogen-1 and nidogen-2, which interact reversibly with the basal lamina, are protein receptors that, along with polysialogangliosides, are responsible for directing tetanus neurotoxin to signal endosomes [19, 34]. These organelles move via retrograde axonal transport to the soma and release tetanus neurotoxin into the cerebrospinal fluid near the presynaptic membrane of inhibitory interneurons, which subsequently bind and endocytose the toxin within synaptic vesicles, similar to botulinum neurotoxins [26].

Step 3: Translocation of the L-chain into the cytosol

After neurotransmitter release, synaptic vesicles are extracted from the plasma membrane and replenished with neurotransmitters. The latter process is influenced by a transmembrane pH gradient generated by the proton pump vesicular adenosine triphosphatase, which acidifies the lumen of synaptic vesicles. The low pH in the lumen is used by tetanus and botulinum neurotoxins to translocate their L-chains into the cytosol through a conformational change of the toxin molecule. An HN domain is involved in this process, which inserts into the membrane of the synaptic vesicle, forms an ion channel and promotes the translocation of the L-chain to the cytosolic side of the membrane [8]. Exactly how this occurs has not yet been precisely established. Two models have been proposed, which differ according to the presumed function of the transmembrane HN channel. According to the first model, the formation of the HN channel is a precondition for L-chain translocation, i.e. the L-chain passes through the pre-formed HN channel [35]. Another model suggests that the channel is formed during (or immediately after) the passage of the L-chain through the membrane, i.e. the appearance of the HN channel is a consequence of L-chain translocation [8].

Step 4: Disruption of the inter-chain disulfide bond with release of the L-chain for expression of its catalytic activity in the cytosol

During and after translocation, the L-chain must undergo conformational changes, and Hsp90, the main cytosolic chaperone protein involved in protein folding, was found to be involved in this process. Accordingly, specific inhibitors of Hsp90 prevent intoxication of nerve terminals caused by tetanus and botulinum neurotoxins [36, 37]. On the cytosolic surface of synaptic vesicles, the L-chain remains disulfide-bound to the H-chain. At the end of the translocation process, the disulfide bond is degraded by the intracellular thioredoxin reductase (TrxR)-thioredoxin (Trx) redox system, which has been found on the cytosolic side of the membrane of synaptic vesicles [38]. The TrxR-Trx system interacts with Hsp90 and releases the L-chain to express its catalytic activity in the cytosol [37]. Specific inhibitors of this redox system prevent the development of tetanus and botulism in experiments on mice and act against all serotypes of botulinum neurotoxin [39–41].

Step 5: Selective cleavage of SNARE complex proteins followed by blockade of neurotransmitter release

Upon release into the cytosol of the neuron, the L-chain acts as a metalloprotease and cleaves one or more proteins of the SNARE complex: VAMP is an integral protein of the synaptic vesicle membrane, while SNAP-25. synaptosomal-associated protein, 25kD) and syntaxin are located on the cytosolic surface of the presynaptic membrane. They form a heterotrimeric complex, which serves as the main mechanism for membrane fusion, ensuring the release of the neurotransmitter into the intersynaptic space [42]. The discovery that tetanus and botulinum neurotoxins cleave SNARE complex proteins, preventing neuroexocytosis of mediators, was the most convincing experimental evidence of the essential role played by SNARE complex in neuroexocytosis [43]. Synaptic activity was found to be extremely sensitive to the cleavage of even minimal amounts of SNAP-25. Experiments show that cleavage of 10–15% of the total intracellular pool of SNAP-25 is sufficient for complete blockade of neurotransmitter release [22].

TOXICOLOGY OF TETANUS NEUROTOXIN AND BOTULINUM NEUROTOXINS

Both neurotoxins studied affect different parts of the nervous system whose normal functioning is essential for survival. In all cases, they deliver a metalloprotease inside the target neurons that specifically cleaves, one by one,

the proteins required for normal neurotransmission. The combination of neurospecificity, enzymatic activity and the importance of target neurons for survival makes tetanus and botulinum neurotoxins the most potent mammalian poisons [11]. Their toxicity varies depending on the botulinum neurotoxin isoform in question and the route of entry. The oral and respiratory routes are less effective than the intramuscular, intravenous or intraperitoneal routes. Only one serotype of tetanus neurotoxin is currently known, and the lowest semi-lethal dose for intraperitoneal administration in mice is about 0.2 ng/kg, corresponding to a femtomolar concentration, assuming uniform distribution in circulating fluids [44].

A recent review of the available literature showed that for botulinum neurotoxins, the half-lethal dose in mice ranged from 0.02 to 5 ng/kg [44]. The half-lethal dose of recombinant botulinum neurotoxin type D for mice is 0.02 ng/kg for intraperitoneal administration, whereas for humans botulinum neurotoxin type D has low toxicity [45], which illustrates the dependence of toxicity of tetanus and botulinum neurotoxins on animal species. This issue has previously been extensively studied for tetanus neurotoxin [44], but a comparable data set for botulinum neurotoxins is still lacking to date. In fact, few of the many dozens of isoforms of botulinum neurotoxins have been studied in terms of toxicity to various animals, also because many of them have only been identified by advances in computational genomics [46]. The evolution of the different isoforms of botulinum neurotoxins is likely to be related to the physiology and ecology of each animal species, including invertebrates [47].

CONCLUSION

The toxic potential of tetanus and botulinum neurotoxins results from targeting a physiological function that is essential to the life of all vertebrates. Research to date has uncovered the molecular basis of action of tetanus and botulinum neurotoxins, including neurospecific binding and mechanisms mediating the catalytic cleavage of neuroexocytosis proteins. Nevertheless, several questions remain unresolved, in particular concerning the details of toxin endocytosis into synaptic vesicles as well as the process of L-chain translocation across the synaptic vesicle membrane and its subsequent release into the cytosol.

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