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INDIRIZZO DI BIOLOGIA CELLULARE

CICLO XXV

The entry of tetanus and botulinum neurotoxins into neurons

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Preface

Even if the modern concept of “toxin” was not described until the 19th century, the lethal effects of some bacterial diseases were well known even in the past. Accordingly, ancient texts report the symptoms of some pathologic syndromes which are nowadays known as plague, tetanus, botulism, diphtheria, cholera or carbuncle. Plague became particularly notorious thanks to the description of its symptoms along chronicles of history but also in many and many novels or holy texts. Not only, the historical documentations of toxins as well as bacterial agents employed as weapons are also described. Often these episodes were interpreted as divine signs of teaching as reported in the Bible or castigation as believed about the death of “Alexander the Great” who consider himself as a god.

Nowadays, it is clear that gods are foreign to such proceedings and that infective diseases are caused by microorganisms, namely viruses or bacteria. Anton van Leeuwenhoek, during the 1670s, was the first person to see bacteria thanks to a self assembled handcrafted microscope. He sent a report of his observation to the Royal Society of London with many detailed drawings but his work was considered with skepticism, probably because the existence of unicellular organisms was entirely unknown and somehow anachronistic. Therefore, in the next period, the knowledge that the world was crowded with small organisms was considered interesting but rather irrelevant. It took a long time before people figured out that bacteria and viruses were the cause of some diseases and were at the same time involved in their spreading from one person to another. Despite this, the idea of infectious diseases has been entertained during the 18th century by some enlightened people, like Antonio Vallisneri, who foresaw the “poisonous nature of some little worms”. Eventually, the proof that microorganisms not only exist but actively take part in life and health of people, was achieved with the works of Louis Pasteur and Robert Koch, even if only one century later. Their experiments, through which the existence of bacteria and pathogens was stated, represent the cornerstones of the modern microbiology and their principles are still used as paradigms (pasteurization and Koch’s postulates). Moreover, in 1884, Koch had the intuition, later confirmed, that pathogenic bacteria, i.e. anthrax bacilli, could elicit their harmful effects due to “poisons” produced inside

them and then released into the host. In the same year, Friedrich Loeffler guessed that another “soluble poison” was the etiologic agent of diphtheria. Such idea opened the doors to the modern concept of toxin and toxicology. Almost 100 years later, in 1959, two independent scientists confirmed Koch hypothesis demonstrating that bacteria-free preparation from *V. cholerae* injected in animal models mimicked the bacterium deadly symptoms. Ten years later the causative agent of cholera was isolated and biochemically characterized, becoming the prototype of a big family of biochemically, immunologically and pharmacologically related toxins[1].

Starting from the observation of pioneers as Koch, Pasteur, Loeffler and many others, microbiology and toxicology have become two of the most important branches of “life sciences” and their breakthroughs along the time not only have contributed to human and animal health, but also have equipped modern science with new “instruments which dissociate and analyze the most delicate phenomenon of living structures”, therefore “by attending carefully to their mechanism in causing death, scientists can learn indirectly much about the physiological processes of life[2]”.

The last sentence, stated by Claude Bernard in 1866, well fits with the properties of Clostridial Neurotoxins[3] (CNTs). In a recent review, in light of the last discoveries, Prof. Mauricio Montal has defined these proteinaceous toxins as a marvel[4], because of their structural architecture and intramolecular partnership which allow very accurate binding, penetration and eventually modification of a very specific process of host physiology. Exactly due to these features, such bacterial exotoxins are the most poisonous substances known and are therefore considered as potential bioweapons. On the other hand, the same properties have allowed a safe use for therapeutic as well as for cosmetic purposes[5]. Moreover, they are widely used by researcher as a tool to accurately manipulate certain physiological processes in a very specific manner, avoiding some drawbacks which could be generated by other kind of approach[3].

In this scenario, the study of clostridial neurotoxins not only has represented a great goal for human health and safety, but had also influenced the science by providing a very useful instrument to aliment and create new knowledge.

Abstract

Tetanus and botulinum neurotoxins cause neuroparalysis by inhibiting neuroexocytosis. They are composed by two main chains: the 100 kDa heavy chain (H) mediates the neurospecific binding to target cells and chaperons the entry of the 50 kDa light chain (L). After binding on the plasma membrane, these neurotoxins enter into nerve terminals via endocytosis inside synaptic vesicles, as shown here for the first time by immuno-electron microscopy. The lumenal acidic pH induces a structural change of the neurotoxin molecule that becomes capable of translocating its L chain into the cytosol, via a transmembrane protein-conducting channel made by the H chain. This is the least understood step of the intoxication process primarily because it takes place inside vesicles within the cytosol. In the present study, we describe how this passage can be made accessible to investigation by making it to occur at the plasma membrane of neurons. The neurotoxin, bound to the plasma membrane of cerebellar granular neurons in the cold, was exposed to a low pH extracellular medium and the entry of the L chain was monitored by measuring its specific metalloprotease activity with a ratiometric method. We found that the neurotoxin has to be bound to the membrane via at least two anchorage sites in order for a productive low-pH induced structural change to take place. In addition, this process can only occur if the single inter-chain disulfide bond is intact. The pH dependence of the conformational change of tetanus neurotoxin (TeNT) and botulinum neurotoxin (BoNT) /B, /C and /D is similar and takes place in the same slightly acidic range, which comprises that present inside synaptic vesicles. Thanks to this reliable method we have also studied the temperature dependence and the time course of TeNT, BoNT/C and BoNT/D L chain entry across the plasma membrane. The time course of translocation of the L chain varies for the three neurotoxins, but remains in the range of minutes at 37 °C, whilst it takes much longer at °20 C. BoNT/C does not enter neurons at 20 °C. Translocation also depends on the dimension of the pH gradient. These data are discussed with respect to the contribution of the membrane translocation step to the total time to paralysis and to the low toxicity of these neurotoxins in cold-blood vertebrates.

Another fundamental event along CNTs neuron intoxication process is the reduction of the interchain disulphide bond. This is a *conditio sine qua non* to free the catalytic part of the molecule in the cytosol of neurons. By using specific inhibitors of the various cytosolic protein disulfides reducing systems, we show here that the NADPH-Thioredoxin reductase-Thioredoxin redox system is the main responsible for this disulfide reduction. In addition, we indicate auranofin, as a possible basis for the design of novel inhibitors of these neurotoxins.

BoNT/A is the most frequent cause of human botulism and at the same time is largely used in human therapy. Some evidences indicate that it enters inside nerve terminals via endocytosis of synaptic vesicles, though this has not been formally proven. The metalloprotease L chain of the neurotoxin then reaches the cytosol in a process driven by low pH, but the acidic compartment wherefrom it translocates has not been identified. Using immunoelectron microscopy, we show that BoNT/A does indeed enter inside synaptic vesicles and that each vesicle contains either one or two toxin molecules. This finding indicates that it is the BoNT/A protein receptor SV2, and not its polysialoganglioside receptor that determines the number of toxin molecules taken up by a single vesicle. In addition, by rapid quenching the vesicle transmembrane pH gradient, we show that translocation of the neurotoxin into the cytosol is a fast process. Taken together, these results strongly indicate that translocation of BoNT/A takes place from synaptic vesicles, and not from endosomal compartments, and that the translocation machinery is operated by one or two neurotoxin molecules.

Another important aspect regarding CNTs research is their employment in human therapy. Accordingly, BoNT/A is almost invariably used in the treatment of many human diseases characterized by hyperactivity of peripheral cholinergic nerve terminals. Unfortunately, some patients are or become resistant to it. This drawback can be overcome by using other botulinum toxins, and pre-clinical studies have been performed with different toxin serotypes. Botulinum neurotoxin type D has never been tested in human muscles in vivo. Here we show that BoNT/D is very effective upon injection in mice, on the mouse hemidiaphragm preparation and on different rat primary neuronal cultures. From these experiments, doses to be injected in human volunteers were determined. The effect of the injection into the human Extensor Digitorum Brevis muscle was

assayed by measuring the compound muscle action potential at different times after injection. Botulinum toxin type D was found to be very uneffective in inducing human skeletal muscle paralysis. These results are interpreted in terms of recent reports on neuronal surface receptors of this neurotoxin and of the established double receptor model of binding.

Riassunto

Le Neurotossine clostridiali (CNT), sono esotossine di origine batterica che causano le due note sindromi neuroparalitiche tetano e botulismo attraverso il blocco della neuroesocitosi. Sono composte da due catene principali legate covalentemente da un unico ponte disolfuro. La catena pesante di 100 kDa (H) fornisce il legame neurospecifico e media l'ingresso della catena leggera (L) di 50 kDa nei neuroni bersaglio. Dopo il legame sulla membrana plasmatica, queste neurotossine entrano nei terminali nervosi all'interno di vescicole sinaptiche tramite endocitosi. Qui il pH acido induce un cambiamento strutturale della molecola che diventa capace di traslocare la catena L nel citosol, grazie ad un canale predisposto dalla catena H. Questo è il passaggio meno conosciuto lungo tutto il processo perlopiù a causa della sede dove avviene, ovvero piccoli compartimenti endocitici scarsamente manipolabili dall'esterno. Nel presente studio si descrive come questo passaggio sia stato reso accessibile all'indagine facendolo verificare sulla superficie dei neuroni. La neurotossina, legata a freddo alla membrana plasmatica di neuroni primari di cervelletto, è stata esposta ad un mezzo tamponato a basso pH per simulare quanto avviene nelle vescicole sinaptiche. L'ingresso della catena L è stato monitorato misurando l'attività metalloproteasica specifica con un metodo raziometrico. Abbiamo trovato che la neurotossina deve essere necessariamente legata alla membrana con almeno due siti di ancoraggio al fine di andare incontro ad un cambiamento strutturale funzionale alla traslocazione. Inoltre, questo processo può avvenire solo se il disolfuro intercatena è intatto. La pH-dipendenza del riarrangiamento conformazionale della tossina tetanica (TeNT) e delle tossine botuliniche (BoNT) B, C e D è simile e avviene nello stesso intervallo, in una condizione di media acidità, tuttosommato simile a quella che si pensa esistere all'interno vescicole sinaptiche.

Grazie a questo affidabile metodo di indagine, abbiamo proceduto studiando la dipendenza dalla temperatura e la cinetica della traslocazione di TeNT, BoNT/C e BoNT/D. A 37 °C, la traslocazione delle tre diverse tossine varia nel tempo, ma rimane sostanzialmente nell'intervallo di minuti minuti, mentre ne richiede molto più a 20 °C. BoNT/C non trasloca a 20 °C. La traslocazione, come precedentemente visto, dipende anche dalla dimensione del gradiente di pH. Questi dati vengono discussi considerando l'intero arco di tempo necessario alla tossine per intossicare i neuroni, così come la scarsa tossicità delle tossine nei vertebrati a sangue freddo.

Un altro evento fondamentale lungo il processo di intossicazione è la riduzione del legame disolfuro intercatena. Questa è una *conditio sine qua non* per liberare la parte catalitica della molecola nel citosol dei neuroni. Utilizzando inibitori specifici dei diversi sistemi ossidoreduttivi citoplasmatici, si è dimostrato che il sistema NADPH-tioredossina reduttasi-tioredossina, è il principale responsabile di questo evento. Inoltre, auranofin viene indicato come possibile molecola *lead* per la progettazione di nuovi inibitori di queste neurotossine.

BoNT/A è responsabile della maggior parte dei casi di botulismo nell'uomo e allo stesso tempo è indicata, quasi senza alternative, come agente terapeutico per il trattamento di numerose condizione patologiche. Alcune prove indicano che essa penetra all'interno dei terminali nervosi via endocitosi di vescicole sinaptiche, ma questo non è mai stato formalmente provato. La subunità catalitica accede quindi al citosol grazie ad un cambiamento conformazionale guidato da un gradient di pH. Tuttavia, quale sia il compartimento acido sfruttato dalla tossina per innescare il cambiamento conformazionale non è stato ancora determinato. Attraverso esperimenti di immuno detezione e miscroscopia elettronica, abbiamo dimostrato che BoNT/A sfrutta il riciclo di vescicole sinaptiche per essere internalizzata e che il numero massimo di tossine per vescicola è dettato dal numero di recettori proteici, nella fattispecie SV2, presenti all'interno della stessa, piuttosto che dal recettore glicolipidico presente sulla membrane esterna. A suffragio di ciò, la rapida inibizione dell'acidificazione dei compartimenti acidi attraverso specifici inibitori, mostra una cinetica di traslocazione di BoNT/A talmente rapida da poter escludere con certezza che tale evento possa avvenire a livello di organelli acidificabili diversi dalle vescicole sinaptiche, quali possono

essere ad esempio gli endosomi. Presi nel loro insieme, questi risultati propendono per un funzionamento di BoNT/A come una nanomacchina capace di traslocare la subunità catalitica attraverso l'impiego di una o tuttalpiù due molecole.

Un altro aspetto per cui la ricerca sulle CNT è importante è sicuramente il loro impiego in terapia umana. BoNT/A è usata nel trattamento di molte malattie umane caratterizzate da iperattività dei terminali nervosi periferici colinergici. Purtroppo, alcuni pazienti sono o diventano resistenti alla terapia. Questo inconveniente può essere superato utilizzando altre tossine botuliniche ed infatti studi pre-clinici sono stati condotti con differenti sierotipi di tossina. La neurotossina botulinica di tipo D non è mai stato testata *in vivo* su muscoli umani. Qui viene mostrato che BoNT/D rappresenta la tossina più efficace in preparazioni *in vivo* ed *ex vivo* nei topi. Da questi esperimenti preliminari, sono state determinate le dosi da testare in volontari umani. L'effetto della iniezione nel muscolo delle dita umano *Extensor Digitorum Brevis* è stata saggiata misurando la risposta del potenziale d'azione evocato nei muscoli a diversi tempi dopo l'iniezione. BoNT/D è risultata essere scarsamente efficace nella neuroparalisi del muscolo scheletrico umano ed è per cui poco appetibile per l'uso umano. Questi risultati sono stati interpretati considerando i recenti risultati in merito ai recettori sfruttati da tale sierotipo e il modo con cui la stessa si lega ad essi.

Keywords

Botulinum neurotoxins, Tetanus neurotoxin, translocation domain, interchain disulphide, neuromuscular junction

1. INTRODUCTION

1.1 Clostridial neurotoxins

The nervous system provides an essential function in animal physiology and even a minor modification within few neurons may result in a profound behavioral alteration. Accordingly, synaptic transmission is a key process in nervous system physiology where synaptic vesicles (SVs) play a major role. Many toxins act selectively on the nervous tissue. Interestingly, the most dangerous pathogens or animals, to subvert host functions at their advantage, have evolved different strategies aimed to impair the mechanisms involved in the neurotransmitters machinery. In general, neurotoxins specifically act on ion channels blocking the transmission of nerve impulse through the alteration of ion permeability. On the other hand, some bacteria of genus *Clostridium* have evolved an elegant as well as sophisticated mechanism, which affects synaptic transmission in another way. Such bacteria, among other, produce neurotoxins which are endowed with specific features aimed to accurately target nerve endings and efficiently catalyze the degradation of fundamental proteins within them. Such diabolic strategy results in the impairment of neurotransmitter release in some essential synapses, eventually causing two very remarkable neuroparalytic syndromes: tetanus and botulism.

1.2 General considerations on CNTs

Clostridial neurotoxins are the most poisonous substances known. One single isoform of TeNT is known, with an estimated human lethal dose of 2.5 ng/kg. On the other hand, many isoforms of BoNTs have been discovered, whose lethality is again supposed to be in the low nanograms per kilogram of body weight range (0.2–1 ng/kg). They are hundreds/thousands times more toxic than other well known bacterial protein killers like diphtheria toxin, anthrax toxin or cholera toxin[6]. Despite the very similar molecular and biochemical features of TeNT and BoNTs, which will be described in details in chapter 1.6, they elicit two very different clinical manifestations, paradoxically opposite, namely a spastic and a flaccid paralysis respectively. Their extreme toxicity depends on their selective action toward specific target cells, neurons. Tetanus and botulinum neurotoxins indeed work in the same way and likewise impair presynaptic neurotransmission. The neurotransmission blockade is caused by the cytosolic modification of the machinery which governs the neurotransmitter release. CNTs are indeed zinc dependent metalloproteases, which specifically reach and hydrolyze SNARE proteins, a family of fundamental proteins that coordinates the regulated neurotransmitter exocytosis mechanism. This cleavage impairs the

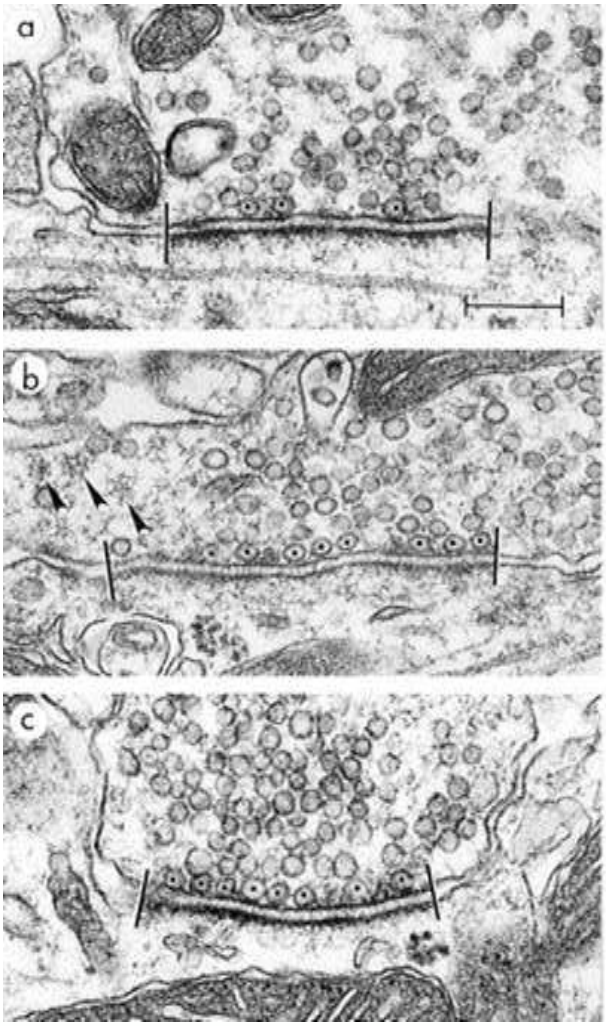

Figure 1: electron microscopy images of presynaptic active zone in control sample and in samples pretreated with CNTs. In (a) are visible docking vesicles within a control experiment, while in b and c are reported images from a culture treated with TeNT and BoNT A, respectively. The number of synaptic vesicles at the active zone is increased in toxin-treated terminals (from Neale et al, journal cell biology, 1999).

ability of SNAREs to bridge vesicles to the correct target membrane and therefore the fusion of neurotransmitter containing vesicles to the plasma membrane. Figure 1B-C shows the typical accumulation of vesicles on the cytosolic side of the presynaptic plasma membrane within the active zones caused by TeNT and BoNT/A respectively[7].

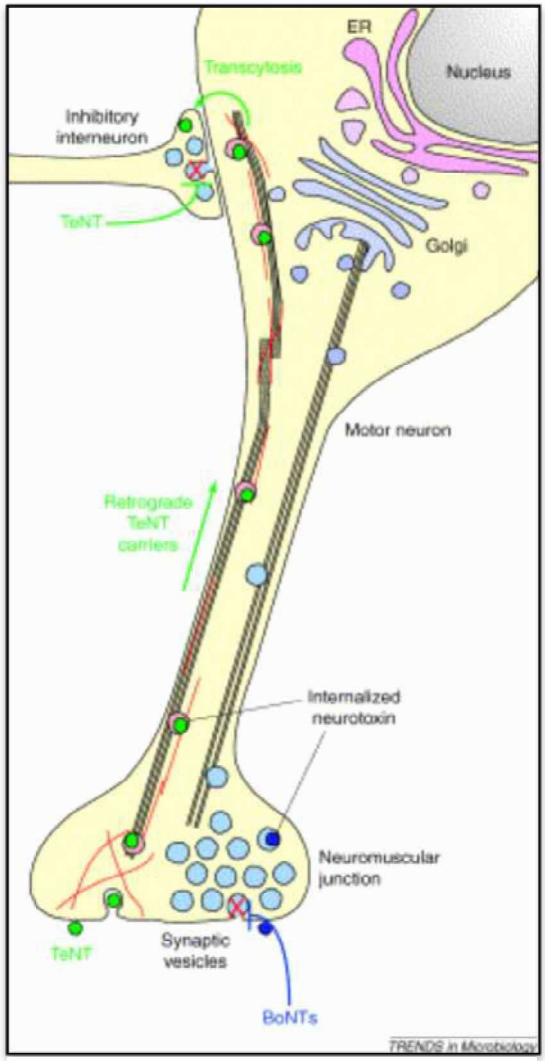

Figure 2: Site of action for BoNTs and retroaxonal transport of TeNT till inhibitory interneurons. interneurons (from Lalli et al, Trends in Microbilogy, 2003).

The different clinical outcomes depend from the different trafficking of TeNT with respect of BoNTs. As shown in fig 2, BoNTs bind and act into α-motoneurons, thereby inhibiting acetylcholine release from motor nerve endings which results in the impossibility of muscles stimulation and therefore contraction (flaccid paralysis).

On the other hand, also TeNT binds motoneurons at the neuromuscular junction but it is somehow hijacked in a protective environment[8, 9, 10] till the cytosol of motoneurons-afferent inhibitory interneurons through a retroaxonal transport[11]. Here, similarly to BoNTs, it blocks the release of synaptic vesicles filled of GABA or glycine, impairing the modulation of motoneurons excitability. This results in an uncontrolled activity of all muscles and therefore in the spastic paralysis typical of tetanus syndrome.

Even if cases of spastic paralysis had never been reported, central effects due to BoNTs intoxication have been recorded in many occasions, reviewed by M. Caleo and G.P. Schiavo in[12]. Consistently, BoNTs efficiently intoxicate central nervous system neurons *in vitro*[13] as well as *in vivo* after local administration[14]. Eventually, retroaxonal transport of BoNT/A has been elegantly described in 2008 by F. Antonucci[15].

1.3 Clinical features of botulism and tetanus

Botulinum neurotoxins are produced by different neurotoxigenic strains of the *genus Clostridium*, namely *C. botulinum*, *C. barati* and *C. butiricum*, even if the largest part of botulism outbreaks is caused by *C. botulinum*. Seven main BoNTs have been identified and classified as the seven serotypes BoNT/A, /B, /C, /D, /E, /F and /G because antigenically distinguishable[5]. Recently it has been found hybrid serotypes between BoNT/C and /D which display a mosaic composition, probably originated from recombination events, presumably through a phage-mediated mechanism. Accordingly they have been named BoNT/C-D and BoNT/D-C (BoNT/D South Africa). In addition to these, other botulinal toxins have been recently described and categorized as subserotypes. In total, more than 30 botulinum neurotoxins isoforms are described[16, 17]. Serotypes A, B, E, and F are associated with human botulism, while few records of human intoxication are reported for botulinum serotype C and D[18, 19], which are generally associated with avian or animal botulism[20-22].

C botulinum is ubiquitously present in the environment as spores. Germination can occur only in anaerobiosis and in a condition of low acidity (pH > 4.5). The intoxication is mainly caused by the ingestion of the mature toxin contained in contaminated food[23, 24] since clostridia colonization of the intestinal tract is quite difficult in adults. Unfortunately, it is more likely in babies and accordingly it has been recently claimed as the main cause of infant botulism[23, 24]. Similarly to tetanus, botulism can be caused also by the growing and the production of BoNTs in anaerobic wounds (wound botulism)[23, 24]. Iatrogenic botulism is recognized as another kind of the disease and it is due to the inappropriate administration or the abuse of the toxin during pharmaceutical or cosmetic treatments.

Despite the different serotypes, the signs and the symptoms of the disease are the same. The facial and throat muscles are the first affected, becoming progressively weak till the paralysis (figure 3). This results in diplopia, ptosis, and dysphagia. The paralysis continues its descent to the trunk. Autonomic nervous system is also involved, resulting in reduced salivation and lacrimation, nausea, vomiting and abdominal pain. When respiratory muscles are concerned, breathing is compromised and death comes through respiratory failure[25]. Therapy

is aimed to neutralize circulating toxin through anti-botulinum serum and keep alive patients through artificial ventilation if necessary. Unfortunately, no countermeasures against BoNTs are available once entered in target cells, even if many attempts had been explored[26-33]. The severity of the disease is therefore proportional to the amount of toxins, which have intoxicated the neurons before antitoxin administration. Accordingly, the duration of paralysis can last for weeks to months[20].

The single tetanus neurotoxin is produced by *Clostridium tetani*. It is strictly anaerobe because it lacks the redox enzymes necessary to reduce oxygen and therefore is disseminated in the ambient as spores. It can germinate only under appropriate environment, where oxygen tension is low, nutrients are present and pH is slightly acid. The best environment in humans is therefore represented by necrotic tissues of skin wounds, ruptures or abrasion. Here the spore germinates and produce the neurotoxin which accumulates in bacterium cytosol and is released only after cell autolysis. Therefore it is not surprising that the first clinical manifestation could arise even after 20 days after skin laceration. The hallmarks of the syndrome are a progressive rigidity and spasticity of muscles. Generally, facial muscles are firstly affected. The presence of *risus sardonicus* (fig.3) is very common and due to facial muscles spasms. The classical arching of the back (opisthotonus) is the consequence of trunk muscles affection along disease progression. Spasms can be so intense to cause bones breaking or joints dislocation. Whether breath muscles are affected, death for asphyxiation is likely, unless artificial respiration is readily available.

The medical treatment is firstly aimed to block the production of toxins, through antibiotics, and to neutralize any circulating toxins with appropriate serum, before they can enter into target cells. Whether toxins have not been neutralized in time, patients certainly will have the symptoms and the syndrome will be the more severe the more quantity of tetanus has been internalized. In such conditions, medical care is aimed to control muscle spasms through sedatives like benzodiazepines as well as to control pain. The best way to fight tetanus is for sure prevention, since prophylaxis is available and well tolerated. Accordingly, tetanus had been a major killer in the past but after the introduction of a systematic vaccination it is nowadays under control, at least in the developed

countries[34]. In poor countries a severe and unfortunately recurrent form of the disease is the neonatal tetanus, generally caused by the employment of not well-sterilized surgical instruments during the delivery.

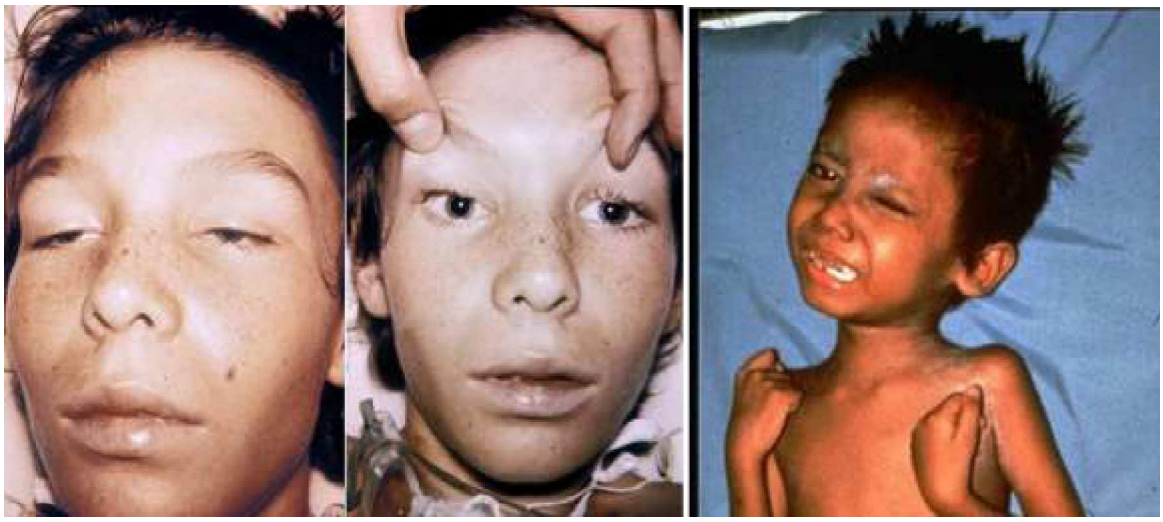

Figure 3: images of patients affected by botulism (left and central photos) **and tetanus** (right photo). Images collected on internet.

1.4 Tetanus and botulism: historical

Neurotoxigenic Clostridia producing tetanus and botulinum neurotoxins are ubiquitous bacteria disseminated in the environment. Generally speaking, shoddy methods in food conservation, inappropriate treatment of wounds and absence of organized health service had been the main causes of botulism and tetanus outbreaks along history, which probably has accompanied mankind since its beginning. Accordingly, reports of tetanus clinical symptoms had been described in the 5th century B.C. by Hippocrates, as well as ancient dietary law and taboos about the risk of consuming poisonous food may reflect the knowledge of botulism in remote time. On the other hand, scientific studies of such diseases and the discovery of their relationships with *Clostridia* have emerged only in a relatively recent past.

Carle and Rattone have described for the first time in 1884 the artificial transmission of spastic paralysis from a tetanized human to a rabbit and some years later, in 1889, Kitasato demonstrated that *C. tetani* spores reproducibly germinated under anaerobic condition and were able to reproduce tetanus in injected animals. The next year, he also showed that the inoculation of animals

with small amount of purified toxin resulted in protective sera production. In 1924 Descombey reported the development of neutralizing antibody induced by a chemically inactivated tetanus toxin[35] (tetanus toxoid).

The first scientific report which officially recognized botulism symptoms in correlation with food poisoning had been published in 1815 by a german health officer, Steinbuch, concurrently with a romantic poet became hereafter a medical officer, Kerner. The last one reported the complete description of symptoms, hypothesis on the etiology and pathophysiology of the toxin's action as well as the primitive idea of a possible therapeutic use of it. In 1895 Van Ermengem isolated C. botulinum from contaminated food and collected sera from died victims. These samples were compared with those gotten from a subsequent botulism outbreak, ten years later. Leuchs found that such strains as well as victims' sera were different. Later in 1919 Burke, at Stanford University, designated them as type A and B, opening the modern area of BoNTs research[35]. Hereafter all the other serotypes were identified. In 1949, Burgen has discovered in London that BoNTs block the acetylcholine release at the neuromuscular junction. Eventually, Schiavo and Montecucco in 1992 first have demonstrated that BoNTs and TeNT are metalloprotease which target SNARE proteins[36], causing the blockade of neurotransmitter release. The last discovery was the greatest evidence that BoNTs and TeNT, even if cause opposite clinical effect, are actually very similar at the molecular level. Accordingly, in the last twenty years, several groups has made many efforts in order to characterize in detail the structural and molecular details of CNTs, discovering that such toxins are indeed absolutely related.

1.5 Synapses and neuromuscular junction: CNTs battlefield

To better understand the reason of CNTs extraordinary toxicity, a highlight on synapse architecture and neuroexocytosis is needed.

The most well characterized chemical synapse is the neuromuscular junction (NMJ), which is a fundamental synapse within the peripheral nervous system. Here, electric impulses flowing from the central nervous system are translated in

a mechanic movement in the periphery, at the level of muscles, thereby permitting the locomotion. According to its fundamental role, NMJ is the target of BoNTs.

The presynaptic element of NMJ is the efferent motoneuron, while the post synaptic element is the skeletal muscle fiber. Motoneurons are very particular neurons. Their soma is within the spinal cord, therefore inside the central nervous system, but they extend proper axons outside, reaching muscles fibers in the peripheral nervous system. One motoneuron generally splits its myelinated axon in many (20-100) non–myelinated terminal fibers and each of them innervates a single muscle fiber, forming a motor end plate. Together, motor end plates belonging to a single motoneuron form the so-called motor unit, as seen in figure 3.

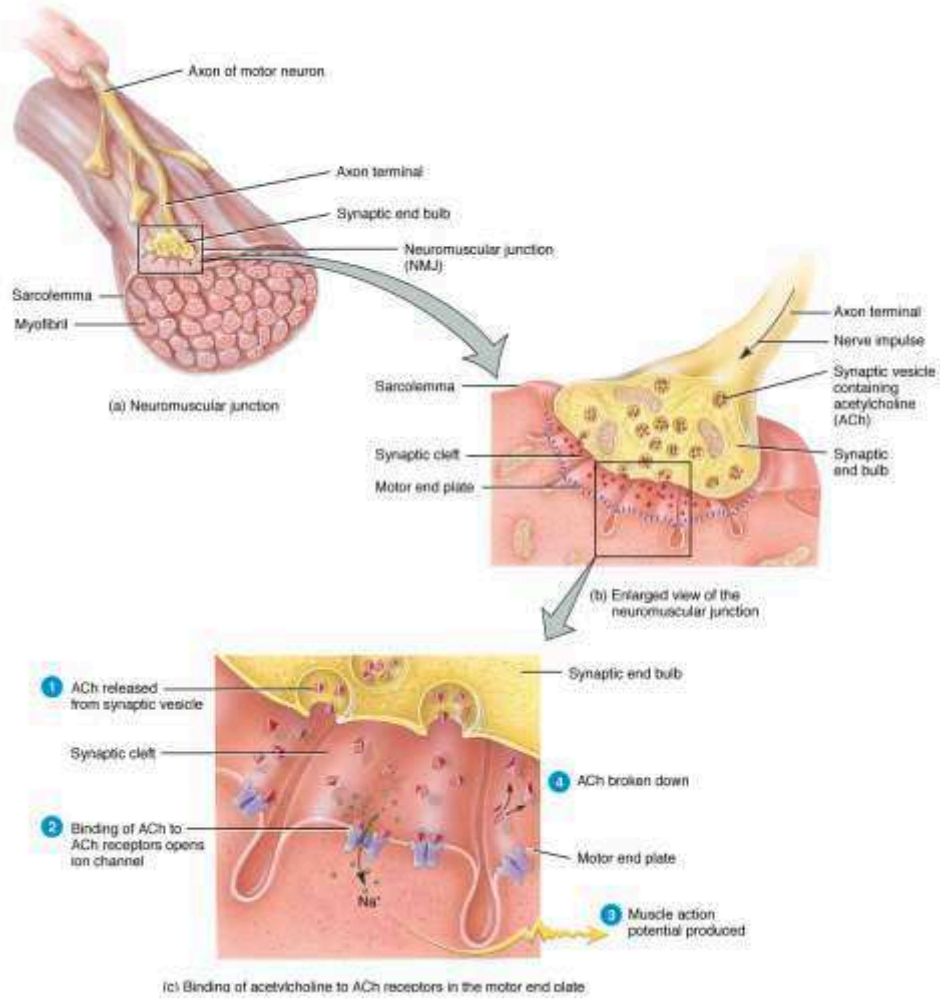

Figure 4: **organization of neuromuscular junction**. (a) motor end plates belonging to a motoneuron; (b) magnification of a single motor end plate; (c) synapse of the neuromuscular junction composed by both presynaptic and post synaptic elements (image from internet).

Besides NMJ, each chemical synapse contains synaptic vesicles (SVs), specialized cytoplasmic carriers, which store neurotransmitters, the molecules responsible of postsynaptic excitation. SVs are held in an actin framework in close proximity of the presynaptic membrane, in zones dedicated to the neurotransmitter release, i.e. the active zones. Upon the arrival of an action potential, SVs undergo to Ca^{2+} dependent exocytosis, after which they are retrieved from plasma membrane and recycled to regenerate exocytosis competent vesicles. Accordingly, SVs contain the machinery required for neurotransmitter uptake and storage, such as transporters, ion channels and the vesicular H^{+} ATPase, which provides the chemical energy to fuel the other proteins. Actually, SV are considered "dirty" nanostructures. Indeed, since they are very small, 40nm, their composition is probably not fixed and can be varied by cytoplasmic and plasma membrane elements. In 2006, Takamori and colleagues tried to dissect the protein and lipid composition of SVs. Interestingly, among the others, they found molecules like synaptobrevins, syntaxins, SNAP25, SV2, synaptotagmins and gangliosides, which are directly involved in CNTs activity. In figure 4 is reported the molecular model of a SV[37].

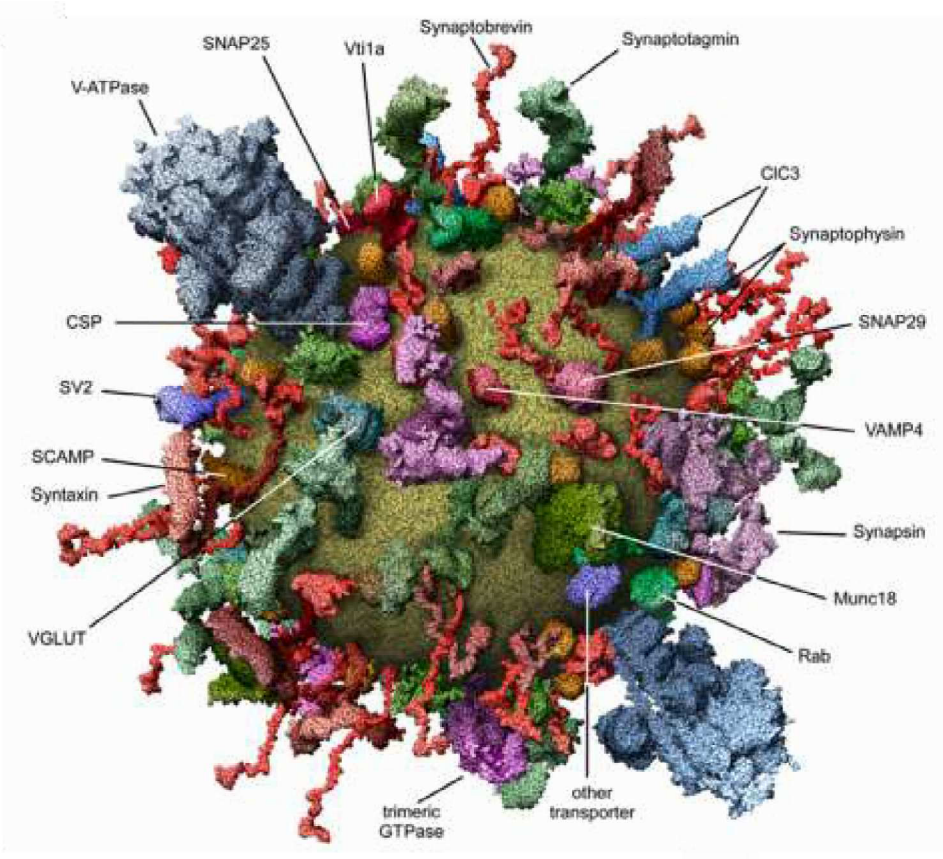

Figure 5: molecular model of a synaptic vesicle (from Takamori et al, Cell, 2006).

SVs are moved from their actin matrix to the active zones upon action potential formation. Here vesicles dock and fuse with the plasma membrane to deliver the neurotransmitter in the synaptic cleft. Such event is promoted by a concerted mobilization of proteins, which eventually results in the fundamental interaction of a highly conserved superfamily of proteins known as SNAREs (Soluble NSF Attachment Protein Receptors). Such proteins are supposed to control recognition and fusion of almost all the vesicular carriers within the cell. Generally, two kinds of SNARE proteins can be distinguished: v–SNAREs and t–SNAREs. The first are present on the vesicle, which is going to fuse, while the latters are present on the target membrane. In SVs, SNARE parterre is composed by the v–SNARE synaptobrevin 1/2 (also known as VAMP1/2 or Vesicle Associated Membrane Protein 1/2) and by the t–SNAREs syntaxin 1A and SNAP25. When fusion occurs, these three proteins interact together thanks to a highly conserved cytosolic domain, known as SNARE motif. It consists in 60-70 aminoacids segment, which can reversibly assemble a parallel four alpha helical bundle, called SNARE complex. As seen in figure 6, VAMP and syntaxin 1A contribute with one SNARE motif, while SNAP25 zip the complex with two[38].

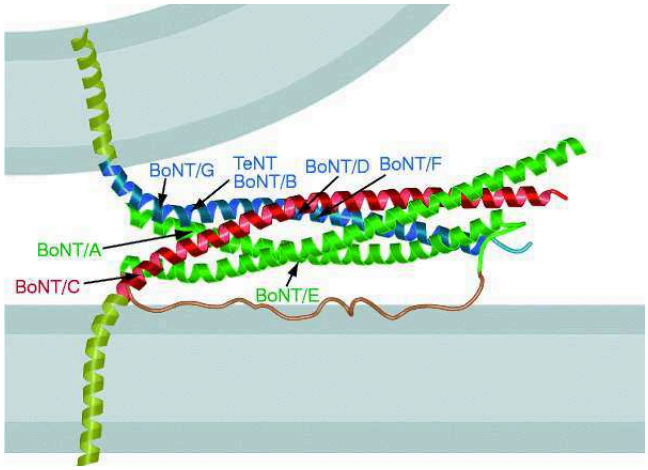

Figure 6: Hypothetical model of the synaptic fusion complex CNTs cleavage sites. In blue VAMP, in green SNAP25 and in red syntaxin 1A (from Sutton et al, Nature, 1998)

It is supposed that the formation of such complex releases a quantum of energy necessary to overcome the repulsive forces among opposed membranes and therefore provides the energy necessary for membrane fusion. Anyway, even if such phenomenon is of great interest, the detailed mechanism is still ambiguous and many players present either on SV and on plasma membrane are probably involved[39]. Nevertheless, what is important to consider for our purposes is that SNARE proteins assemble the minimal machinery compatible for fusion, suggesting that they are absolutely fundamental for neurotransmitter release and therefore in neuroexocytosis[40, 41]. Accordingly, it cannot be considered a coincidence that CNTs have evolved a strategy to specifically block neurotransmission by acting on SNARE proteins.

1.6 CNTs structure: intramolecular partnership

The clinical outcomes of TeNT and BoNTs are very different, almost opposite in their manifestations. Despite this, all CNTs intoxicate their target cells and produce the same intracellular modification, which is responsible for the final phenotype in a very similar manner. Moreover, the discovery that also BoNTs and not only TeNT can be retrotransported[12, 15, 42], has definitely demonstrated that such neurotoxins are more similar than how much was thought in the past and therefore TeNT cannot be considered as the "black sheep" of the flock.

Accordingly, from the structural point of view, CNTs are absolutely related. They are produced by the bacteria as a single polypeptidic chain of 150 kDa which are subsequently cleaved by specific bacterial or host proteases into the final, active toxins[43]. As schematized in figure 3, the mature toxins are composed of two main chains, linked by a unique disulphide bond.

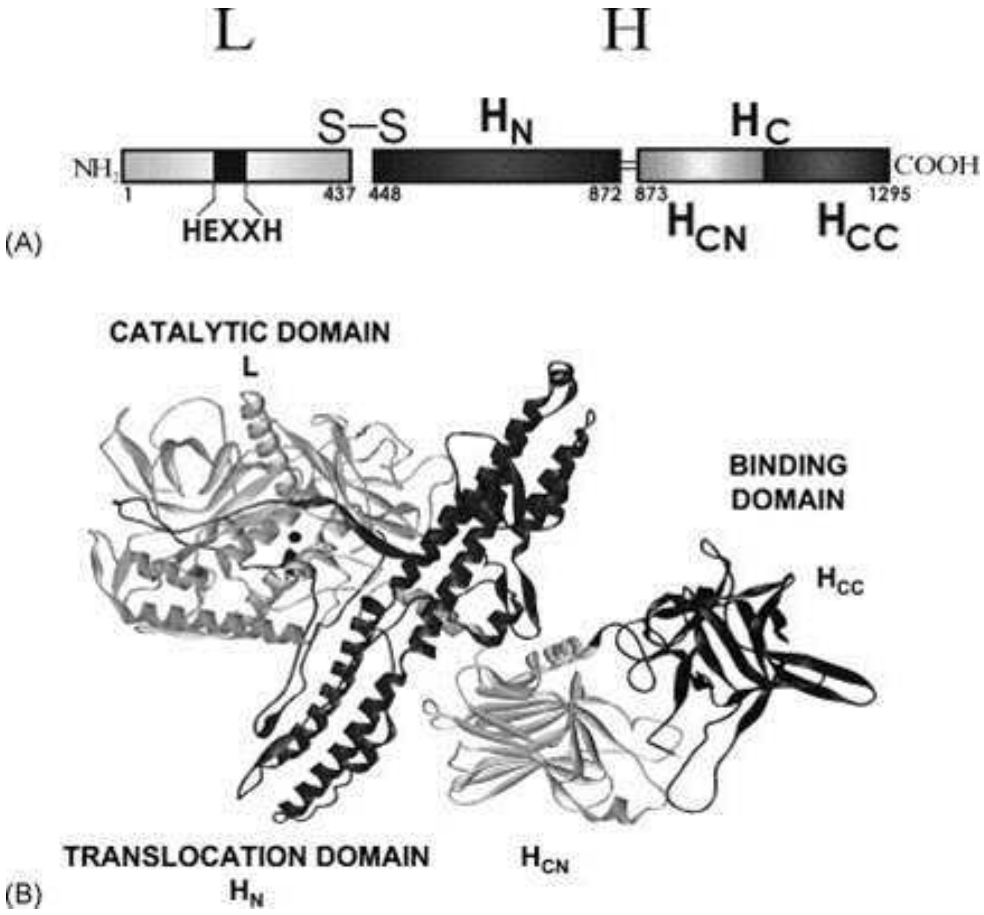

Figure 7: CNTs structure. (a) Schematization of modular organization of CNTs; (b) ribbon representation of BoNT/A crystallographic structure (from Rossetto et al, Toxicology Letters, 2004)

The smaller chain, 50 kDa, called light chain (L), is the catalytic part of the toxin and is responsible for the protease activity toward SNARE proteins. The bigger chain, 100 kDa, is called heavy chain (H) and can be functionally divided in two subdomains. The amino-terminal part (H_N, 50 kDa), also known as translocation domain, in response to low pH, inserts inside membrane and forms cation-selective channels[44-47] and has been ascribed to the L chain delivery from the uptake compartment to the cytoplasm, across an intracellular membrane[4, 48-50]. The 50 kDa carboxy-terminal part (H_C) is responsible for the specific binding to motoneurons plasma membrane[43].

The molecule can be considered as a nanomachine[4]. The crystal structures of BoNT/A, BoNT/B and BoNT/E reveal that the structure is modular[51]. Each part interplay with the others, resulting in a wonderful example of intramolecular partnership[4]. The intoxication process consists of 4 main steps[24, 52]: (1) the neurospecific binding, (2) the cytosolic internalization, (3) the membrane translocation and eventually (4) the SNARE degradation. Another fundamental event, which can be considered as an extra step, is the disulphide reduction of the interchain disulphide bridge, necessary in order to free the L chain into the cytoplasm. Besides the very similar tridimensional structure and functional organization, which result in a very similar intoxication mechanism, each neurotoxin maintains proper features, which make them reciprocally unique. These differences are mainly evident in some properties, such as binding modalities and target specificity, which will be described in the next sections.

1.6.1 H_C and binding

As previously said, the H_C is responsible and sufficient for the neurospecific binding at the neuromuscular junction[53-57]. As depicted in figure 7, it can be structurally subdivided into two parts, the 25 kDa C-terminal one (H_{CC}) and the 25 kDa N-terminal one (H_{CN}).

H_{CN} is a lectin like domain, highly conserved among CNTs, with a jelly role motif similar to that of carbohydrate binding protein (legume lectins). Actually, no carbohydrates binding properties have been reported in this domain and its function remains therefore still elusive. It has been reported its ability to bind

phosphatidylinositols and phosphoinositides, at least for BoNT/A and BoNT/C. it has been proposed that such interaction could assist the correct positioning of HN for the membrane insertion[58, 59].

Nevertheless, it is H_{CC} that harbors the fundamental features for the specific binding to motoneurons as well as for the different trafficking of TeNT with respect to BoNTs[43].

H_{CC} is a β-trefoil domain and its selectivity for neuronal membranes is one of the hallmarks, which makes CNTs the most poisonous toxins known. Accordingly, along the last 15 years, the structural and molecular details on the binding strategy of each toxin had been investigated.

Many groups have identified gangliosides as critical molecules for the specific binding to neuronal cells, reviewed by Binz and Rummel[43]. Gangliosides are a large family of glycolipids (glycosphingolipids) present on the external plasma membrane of all animal cells which are involved in many pathways like cell signaling, cell adhesion, protein sorting and are very important for membrane domain formation, existence and organization[60]. Neurons are particularly enriched in gangliosides, especially in axons and dendrites where they govern membrane curvature. The molecule is based on a ceramide-oligosaccharide framework modified with one or more sialic acids (monosialogangliosides and polysialogangliosides respectively) (PSG). Figure 8 shows an example of such molecules[61].

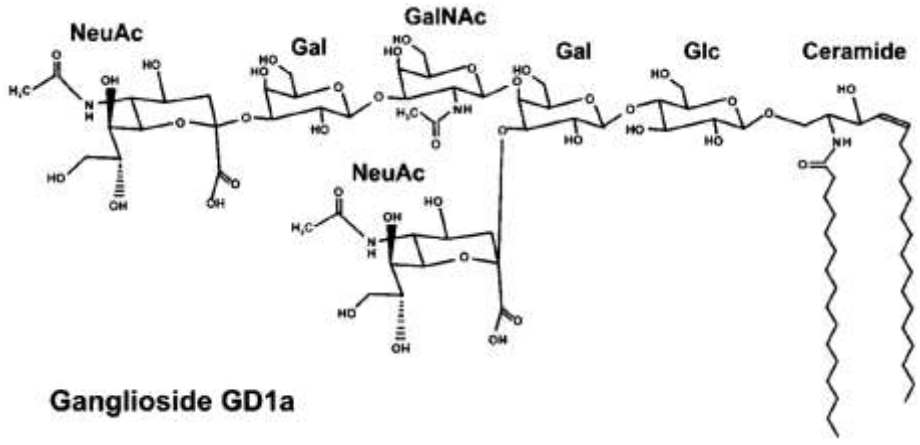

Figure 8: molecular structure of complex ganglioside GD1a,(modified from Vyas et al. PNAS 2002)

Many attempts have been explored to demonstrate the role of gangliosides in CNTs toxicity. TeNT and many BoNTs show high affinity toward PSG

immobilized on a solid substrate. Furthermore, removal of sialic acid residues with neuraminidase decreases BoNT/A and TeNT potency in spinal cord neurons[62], while insensitive chromaffine cells become sensitive to TeNT and BoNT/A by preventive decoration with gangliosides mixture[63-65].

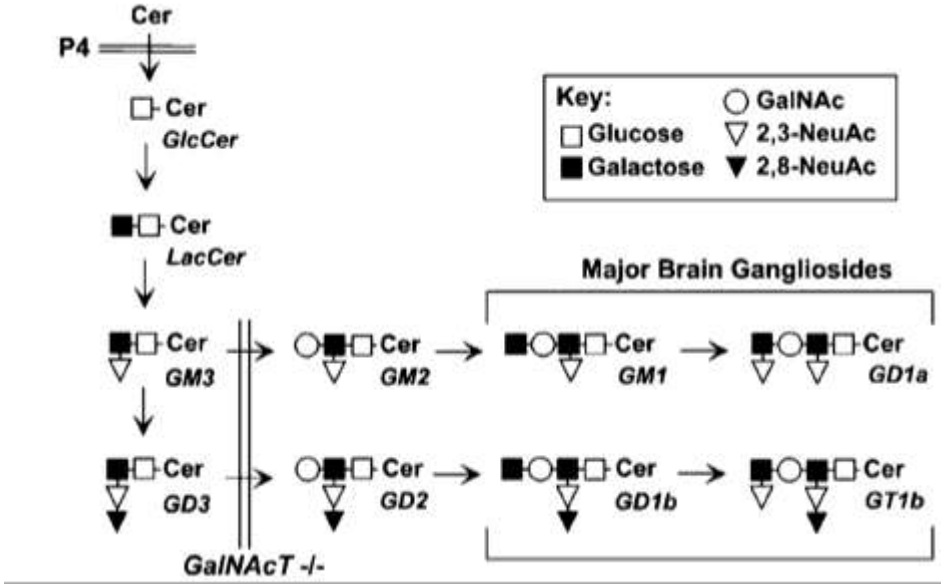

Figure 9: biosynthetic pathway for major brain gangliosides. Gangliosides are biosynthesized thanks to sequential addition of saccharides to the ceramide framework by specific enzymes (glycosyltransferases). Ganglioside nomenclature is here reported in accordance to Svennerholm[66] (modified from Vyas et al. PNAS 2002)

Accordingly, the inhibition of gangliosides biosynthetic chain (figure 9) in primary spinal cord neurons or in neuroblastoma cell line confers resistance to TeNT and BoNT/A respectively[67, 68]. Moreover, genetic ablation of NAcGal-tranferase, which blocks the biosynthetic pathway and limits gangliosides expression to GM3, GD3 or their precursor, reduces binding and entry of BoNT/A, /B, /E and /G in hippocampal neurons which can be restored by adding exogenous gangliosides[69, 70]. The same genetic ablation blocks TeNT and decreases BoNT/A and BoNT/B toxicity *in vivo*[71, 72].Similarly, mice expressing only Lac-Cer, GM3, GM2, GM1 and GD1 because GD3-synthase ablated, are TeNT resistant, but BoNT/A, /B, /E sensitive[73]. Knocking out both genes, double knock out mice, which can express only GM3 ganglioside, display resistance to BoNT/B and /G[74], while GM3 synthase knock out mice, which can express Lac-Cer solely, are refractory to BoNT/C[75]. It is therefore clear that complex gangliosides such as GD1a, GD1b and GT1b are fundamental components, which mediate the anchorage of CNT to neuronal membranes.

The interaction between CNTs and gangliosides at the molecular level has been extensively studied. Co-crystallization experiments of TeNT H_{CC} with GT1b have shown two main interaction sites. In one a lactose moiety interacts with the residues D1222, T1270, S1287, W1289, Y1290 and G1300, while in a second one a sialic acid interact with R1226[76]. In these interactions, W1289 and R1226 play a pivotal role for TeNT anchorage[77], and therefore these two sites had been named the W lactose (lactose binding pocket) and the R sialic site (sialic binding pocket)[78]. Chen and colleagues resolved the anchorage properties of each binding sites through solid phase binding assays. It was reported that the sialic site preferentially binds b series gangliosides, while the lactose binding site preferentially binds a series gangliosides[79]. Figure 10 schematically summarizes such interaction. It can be therefore concluded that gangliosides are necessary and sufficient for TeNT binding[78].

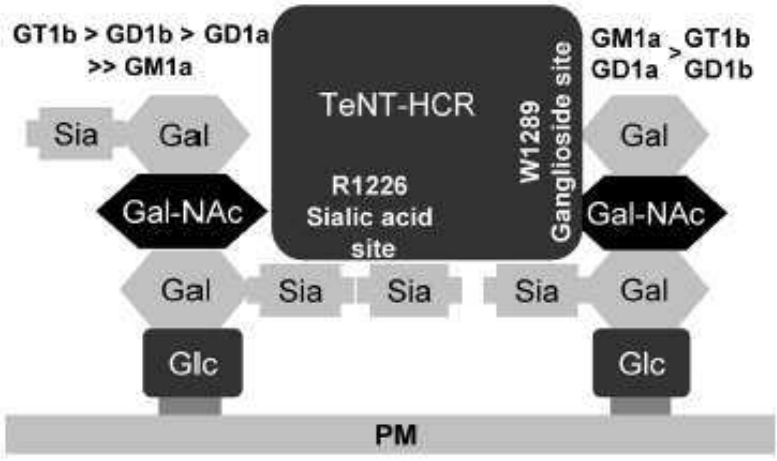

Figure 10: lactose and sialic binding sites in TeNT HCC and their interaction with different gangliosides (from Chen et al, biochemistry, 2008)

The lactose site in TeNT is composed by the sequence H...SXWY, which is typical for carbohydrate engagement sites and is found also in other bacterial toxins like ricin and cholera. In this kind of pocket, histidines imidazole ring and tryptophan indole ring are perpendicular to each other, while the tyrosine and serine form the backwall of the pocket. The interaction is generally supported by the aromatic residue which supplies a hydrophobic and planar surface where the ganglioside sugar ring can stack, while the polar residues interact with sugar hydroxyl groups, as shown in figure 11.

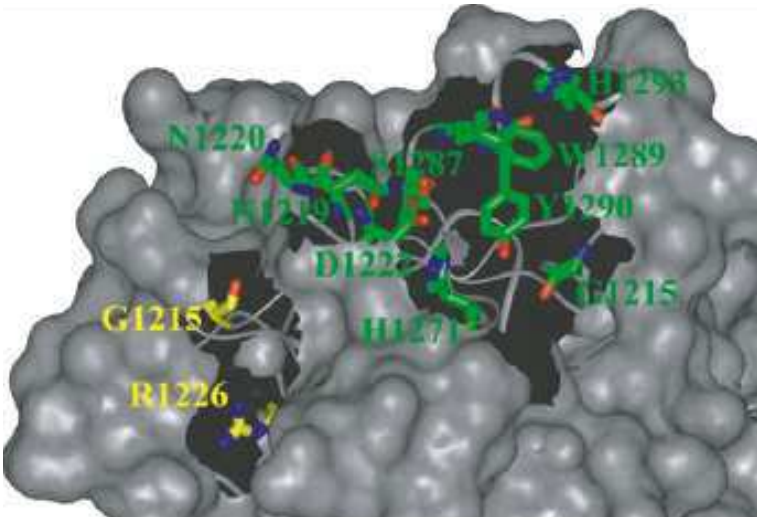

Figure 11: Sialic and lactose binding pockets of TeNT H_CC-domain. Amino acid residues that interact with them are shown in the stick model in yellow and green, respectively. (from A. Rummel et al, J. Mol. Biol. 2003)

BoNT/A, /B, /E, /F and /G share with TeNT the conserved lactose H...SXWY (in BoNT/G H is replaced with G and in BoNT/E H is replaced with K) and therefore, similarly to TeNT, they bind PSG oligosaccharide portion such as GD1b or GT1b[43]. Accordingly, Swaminathan and Eswaramoorthy have shown that BoNT/B interacts with carbohydrates through the lactose binding site[80, 81]. Interestingly, while mass spectrometry analysis with TeNT H_{CC} shows the retention of two carbohydrate molecules[77], the same experiment with BoNT/A and BoNT/B H_{CC} displays the retention of only one carbohydrate molecule[82]. Accordingly, because they bind only one gangliosides, the lactose binding site is called the gangliosides binding pocket (GBP). In figure 7 is reported the GBP of BoNT/A. On the other hand, the sialic binding pocket within such serotypes is not conserved at all. Actually, the fact that neurons stimulation increases the uptake and the entry of BoNT/A[83-85], has raised the possibility that BoNTs could interact with another partner which induces their entry through the endocytic compartment. Because stimulation of neurons increases exo/endocytosis events, and particularly the recycling of synaptic vesicles, the binding to the luminal domain of a SV protein, which is temporarily exposed to the

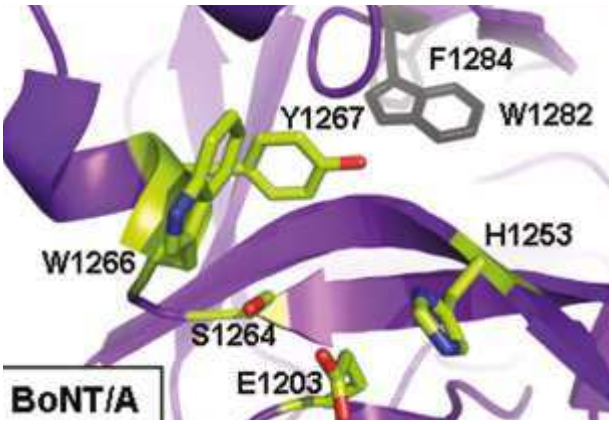

Figure 12: the ganglioside binding pocket of BoNT/A, from Karalewitz et al, Biochemistry 2010

outside and therefore available for the interaction, had been considered a very exciting option as well as the most likely explanation[43].

Montecucco first proposed that BoNTs extraordinary potency could derive from their ability to bind neurons via two receptors, one with low affinity, the gangliosides, which increases toxin density on target membrane, and a secondary one which triggers the internalization through the endocytic compartment[86]. Classical biochemical approaches lead to the discovery of BoNT/B interaction with synaptotagmin I and II[87, 88]. Subsequent studies provided by Dong and colleagues finally proved, through elegant loss/gain of function experiments, that BoNT/B binds gangliosides as low affinity receptor and exploits the interaction with synaptotagmin I/II for the internalization[89], experimentally demonstrating Montecucco's double receptor concept. A little later, it was shown that also BoNT/G could interact with synaptotagmin I/II *in vitro*[90]. This was confirmed some years later by biochemical experiments on neuronal culture as well as *in vivo*[69]. In 2006 Chai and Jin, together with their respective research groups, independently resolved the co-crystal structure of BoNT/B with synaptotagmin II[91, 92]. From the crystals, it has been understood that BoNT/B interacts with a string of 20 aminoacids within the N-terminal domain of synaptotagmin II (44–66), which is the luminal part of this protein, fundamental during SV exocytosis events. Such string is normally devoid of secondary structure, but assumes an helical structure upon interaction with a cleft in BoNT/B binding domain, located in proximity of the gangliosides pocket as shown in figure 13.

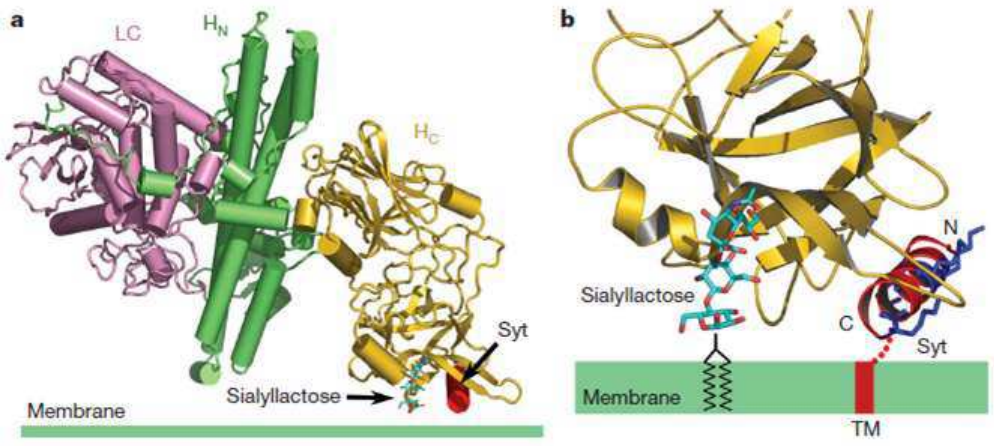

Figure 13: BoNT/B simultaneous interaction with gangliosides and protein receptor. (a) binding mode of BoNT/B on the membrane surface proposed by Jin and colleagues[92](PDB 1F31). In purple the light chain (L), in green the translocation domain (H_N), in green the binding domain (H_C) (b) magnification at the interface between BoNT/B and plasma membrane.

In 2007, Rummel and colleagues demonstrated through computer assisted analysis that, the same cleft within the binding domain of BoNT/G, interacts with the same synaptotagmin I/II aminoacids string[74]. BoNT/B and /G H_{CC} show a good degree of conservation[43], which is not shared by and among the other serotypes. Accordingly, other serotypes don't bind synaptotagmins. However, the discovery of BoNT/B proteinacious partner prompted the research in this direction. Accordingly, with similar approaches employed for BoNT/B, the protein receptors of other BoNTs have been rapidly identified. Immunoprecipitation, pull-down and competition experiments lead to the discovery of synaptic vesicle protein 2 (SV2) as the protein receptor of BoNT/A[93], BoNT/E[70] and BoNT/F[94]. Interestingly very recent studies revealed that BoNT/C and BoNT/D, differently from other BoNTs, bind neurons via two gangliosides, as TeNT does, but with different modalities. One binding site is located in the same position where BoNTs interacts with their protein partner[95, 96]. In BoNT/D the structural shape of the pocket is very similar to the sialic acid binding site of TeNT[95]. The other binding site is in a position related to the GBP of BoNT/A, /B, /E, /F and /G. The peptidic motif responsible of the interaction, similar among these neurotoxins, in BoNT/C is only partially conserved and in BoNT/D isn't conserved at all. Despite this, from the structural point of view the architecture of the pockets is maintained and therefore contribute for ganglioside accomodation[95], even if with peculiar modality. Figure 14a and b show the gangliosides binding sites within BoNT/D and BoNT/C H_{CC} respectively.

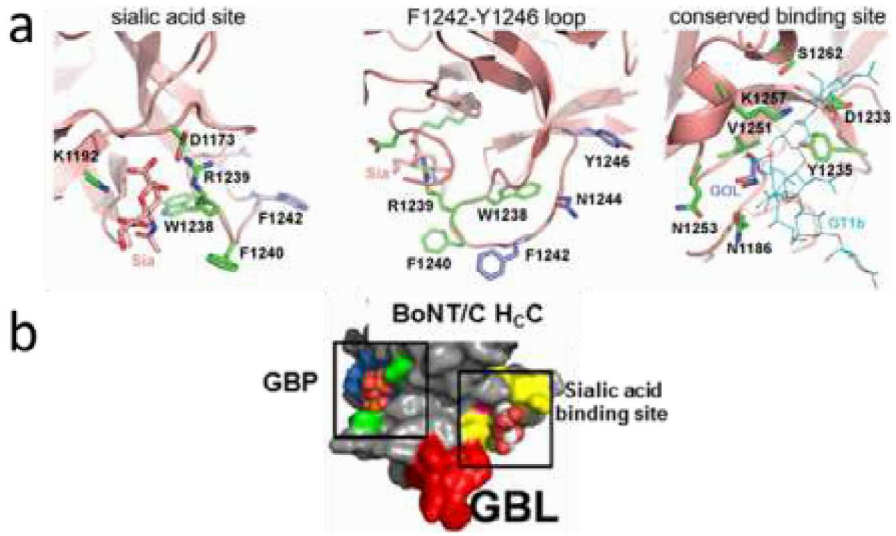

Figure 14: ganglioside binding pockets and binding loop within BoNT/D (a) and BoNT/C (b) H_CC (modified from Strotmeier et al, Biochem. Journal., 2010 and Karalewitz et al, the journal of biological chemistry, 2012)

Moreover, it has been reported that BoNT/C, /D and /D-SA have an extra site of interaction with gangliosides, in a position which is close to the GBP in other BoNTs[97, 98]. In BoNT/C, /D and /D-SA, from the structural point of view, this site is β-hairpin loop. Accordingly, it has been called gangliosides binding loop (GBL)[99]. This region is actually present also in BoNT/A and BoNT/B but it doesn't count for gangliosides interaction, probably because, differently from BoNT/C and /D-SA H_{CC}, they lack a fundamental tryptophan (W1258 and W1252 respectively) which interacts with the ganglioside. Actually, also BoNT/D has a tryptophan (W1238) in the GBL, but it seems to be not essential for the interaction, which is mainly mediated by two phenylalanine residues (F1240[97] and F1242[95]). Figure 15 shows the architecture of BoNT/C, /D and D-SA GBL with respect to BoNT/A and /B, highlighting the aminoacids fundamental for ganglioside interaction. Solid phase immobilization of different gangliosides, demonstrated that BoNT/C and BoNT/D[97] preferentially bind b series gangliosides, such as GD1b and GT1b, while BoNT/D-SA is unique among CNTs because prefers GM1a[99].

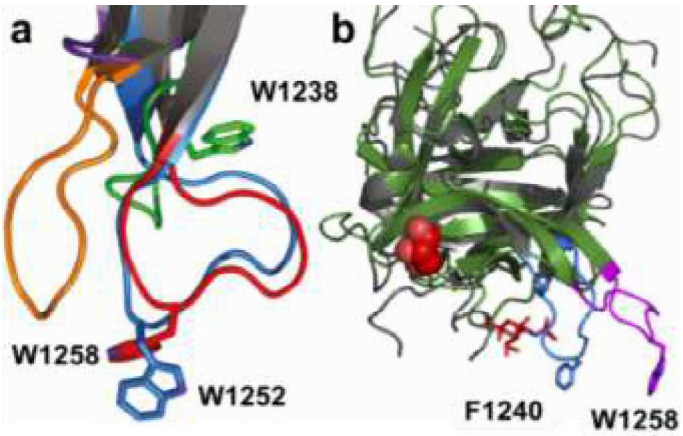

Figure 15: BoNTs molecular architectures of GBL. (a) BoNT/A (purple), BoNT/B (orange), BoNT/C (red), BoNT/D (green) and BoNT/D-SA (cyan) ganglioside binding loop with the tryptophan fundamental for ganglioside interaction (from Karalewitz et al, Biochemistry, 2010); (b) alignment of BoNT/D (gray) and BoNT/C (green) H_CC. Ganglioside binding loops and aminoacids involved in gangliosides interaction are colored in cyan for H_CC/D and purple for H_CC/C (from Kroken et al, the journal of biological chemistry, 2011).

Therefore it is possible summarize the binding properties of CNTs saying that TeNT, BoNT/C and BoNT/D bind to different kinds of ganglioside which are present on the external leaflet of neuronal cells, while other BoNTs, including BoNT/A and BoNT/B exploit at first a low affinity interaction with gangliosides, but, to properly complete their binding, they need the support of the luminal domain of a SV protein, i.e. SV2 and synaptotagmin I/II, respectively.

1.6.2 H_N and translocation

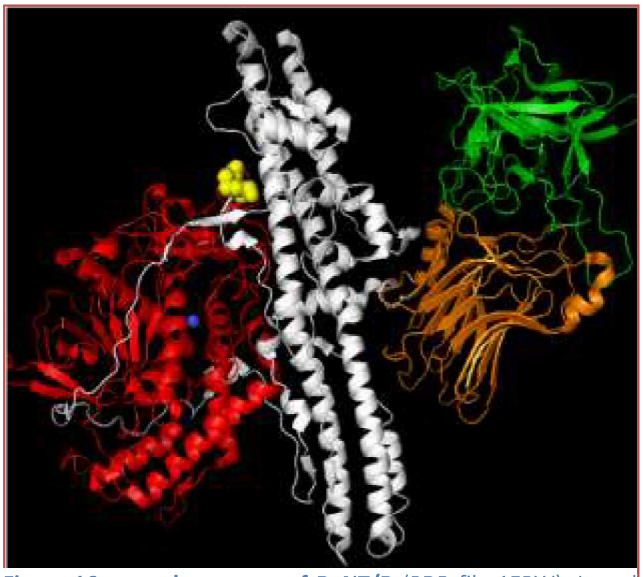

Figure 16: crystal structure of BoNT/B (PDB file 1EPW). In red the L, in white the translocation domain (H_N), in green the binding domain (H_{CC}) and in orange the lectin like domain (H_{CN}).

The N-terminal domain of the heavy chain (H_N) is a fundamental part of CNTs. It is supposed to mediate the translocation of the catalytic part from the endocytosed vesicle inside the cytosol in order to reach SNARE substrates. It is a 50 kDa polypeptide, corresponding to segment 685-827 in BoNT/A primary structure, with an elongated shape designed by two long and parallel α–helices[51, 80]. In addition to them, the H_N domain also consists of a random coil aminoacids string, called belt region that wraps around and partially shield the active site of the L. From the crystal structure of BoNT/A and BoNT/B it is possible to note that H_N confers, together with the other two domains, the characteristic butterfly shape to the toxins, as seen in figure 16. The translocation domain shows high degree of conservation among serotypes with exception of the belt[4]. H_N is similar to the configuration of some viral proteins which responds to acidification by changing their conformation[100, 101]. Accordingly, CNTs exploit the pH gradient, generated by the vacuolar proton pump, to change its molecular structure. This results in the insertion of the translocation domain inside the membrane, with the formation of a transmembrane protein conducting channel[4]. Channel formation and membrane translocation of CNTs have been studied with liposomes and planar lipid bilayers[44-48, 102]. Montal and Koriazova elegantly demonstrated that, upon acidification, BoNT/A was able to translocate across a planar lipid bilayer the catalytic subunit, with the formation of an ion conducting channel[48]. A more direct approach, using cell membranes patch clamping recordings, has been explored by many groups[103, 104]. Moreover Fisher and colleagues, with a similar strategy, demonstrated that BoNT/A heavy chain acts as a chaperone to maintain unfolded the light chain during membrane translocation and predispose the reduction of

the interchains disulphide bridge[4, 50, 105]. Interestingly, such approach demonstrates L chain membrane bypass through the progressive channel activity. Before acidification, current conductance is absent, then, lowering pH, ions leakage starts partially, because the channel is occluded by L transit. Eventually, it enters in an ion-conducting state as L is translocated (figure 17).

Even though such procedure is reliable and prone to external manipulation, one limitation is the lack of information about the efficiency of membrane translocation, because these approaches mainly analyze single channel events. Furthermore, no information about the modality through which translocation domain approaches the membrane can be deducted.

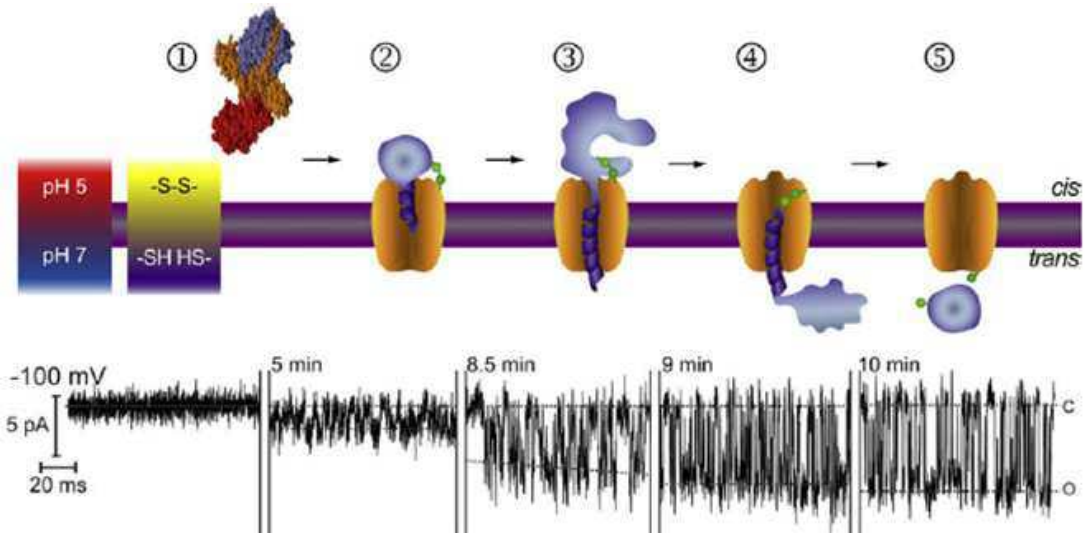

Figure 17: BoNTs translocation model. Sequence of events, which are supposed to happen inside SV to deliver the LC in the cytosol through the HC channel. In the lower part of the panel it is shown the channel conductance of BoNT/A upon acidification (from Montal, Toxicon, 2009).

From the crystal structure of BoNT/B bound to ganglioside and synaptotagmin II, two main hypotheses have been made about H_N positioning with respect to the other parts of the toxin and the membrane. Chai *et al.* proposed that H_N helices stay with a parallel orientation with respect to the membrane, facilitating their interplay[91] by placing hydrophobic segments in proximity to the vesicle membrane surface, (figure 18a). On the other hand, Jin and colleagues have suggested that H_N helices sit orthogonal to the plane of the membrane, as depicted in figure 18b[92].

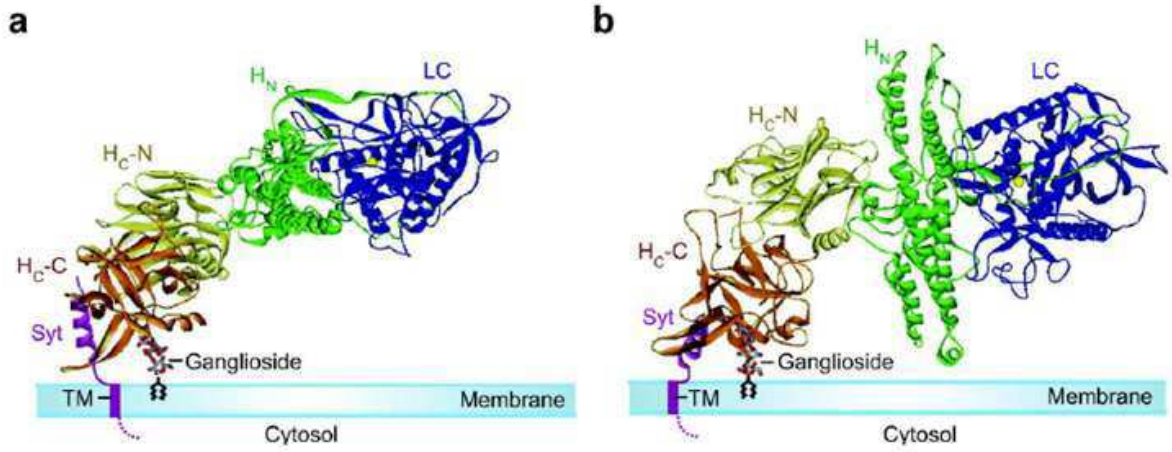

Figure 18: different orientations of BoNT/B H_N (colored in green) **with respect to plasma membrane** (a) Chai model; (b) Jin model (from Rossetto and Montecucco, Handbook of Experimental Pharmacology, 2008).

Translocation step remains the less understood process and little is known concerning the aminoacids, which regulate the conformational change of H_N for membrane insertion. Accordingly, understanding how translocation domain is positioned with respect to the plasma membrane is a question of major interest because could share light on the intramolecular events necessary for channel formation.

1.6.3 L chain and protease activity

Light chain is the catalytically active part of CNTs which is responsible for cytosolic hydrolysis of SNARE proteins and therefore of the blockade of neurotransmitter release. The crystal structures of all CNTs L chains have been determined at atomic resolution. They share a very similar tridimensional structure, which is similar to that of zinc dependent metalloprotease thermolysin. Even if they are very similar in structural organization each L have a unique SNARE specificity. Accordingly, as seen in figure 6, L/B, L/D, L/F, L/G and L/TeNT hydrolyze synaptobrevin–2 (VAMP2), L/A and L/E hydrolyze SNAP25, while L/C is unique and acts either on SNAP25 and syntaxin[4, 106, 107].

They all belong to zinc metalloprotease family but are endowed with a unique structure among them. The active site is related to that of thermolysin and has a core of aminoacids, conserved among CNTs[108] (peptide motif HEXXH, figure 7 and 19), which is essential for catalysis because it coordinates a catalytic zinc.

Figure 19: metalloprotease motif HexxH conserved among CNTs (from Montecucco and Schiavo, Molecular Microbiology, 1994).

Accordingly, the zinc is bound to such motif thanks to a glutamic acid and the imidazole rings of the histidines. A fourth coordination point is provided by a water molecule, bound to the glutamic acid[107] (figure 20). Due to its interaction with the catalytic water, the E residue is particularly important, indeed its mutation leads to complete loss of protease activity[109]. In addition to the catalytic core, there is a secondary layer of residues, less close to the zinc center, including Arg362 and Tyr365 (numbering of BoNT/A), which seems to be directly involved in the hydrolysis of the substrate[110-113].

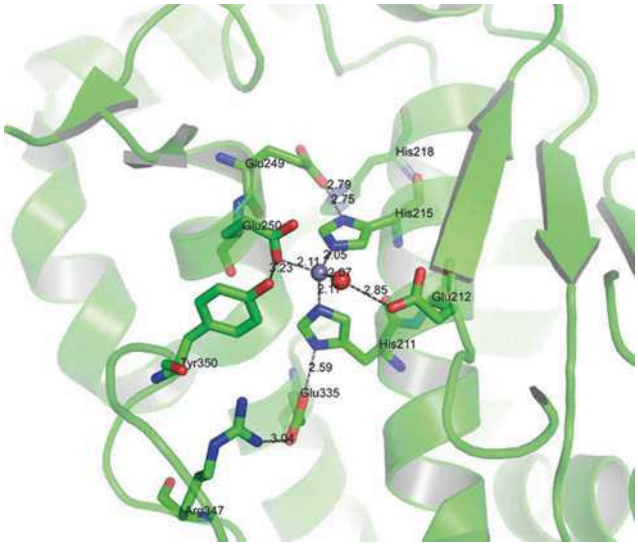

Figure 20: 3D structure of L chain active site with the tetrahedral motif coordinating the zinc atom. Aminoacids numbering is relative to BoNT/E sequence (from Swaminathan, FEBS journal, 2011).

As seen in figure 16, L is globular with a mixture of both α-helix and β-strand. The active site is located in a buried cleft where substrate can access along a groove in order to complementary fit for the processing, as depicted in figure 21. Interestingly, such groove can be occupied also by the belt region, suggesting that perhaps such structure could work as a false substrate to inhibit protease activity till the L is released in the cytosol through the reduction of the disulphide bridge[114]. Moreover, the crystallization of L in complex with its substrates has

provided practical evidence of an array of substrate-binding sites remote from the active site (so-called exosites)[115, 116]. A major role is played by a string of 9 aminoacids within the SNARE substrate. The motif is featured by the alternation of three acid aminoacids with hydrophobic and hydrophilic residues[116-119]. There are two copies of such motif in synaptobrevin and syntaxin, while there are four in SNAP25. Such exosites are fundamental for CNTs cleavage and accordingly the presence of at least one copy is a stringent condition for toxins catalytic activity. Accordingly, the high specificity for the substrates is mainly due to such interactions rather than active site architecture.

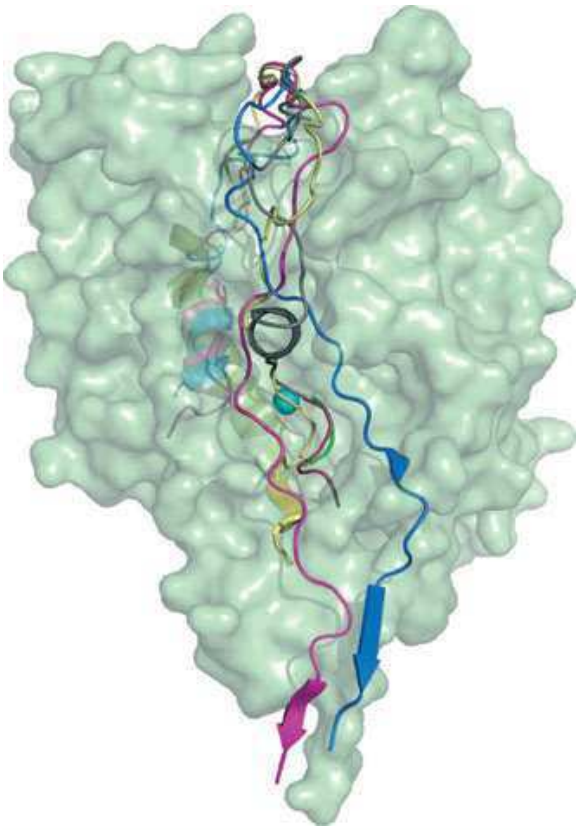

Figure 21: L chain and belt region. Substrates and belt region occupy the same groove. Belts of BoNT/A and E are shown in magenta and blue. SNAP-25 and VAMP are in yellow and grey. In orange the BoNT/A cleavable peptide QRATKM and in green the BoNT/E cleavable peptide RIME. All of them occupy the same groove (from Swaminathan, FEBS journal, 2011).

1.7 Mechanism of action

As said before, CNTs have a multi-modular architecture, which is functional to their mechanism of action. CNTs exert their neurotoxic effect through an intramolecular partnership among their different domains, which results in a multistep mechanism[24, 52]: binding, internalization, intracellular trafficking, membrane translocation and eventually proteolytic degradation of SNAREs. Figure 22 summarizes such main steps.

It is important to underline that what determines the CNTs trafficking inside nerve endings is the H_{CC}. As discussed above, CNTs have unique binding properties, which mediate their intracellular fate. The current accepted model, which describes CNTs binding and internalization, is the double receptor model. It consists in the idea that CNTs initially bind a molecule present on the extracellular leaflet of the plasma membrane and, only subsequently, interact with

another partner, possibly a protein, which mediates their penetration inside target neurons[4, 43, 120]. Accordingly, it has been proposed that the low affinity interaction with polysialogangliosides provides the accumulation of CNTs on peripheral neurons. Hereafter, the destiny of BoNTs and TeNT somehow diverges. Some BoNTs bind the luminal portion of a SV protein, strongly supporting the idea that they penetrate inside neurons *via* endocytosis of synaptic vesicles, though this has not been formally proven. Different groups have reported convincing evidences about BoNT/A, /E and /F binding to SV2[70, 93, 121-123] as well as BoNT/B and /G binding to synaptotagmins I/II[69, 74, 87, 88], which are SV proteins with a luminal portion.

The protein receptor of the other BoNT serotypes has not been conclusively determined. Recently, it has been proposed that BoNT/D and BoNT/D-C are internalized in central nervous system neurons thanks to SV2 and synaptotagmin I/II interaction, respectively[124, 125]. In agreement with what said before, it seems that BoNT/C binds neurons only through gangliosides interaction. Moreover, many attempts have been explored in order to identify a potential proteic partner but they have failed. Therefore, it could be that such toxin doesn't need the SV recycling to enter into target cells. Nevertheless, stimulation of cultured neurons, which increases SV recycling, enhances its internalization. Thus, to date it seems that SV recycling helps BoNT/C entry, but it is not clear whether gangliosides alone are sufficient to mediate its internalization[126].

Similarly to BoNTs, TeNT binds polysialogangliosides at peripheral nerve terminals. Nevertheless, its translocation in peripheral neurons cytoplasm is somehow blocked and the internalization vesicle hijacked into a retroaxonal pathway[8-11]. Great efforts have been made in order to understand the modalities of such event. Deinhardt and colleagues have made one major discovery by demonstrating that, at the periphery, TeNT is not internalized in SV as BoNTs, but it is present into clathrin–coated vesicles. Such sorting might be due to glycol–protein carbohydrates which undermine GD1b or other b-series gangliosides which have provided TeNT initial binding through the carbohydrate-binding pockets[127]. Accordingly, TeNT H_{CC} has been reported to interact with a GPI-anchored glycoprotein and to bind to lipid rafts[52, 57, 128, 129]. Therefore, TeNT would exploit the same trafficking of neurotrophins, like BDNF and NGF, in order to be retrotransported from the peripheral nervous system to the central nervous

system[8], where eventually it penetrates inside the cytosol of inhibitory interneurons.

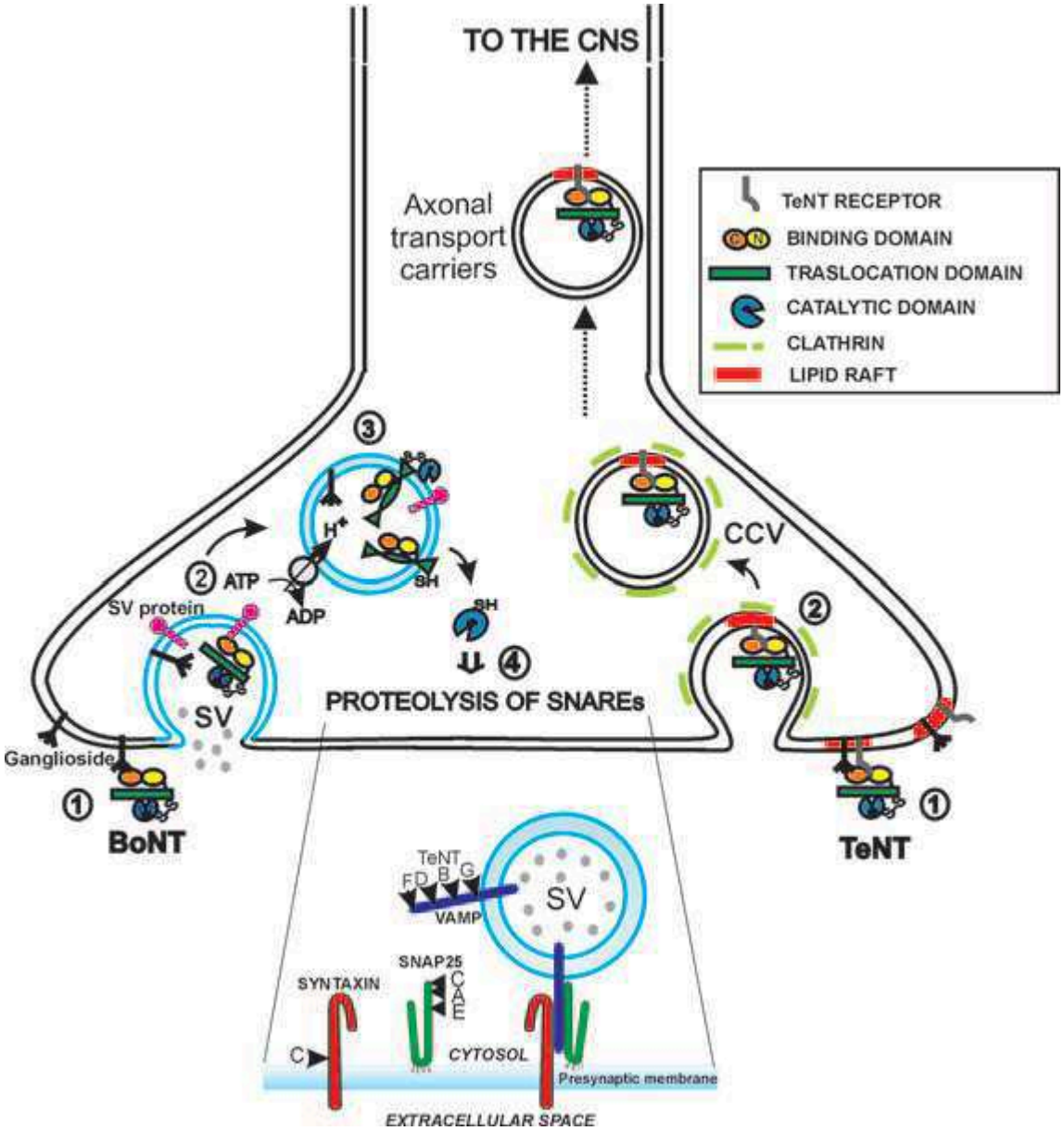

Figure 22: Anchorage and entry of BoNTs and TeNT at peripheral nerve terminal. (1) The BoNT binding domain associates with the presynaptic membrane of α-motoneurons as discussed before. (2) BoNTs are endocytosed within synaptic vesicles via their retrieval in order to be refilled with neurotransmitter. TeNT exploits a pathway requiring lipid rafts and clathrin-coated vesicles (CCV) and then is sorted into vesicle carriers of the axonal retrograde transport pathway. (3) The acidification of SV lumen provided by the v-ATPase, induces major conformational changes in CNTs structure which result in membrane insertion of the vesicle membrane and in the translocation of the L into the cytosol. (4) Inside the cytosol the L chain hydrolyzes of one of the three SNARE proteins VAMP, SNAP-25 and syntaxin, as depicted in the lower panel. The same four-step pathway of entry of BoNTs into peripheral nerve terminals is believed to be undertaken by TeNT in spinal cord inhibitory interneurons, which are reached after retroaxonal transport and release from the peripheral motoneurons. (from Rossetto and Montecucco, Handbook of Experimental Pharmacology, 2008).

Therefore, TeNT H_{CC} can be intended as a nice tool to track *in vivo* retroaxonal transport as well as an efficient system to deliver cargos to the central nervous system, bypassing the blood brain barrier[9]. To prevent the translocation of the L,

the vesicle where TeNT is internalized in nerve endings, displays a protected environment with a controlled pH[10]. Recently, it has been proposed by Yeh and colleagues that, once arrived close to target neurons, also TeNT could exploit the interaction with a SV protein, i.e. SV2 as BoNT/A, /E and /F do, to be internalized in a compartment compatible with the translocation of the L chain in the cytosol[130]. On the other hand, Blum and colleagues demonstrated that TeNT can enter into stimulated and non-stimulated neurons at the same extent. This suggests that the need of synaptic vesicles recycling as well as the presence of a proteic receptor could be not essential for tetanus entry into central nervous system neurons.

Recently, as aforementioned, it has been demonstrated that also some BoNTs may undergo to retroaxonal transport.

Once internalized in the final neuronal target, the CNT-receptor complex is exposed to an acidic milieu provided by the vesicular proton pump v-ATPase, which acidifies SV in order to create a pH gradient necessary for neurotransmitter refilling. Such acid environment induces a conformational change on toxins structure, leading to the insertion of the H_N, into the endosomal membrane[24, 131, 132], thereby forming a transmembrane protein-conducting channel that delivers the L into the cytosol where it acts[4, 105, 107]. Indirect evidence for the need of acidification along translocation is the fact that some drugs, which prevent vesicles acidification by v-ATPase specific inhibition, block CNTs entry and toxicity[85, 133]. Similarly, buffering the vesicular lumen with chemicals, the L cannot translocate and remains entrapped inside vesicle[134]. The low pH-driven toxin conformational change has been reported to take place in a very narrow range of pH: 4.4–4.6 for TeNT, BoNT/A, /B, /C, /E, and /F[132]. Unfortunately such experiments have been made with artificial membranes, which lack the complexity of a biological membrane. Nevertheless, what remains interesting is that all CNTs behave similarly in response to pH drop, suggesting that perhaps key residues involved could be conserved.

The diameter of the channel provided by the H_N is estimated to be around 15 Å. Given that the folded L exceeds such dimension, it must undergo to an unfolding process in order to pass through the channel. Circular dicroism and crystallographic evidences[135], demonstrate that the L secondary structure doesn't change significantly, rather, the tridimensional shape passes from a globular

configuration to a more elongated shape, suitable for channel transit. This has been proved by following the exposition of hydrophobic residues within L structure, as shown in figure 23[136].

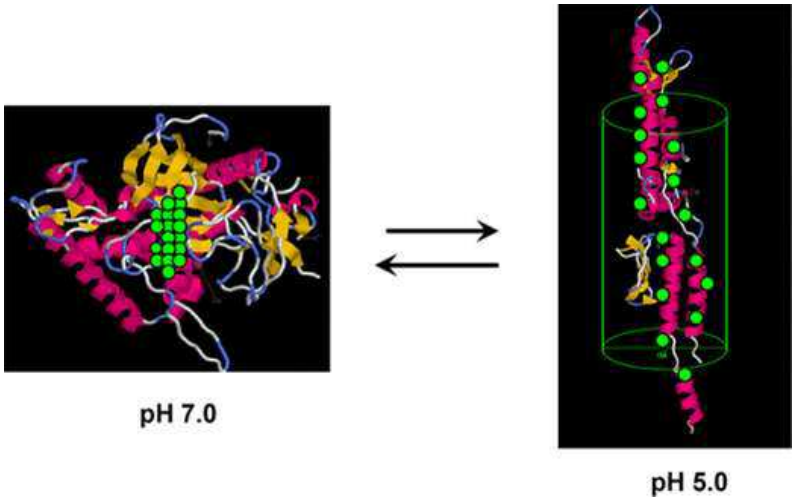

Figure 23: schematic model of BoNT/B LC tridimensional reorganization upon acidification (from Cai et al, Protein journal, 2006).

After translocation, the interchain disulphide must be reduced and the L chain refolded before reaching and processing its substrates. The catalytic activity is in fact achieved only after disulphide reduction, which is a *conditio sine qua non* that precludes toxin functionality. To date no one has understood either which is the cytosolic reducing agent or whether L is able to refold alone or needs the assistance of cytoplasmic proteins. However, once free in the cytosol, the cleavage of the SNAREs can eventually occur. The hydrolysis of one single kind of SNARE protein is sufficient for vesicle fusion abrogation, which thereby causes the severe paralysis pathognomonic of botulism or tetanus[4].

1.8 CNTs: "turning bad guys into good[3]"

As shown in figure 1, the most evident consequence of CNTs intoxication is the accumulation of SVs on the cytosolic face of the plasma membrane[7]. However, from the morphological and anatomical point of view, intoxicated neurons don't result damaged[137, 138]. Anatomically, contacts between muscles and nerves are completely maintained. On the other hand, the number of motor end plates on single muscle fibers and the number of fibers innervated by a single motor axon reciprocally increases, probably because, to prevent muscles atrophy and restore

the NMJ functionality, muscle cells release trophic factors. This results in similar alterations documented in other form of denervation. However, during a secondary late phase of NMJ recovery, newly synthetized elements degenerate and the original endplate recovers completely its functionality. The time of recovery can vary depending on the type of nerve terminal, the BoNT serotype, the animal species and its dosage.

Given that the paralyses of muscles is followed by a full functional recovery devoid of major alteration of the system, the employment of CNTs, especially of BoNTs, has found fertile ground as therapeutics for the treatment of a variety of human diseases. The first toxin used for human therapy was BoNT/A, approved by US Food and Drug Administration in 1989. It has been employed for the treatment of strabismus, blepharospasm and hemifacial spasm. From that day, the number of indications that can be treated with BoNTs has considerably increased. To date many human diseases, characterized by hyperactivity of peripheral cholinergic nerve terminals, as focal dystonias, spasticity, tremors, hyperhidrosis, migraine and tension headaches, but also cosmetic application are indicated for BoNT/A treatment[139]. Next studies, which have compared the duration of many serotypes, demonstrated that BoNT/A has the longer effect, whereas BoNT/B has a similar duration of action only when used at higher dosage. BoNT/F and BoNT/E have the shortest effect[140, 141]. BoNT/C has been reported to have a span comparable to BoNT/A[142]. The investigation of non–A, non–B BoNTs efficacy for human purposes is a very important issue from the therapeutic point of view. Indeed, over many years of clinical recording, the usage of BoNT/A has shown the existence of non–responding subjects as well as the drop of efficacy due to antibodies development. To date, unfortunately, few is known about the employment of other serotypes, such as BoNT/D or other mosaic toxins composed by recombination between BoNT/D and BoNT/C which could represent a reservoir for potential therapeutic employment. Table 1 shows a list of the main diseases treated with BoNTs.

Disease/condition	Clinical Effect	Benefit	Duration (months)
Blepharospasm	Established	+ + +	1–4
Hemifacial spasm	″	+ + +	2–6
Laringeal dysphonia	″	+ + +	1–6
Focal hyperhidrosis	″	+ + +	>12
Hypersalivation	″	+ + +	Several months
Oromandibular dystonia	″	+ +	1–3
Torticollis	″	+ +	1–3
Strabismus	″	+ +	Up to many months
Limbs dystonia	″	+ +	1–3
Occupational cramps	″	+ +	1–3
Myokymia	″	+ +	2
Facial synkinesia	Experimental	+ +	1
Pathological lacrimation	″	+ + +	<6
Achalasia esophagea	″	+ +	1–2
Anal fissure and rectal spasms	″	+ +	1–2
Bruxism	″	+	1–5
Spasticity	″	+	1–3
Urinary retention	″	+	1–2
Essential tremor	″	+	1–3
Nystagmus	″	+/–	?
Dysphagia	″	+/–	Up to many months
Facial wrinkles	″	+/–	?
Myofacial pain-dysfunction	″	+/–	?
Muscle-contraction headache	″	+/–	?
Single injections:			
Anal fissures		very effective	
Vaginism	Experimental	very effective	

Table 1: employment of BoNTs for human therapy (from Rossetto et al, Toxicon 2001).

1.9 Aims of the thesis

The translocation step remains the less understood event along CNTs neuron intoxication mechanism. Available informations indicate that such process is driven by a transmembrane pH gradient inside vesicles[4, 48, 132]. Most studies were performed with model membrane systems[48, 132], whilst *in vivo* experiments used as read out an indirect electrophysiological measurement. Even though such approaches have shared light on several molecular features of the translocation, no information on the efficiency can be inferred, because they report single molecules behaviors. During my PhD, I developed an assay that couples translocation event with its direct effect into neurons cytoplasm, i.e. SNAREs hydrolysis. Conditions that by-pass the SV endocytosis step and induce the entry of the L chain directly from the plasma membrane were found using primary cultures of cerebellar granular neurons (CGNs), which are almost exclusively composed of neurons, and the pheochromocytoma cell line PC12. The protocol consists in: a) binding of the toxin in the cold in order to avoid the canonical internalization, b) rapid change of culture medium pH at different T to induce the translocation of the toxins across the plasma membrane and, c) overnight incubation in normal culture conditions to allow SNARE cleavage. This last step is performed in the presence of bafilomycin A1 to block cell entry of those molecules, which have not translocated across plasma membrane and can therefore intoxicate cells via the canonic route.
The protocol was reliable and has allowed us to study, in addition to the already known pH dependence, the time and the temperature dependence of the translocation, two parameters which strongly influence biological processes.

Moreover, nothing was known about the cytosolic reducing system which can free the L chain after translocation. This is another fundamental event along CNTs entry mechanism and interfering with it could be an interesting strategy to stop intoxication, since the reduction is *a conditio sine qua* non for L protease activity. There are several disulphide bond reducing systems in the cytosol of cells and I have used a pharmacological approach to identify which of them is involved in the reduction of the interchain SS bond of TeNT and BoNTs. The major protein disulfide reducing systems, in addition to the glutathione pool, are

the NADPH-thioredoxin reductase-thioredoxin system and the glutathione-glutaredoxin system. They are implicated in controlling a variety of cell functions altered in a number of human diseases. Accordingly a large number of drugs have been developed to be evaluated as candidate drugs for clinical use and we used specific one to test the role of the various systems.

The internalization step, as well as the trafficking of CNTs inside peripheral nerve endings, is the subject of many studies because toxins tropism represents a potential issue either for the formulation of antitoxins or for the development of neurotoxin based molecular probes. Additionally, it could be useful also in order to shed light on another fundamental issue, i.e. the possible cooperation between many toxin molecules for a productive translocation of the catalytic moiety. In this scenario, the study of BoNT/A is of major interest because this neurotoxin is responsible for the greatest part of human botulism cases and is almost invariably used in human therapy.
Some evidence support the idea that BoNT/A enters *via* synaptic vesicles, but the actual presence of toxins inside vesicles, and the type of vesicles through which the translocation occurs, has not yet been determined. Here, the entry of BoNT/A into the nerve terminal of the mouse NMJ was studied by using a BoNT/A H_C-EGFP chimera and fluorescence microscopy and immunoelectron microscopy with gold-labeled anti-GFP antibodies. Moreover, experiments aimed to quench the pH of endocytic compartments at different time points were performed in order to identify the subcellular compartment where BoNT/A translocation takes place.

Another fundamental aspect concerning CNTs study is their employment in the treatment of human diseases. Even if BoNT/A is the most used as therapeutics, its application is sometime hampered by intrinsic patients resistance or the development of antitoxin antibodies. In the last years, other serotypes have been investigated for their efficiency in humans[142, 146-148]. However, BoNT/D was never tested in human muscles *in vivo*. BoNT/D could be a very interesting alternative to other BoNTs because it is the most potent serotype in mice and therefore it could be used in very little amount limiting antiserum development. Here we compared BoNT/D potency in mice and *Extensor Digitorum Brevis* muscles of human volunteers.

2. RESULTS

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

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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

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Summary

Tetanus and botulinum neurotoxins are di-chain proteins that cause paralysis by inhibiting neuroexocytosis. These neurotoxins enter into nerve terminals via endocytosis inside synaptic vesicles, whose acidic pH induces a structural change of the neurotoxin molecule that becomes capable of translocating its L chain into the cytosol, via a transmembrane protein-conducting channel made by the H chain. This is the least understood step of the intoxication process primarily because it takes place inside vesicles within the cytosol. In the present study, we describe how this passage was made accessible to investigation by making it to occur at the surface of neurons. The neurotoxin, bound to the plasma membrane in the cold, was exposed to a warm low pH extracellular medium and the entry of the L chain was monitored by measuring its specific metalloprotease activity with a ratiometric method. We found that the neurotoxin has to be bound to the membrane via at least two anchorage sites in order for a productive low-pH induced structural change to take place. In addition, this process can only occur if the single inter-chain disulfide bond is intact. The pH dependence of the conformational change of tetanus neurotoxin and botulinum neurotoxin B, C and D is similar and take places in the same slightly acidic range, which comprises that present inside synaptic vesicles. Based on these and previous findings, we propose a stepwise sequence of molecular events that lead from toxin binding to membrane insertion.

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Introduction

Tetanus and botulism are caused by two closely related types of neurotoxins: tetanus neurotoxin (TeNT) and botulinum neurotoxins, which are produced by bacteria of the genus *Clostridium* in seven different serotypes (abbreviated as BoNT/A to BoNT/G) (Rossetto *et al.*, 2006); in addition several BoNT variants have been recently characterized (Morlishi *et al.*, 1996; Arndt *et al.*, 2006). TeNT binds to peripheral motoneurons and migrates retroaxonally to reach and block inhibitory interneurons of the spinal cord, thus causing a spastic paralysis (Schiavo *et al.*, 1994). The BoNTs bind and enter peripheral nerve terminals, and cause a flaccid paralysis with autonomic symptoms (Rossetto *et al.*, 2006; Johnson and Montecucco, 2008).

These neurotoxins consist of two chains: the L chain (50 kDa) and the H chain (100 kDa) linked by a single inter-chain disulfide bond (Lacy *et al.*, 1998; Lacy and Stevens, 1999; Swaminathan and Eswaramoorthy, 2000; Kumaran *et al.*, 2009; Montal, 2010). The H chain is folded into three domains. An N-terminal 50 kDa domain termed HN that, at low pH, inserts in the membrane and forms transmembrane ion channel (Hoch *et al.*, 1985; Donovan and Middlebrook, 1986; Blaustein *et al.*, 1987; Shone *et al.*, 1987), and which acts as a chaperone for the membrane translocation of the L chain (Koriazova and Montal, 2003; Fischer and Montal, 2007a,b; Fischer *et al.*, 2008; Montal, 2010). The 50 kDa C-terminal part of the H chain is responsible for neurospecific binding (Binz and Rummel, 2009) and consists of two 25 kDa domains: the C-terminal one is termed 'Hc-C' and the other one is termed 'Hc-N'. The latter is highly conserved, and has been proposed to assist the correct positioning of HN on the membrane for the subsequent membrane insertion (Muraro *et al.*, 2009). The Hc-C domain of TeNT

contains two closely located sugar binding sites, whose structures were defined by X-rays crystallography (Fotinou *et al.*, 2001; Jayaraman *et al.*, 2005) and biochemical and functional experiments (Rummel *et al.*, 2003; Chen *et al.*, 2008; 2009). Trp-1289 and Arg-1226 are key residues of the lactose and sialic binding sites respectively (Rummel *et al.*, 2003), and were therefore proposed to be termed W and R (Chen *et al.*, 2009); here, we label them as W lactose and R sialic sites.

BoNT/A, /B, /E, /F and /G share a conserved W lactose site (sequence H/G/K. . . . SXWY), which binds the oligosaccharide portion of polysialogangliosides (PSG) such as GD1b or GT1b (Binz and Rummel, 2009). The R sialic site of BoNT/B and /G binds the alpha-helical segment 44–60 of the luminal domain of the synaptic vesicle (SV) protein synaptotagmin (Syt) (Nishiki *et al.*, 1996; Rummel *et al.*, 2004b; 2007), while the R site of BoNT/A, /E and /F binds different luminal segments of the multispanning SV membrane protein SV2 (Dong *et al.*, 2006; 2008; Mahrhold *et al.*, 2006; Fu *et al.*, 2009; Rummel *et al.*, 2009). Very recent studies have revealed that the Hc-C domains of BoNT/C and /D bind two molecules of PSG at two different sites, as TeNT does, but that the W lactose site is not located in the same position (Rummel *et al.*, 2009; Karalewitz *et al.*, 2010; Strotmeier *et al.*, 2010; Zhang *et al.*, 2011). In addition, it has been recently reported that the endocytosis of TeNT and BoNT/D is mediated by SV2 (Yeh *et al.*, 2010; Peng *et al.*, 2011).

In the cytosol of nerve terminals, the L chains cleave the SNARE proteins: VAMP/synaptobrevin (by TeNT, BoNT/B, /D, /F and /G), SNAP-25 (by BoNT/A, /C and /E) and syntaxin (by BoNT/C) (Schiavo *et al.*, 1994; Rossetto *et al.*, 2006; Montal, 2010). Such an activity can be tested in primary cultures of central nervous system neurons (Keller *et al.*, 1999; Lalli *et al.*, 1999; Verderio *et al.*, 1999; Stahl *et al.*, 2007; McNutt *et al.*, 2011) or in cell lines such as pheochromocytoma cells (PC12) differentiated with nerve growth factor (NGF) in culture (Sandberg *et al.*, 1989). After binding, the BoNTs are endocytosed inside SV, whose v-ATPase proton pump renders them acidic. Low pH causes a structural change of the toxin molecule with the formation of a *trans*-membrane protein-conducting channel by the H chain, which chaperons the unfolded L chain into the cytosol (Montal, 2010). Indeed, bafilomycin A1, a specific inhibitor of the v-ATPase, blocks the entry of the L chain into the cytosol (Simpson *et al.*, 1994; Williamson and Neale, 1994).

Channel formation and membrane translocation of TeNT and BoNTs have been studied with liposomes and planar lipid bilayers (Boquet and Duflot, 1982; Hoch *et al.*, 1985; Donovan and Middlebrook, 1986; Blaustein *et al.*, 1987; Shone *et al.*, 1987; Menestrina *et al.*, 1989; Fu *et al.*, 2002; Koriazova and Montal, 2003; Puhar *et al.*, 2004;) or by patch-clamping spinal cord neurons for TeNT (Beise *et al.*, 1994), or PC12 for BoNT/A and /E (Sheridan, 1998) or Neuro2A cells for BoNT/A (Fischer and Montal, 2007a). Using this latter approach it was shown that the BoNT/A H chain acts as a *trans*-membrane chaperone, which maintains the L chain in an unfolded state during translocation and releases it after reduction of the disulfide bridge (Fischer and Montal, 2007b; Montal, 2009; Montal, 2010). Although these methods have cast light on several molecular features of the process, no information about the efficiency of membrane translocation could be given as these approaches mainly analyse single channel events. On the other hand, the lumen of the SV inside the nerve terminal is not accessible to experimentation.

To study the membrane translocation of the clostridial neurotoxins in neurons, we have developed a protocol, based on an experiment first performed with diphtheria toxin (Draper and Simon, 1980; Sandvig and Olsnes, 1980; Collier, 2001). Conditions were found that by-pass the SV endocytosis step and induce the entry of the L chain directly from the plasma membrane. We used primary cultures of cerebellar granular neurons (CGN), which are almost exclusively composed of neurons, and the pheochromocytoma cell line PC12, in the presence of bafilomycin A1 to block cell intoxication via SV. We have thus obtained compelling evidence that: (i) binding to two receptors is a strict pre-requisite for a productive low pH induced conformational change followed by membrane translocation; (ii) different neurotoxins change structure and interact with the membrane in the same range of pH values which includes pH of the SV lumen pH; and (iii) an intact inter-chain disulfide bridge is essential for the deployment of a productive low pH-induced intoxication of neurons.

Results

Low pH fails to induce the entry of BoNT/A and BoNT/B in CGN neurons and PC12 cells

To study the process of the low pH induced membrane translocation of the L chain of the clostridial neurotoxins in the cytosol, we begun with BoNT/A, which is the most studied toxin of this group, as it is the one used in human therapy (Davletov *et al.*, 2005; Montecucco and Molgó, 2005). We tested it on two very different types of excitable cells: granular neuron from the cerebellum and the PC12 cell line, to obtain a more general picture. We used a protocol based on a binding step at 4°C followed by a brief low pH incubation at 37°C. Figure 1 shows that, in both cell types, low pH media did not induce a higher cleavage of SNAP-25, the target of BoNT/A L chain. This result was unexpected as we would have

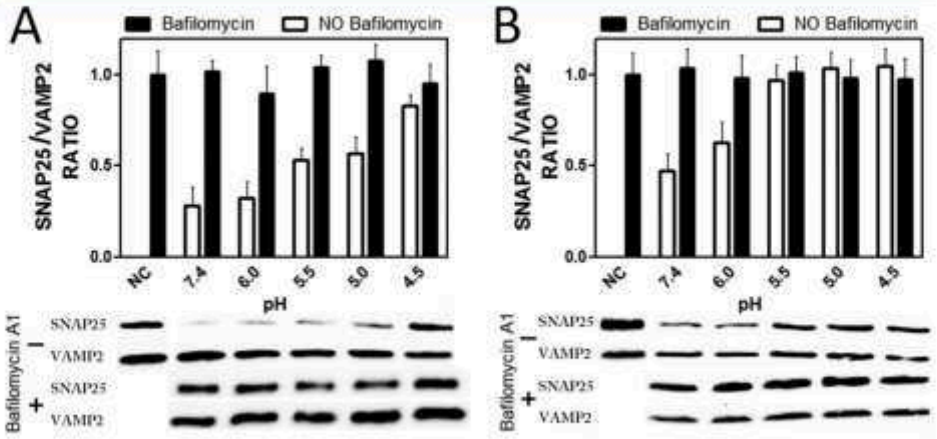

Fig. 1. BoNT/A-mediated cleavage of SNAP-25 in cerebellar granular neurons (A) and in NGF differentiated PC12 cells (B). Cells were incubated with BoNT/A (1 nM in A and 5 nM in B) at 4°C for 15 min, washed and incubated at 37°C with buffers of the indicated pH value (abscissa) for 5 min, washed again and incubated for 12 h with cell culture medium in the presence (filled bars), or absence (open bars) of bafilomycin A1 (100 nM). Cells were lysed and their SNARE proteins were estimated by immunoblotting with specific antibodies (lower panels); SNAP-25 and VAMP-2 were quantified by densitometric analysis, and their ratios are plotted, taking the value in non-treated cells as 1 (upper panels); VAMP which is not cleaved by BoNT/A is taken as an internal standard. The lower panels show the immunoblotting obtained in a representative experiment. SD values are derived from three different experiments performed in duplicates.

anticipated that SNAP-25 cleavage would have become apparent in neurons exposed to acidic pH media. We interpreted this finding as due to the entry of membrane bound toxin via SV endocytosis taking place during the long incubation at neutral pH, after the pH jump, needed to allow the L chain to cleave the cytosolic SNAP-25. Consistently, if bafilomycin A1 was present, no SNAP-25 cleavage was detected (filled bars in Fig. 1). Similar data where obtained with BoNT/B (see Fig. 5). The lower effect of BoNT/A in the pH 4.5 and 5.0 samples are likely to be attributed to a non-productive change of structure of a fraction of the BoNT/A bound to the cell surface. These data were obtained using the uncleaved SNARE, in this case VAMP2, as an internal standard in each sample (VAMP2 for BoNT/A and SNAP-25 for BoNT/B). This 'ratiometric SNARE' quantification of cleavage allows consistent and accurate data to be generated as it effectively normalizes the differences in SDS-PAGE lanes and different experiments.

Low pH induces the TeNT-mediated cleavage of VAMP/synaptobrevin

The simplest explanation for the failure of low pH to induce entry of the L chain of BoNT/A and BoNT/B is that there is not enough SV2 or synaptotagmin on the surface of CGN and of NGF differentiated PC12 cells, which is consistent with recent experiments (Yeh *et al.*, 2010; Peng *et al.*, 2011) and with the lack of labelling of non-permeabilized CGN with an antibody specific for the lumenal domain of SV2 (not shown). The sole binding of BoNT/A and BoNT/B to PSG via the W lactose site may not provide a sufficiently high affinity (Rummel *et al.*, 2004a) to ensure binding to neurons. If this is the case, low pH jump should be effective with TeNT, which binds two PSG molecules via its W lactose and R sialic sites. Indeed, Fig. 2 shows that both cell lines, when exposed to TeNT and then to low pH, in the presence of bafilomycin A1, exhibit an increasing cleavage of VAMP as the pH of the external medium is lowered. In the absence of bafilomycin A1, TeNT cleaved VAMP irrespective of the pH of the buffer of incubation used after binding, as in the case of BoNT/A (not shown). This result supports the interpretation that a clostridial neurotoxin has to be bound to the membrane via two binding sites in order to undergo a productive low pH-driven entry of the L chain into the cytosol. This result was unexpected on the basis of previous biophysical experiments, which have shown channel formation by the HN domain, devoid of the HC binding domain (Blaustein *et al.*, 1987; Hoch *et al.*, 1985; Shone *et al.*, 1987; Fischer *et al.*, 2008). The difference here is that we are not looking at channel formation only, but to the complete event of channel formation plus L chain translocation through the channel. We term this effect hereafter the 'two feet on the ground' effect.

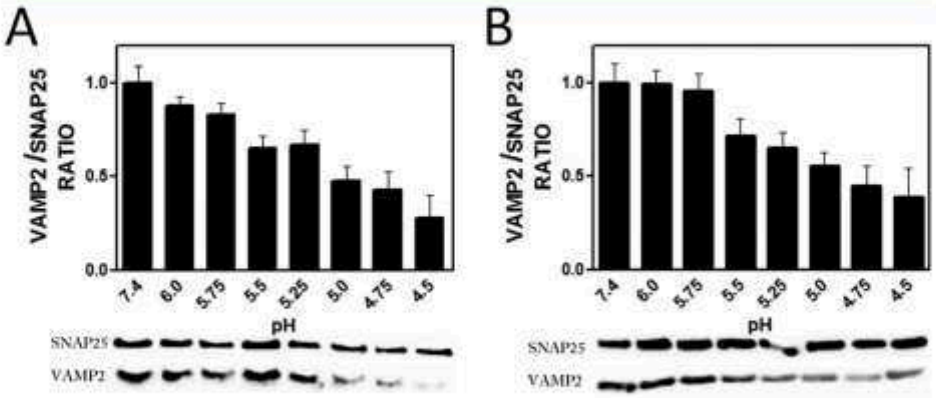

Fig. 2. Cleavage of VAMP2 by TeNT in cerebellar granular neurons (A) and in NGF differentiated PC12 cells (B) as a function of the pH of external media in the presence of bafilomycin A1. Cells were incubated with TeNT (1 nM in A and 5 nM in B) and analysed as described in the legend of Fig. 1; The ratio between the amounts of VAMP and of SNAP-25 determined in the samples treated at pH 7.4 was taken as 1; SNAP-25 which is not cleaved by TeNT is used as an internal standard. Bafilomycin A1 (100 nM) was present during incubation of the cells in normal medium after the low pH jump in order to eliminate the contribution of the toxin entering via endocytosis afterwards. The lower panels show the immunoblotting obtained in a representative experiment. SD values are derived from three different experiments performed in duplicates.

Low pH induces the BoNT/C- and BoNT/D-mediated cleavage of their respective SNARE targets

If the two receptors toxin binding ensuring high affinity interpretation is correct, it should be true also for BoNT/C and /D, which were recently shown to bind two PSG molecules via the W lactose and the R sialic sites (Karalewitz *et al.*, 2010; Strotmeier *et al.*, 2010). Figure 3 shows that indeed, these two neurotoxins cleave their respective substrates in a way dependent on the acidity of the external medium present during the low pH jump in CGNs. This conclusion is reinforced by the fact that the analysis of the

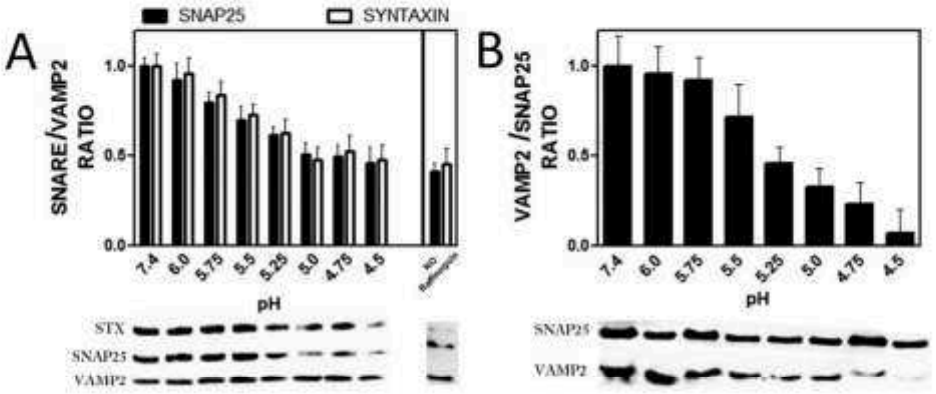

Fig. 3. BoNT/C (A) and BoNT/D (B) cleavage of their SNARE target proteins in cerebellar granular neurons in the presence of bafilomycin A1. Cells were incubated with BoNT/C (1 nM) or BoNT/D (0.1 nM) and analysed as described in the legends of Fig. 2, using VAMP2 (A) and SNAP-25 (B) respectively, as internal standards (filled bars). In (A), the empty bars are the values of the ratios between the staining with an antibody specific for syntaxin with that of an antibody specific for VAMP. The box of (A) reports the values of the ratios of SNAP-25 and of syntaxin with respect to VAMP in samples of CGN treated with BoNT/C, without low pH step and without bafilomycin A1. The lower panels show the immunoblotting obtained in a representative experiment. SD values refer to three different experiments performed in duplicates.

cleavage of syntaxin and SNAP-25, the two substrates of BoNT/C, using VAMP as internal standard, shows a very similar pH dependence. Figure 3 also shows that a fraction of cellular SNAP-25 and syntaxin appears to be not accessible to cleavage by BoNT/C irrespectively from the mode of entry, i.e. via a low pH jump from the plasma membrane or via endocytosis in the absence of bafilomycin A1 (right bars of Fig. 3A).

The comparison of the pH dependence of the effect on cells of TeNT, BoNT/C and /D indicates that these three neurotoxins change conformation upon exposure to acidic pH in a similar way, suggesting that the residues and structural elements involved in this structural transition are closely conserved. It should be noted that the range of pH values 4.5–6 determined here is higher than those determined using liposomes (Shone *et al.*, 1987; Menestrina *et al.*, 1989; Montecucco *et al.*, 1989; Fu and Singh, 1999; Fu *et al.*, 2002; Puhar *et al.*, 2004;), monolayers (Schiavo *et al.*, 1991) or *planar* lipid bilayers (Hoch *et al.*, 1985; Donovan and Middlebrook, 1986; Blaustein *et al.*, 1987) where the channel activity was induced at pH 5 or lower. This emphasizes the importance of using a cell membrane system to obtain figures that are of significance for the *in vivo* situation. In fact, it should be noted that the 4.5–6 pH interval found here includes the pH values measured in the lumen of SV (Miesenböck *et al.*, 1998; Sankaranarayanan and Ryan, 2000).

Low pH induces BoNT/B-mediated cleavage of VAMP/synaptobrevin in PC12 cells stably expressing its protein receptor on the cell surface

The 'two feet on the ground' hypothesis also suggests that a cell refractory to the low pH jump experiment due to lack of the toxin protein receptor should be rendered toxin sensitive by expressing the appropriate protein receptor on the plasma membrane. The binding of the Hc-C of BoNT/B to the fragment 44–60 of Syt has been characterized in detail (Chai *et al.*, 2006; Jin *et al.*, 2006). Thus, we decided to generate a PC12 cell line stably expressing on the cell surface the BoNT/B receptor Syt segment 1–140 linked via its transmembrane region to a cytosolic EGFP to monitor expression and its localization on the plasma membrane. A point mutation N24Q at the N-terminal glycosylation site of Syt was inserted in order to direct the fusion protein to the plasma membrane rather than to SV (Han *et al.*, 2004). PC12 cells were transfected with DNA encoding for this construct and examined by fluorescence microscopy. In order to avoid possible artefacts due to protein overexpression, only individual cells expressing a moderate amount of fluorescence on the plasma membrane were selected and expanded. A cell line stably expressing the fusion protein at the plasma membrane was selected and termed PC12-N24Q (Fig. 4).

When CGN or wild-type PC12 cells were treated with BoNT/B (as done before with BoNT/A) either in the presence or absence of bafilomycin A1, no pH dependent-cleavage of VAMP was apparent (Fig. 5A). In contrast, the left panel of Fig. 5B shows that in PC12-N24Q cells, BoNT/B cleaves more VAMP when exposed to external acidic media, though the effect is not as evident as for TeNT or BoNT/C and /D. A previous report showed that pretreatment of PC12 cells with PSG makes them more sensitive to BoNT/B (Jin *et al.*, 2006). In agreement, we found that the pre-incubation of PC12-N24Q cells with PSG enhances the low pH effect of BoNT/B (central panel of Fig. 5B), while the same pre-incubation of parent PC12 cells is not effective (right panel of Fig. 5C). These results strongly support the 'two feet on the ground' interpretation, which can be extended to BoNT/A and /E following the recent findings obtained by expressing SV2 on the cell surface of test cells (Peng *et al.*, 2011).

The inter-chain disulfide bridge is essential for the low pH induced SNARE cleavage by clostridial neurotoxins

It has long been known that the conserved disulfide bond linking the L to the H chain is essential for neurotoxicity (Schiavo *et al.*, 1990; De Paiva *et al.*, 1993; Kistner *et al.*, 1993; Simpson *et al.*, 2004; Shi *et al.*, 2009). More recent studies performed with BoNT/A and /E on Neuro 2A cells have suggested that the disulfide bridge must remain intact throughout the membrane translocation of the L chain and that premature reduction of the disulfide bond after channel formation arrests translocation (Fischer and Montal, 2007b). In accordance with these data, we found that the treatment of TeNT, BoNT/C and /D treated with DTT prior to incubation with neurons as in Figs 2 and 3 abolishes the low pH-dependent cleavage of their targets (Fig. 6). This result suggests that the integrity of the inter-chain disulfide bond is required before the L chain translocation, during the low pH driven structural change of the clostridial neurotoxins.

Discussion

The membrane translocation of the clostridial neurotoxins from the acidic lumen of synaptic vesicles to the cytosol is the least understood of the four steps of their process of nerve terminal intoxication (Schiavo *et al.*, 1994; Davletov *et al.*, 2005; Binz and Rummel, 2009; Montal, 2010). Here we have shown that these neurotoxins have to be bound to the membrane via two anchorage points, which ensures a high affinity binding, in order to undergo a productive low pH induced conformational change with the formation of a H chain protein-conducting channel and consequent translocation of the L chain in the cytosol. This result was not expected on the basis of previous

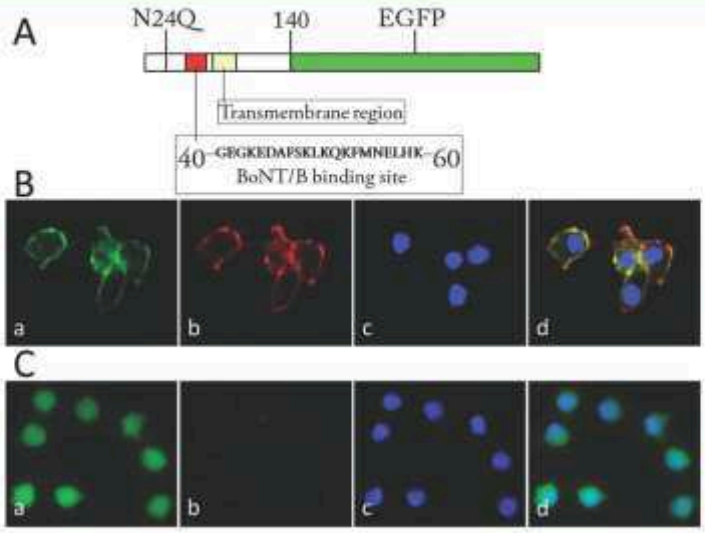

Fig. 4. Fluorescence microscopic images of PC12 cells stably expressing on their surface the BoNT/B protein receptor synaptotagmin fragment 1–140, linked via a transmembrane segment to a cytosolic EGFP.
A. Schematic diagram of the EGFP-synaptotagmin-1–140 chimera.
B. PC12 cell line expressing synaptotagmin 1–140-EGFP on its plasma membrane: (a) EGFP fluorescence; (b) fluorescent staining with an extracellular antibody specific for the luminal domain of synaptotagmin, showing that this domain is exposed on the cells surface and therefore can act as BoNT/B receptor; (c) DAPI staining of nuclei; and (d) merging of the (a), (b) and (c) panels.
C. Confocal images of PC12 cells expressing cytosolic EGFP produced as a control of the fluorescence localization: (a) EGFP fluorescence; (b) fluorescent staining with an extracellular antibody specific for the luminal domain of synaptotagmin; (c) DAPI staining of nuclei; and (d) merging of the (a), (b) and (c) panels. Fluorescence images were obtained without cell permeabilization.

results obtained with various model systems that had shown that the transmembrane ion channels could be formed by L-HN or HN, i.e. in the absence of the Hc binding domain (Hoch *et al.*, 1985; Donovan and Middlebrook, 1986; Blaustein *et al.*, 1987; Shone *et al.*, 1987; Fischer *et al.*, 2008). It could therefore have been possible to envisage that, inside the SV lumen, the low pH could cause the detachment of the H chain from one or both its two receptors before its penetration into the lipid bilayer. Hydrophobic fluorescent probes binding (Puhar *et al.*, 2004), membrane photolabelling (Montecucco *et al.*, 1989) and interaction with lipid monolayers (Schiavo *et al.*, 1991) had indicated that low pH induces the exposure of hydrophobic segments that presumably mediate the membrane insertion of the toxin. More importantly, the interplay between the H chain ion channel and the L chain of BoNT/A suggested that the H chain acts as a transmembrane chaperone that conduces the L chain to the other side of the membrane (Koriazova and Montal, 2003; Montal, 2010). These studies were performed with model systems not directly coupled to a readout consequent to membrane translocation such as the measurement of SNARE cleavage. The present data were obtained with intact neurons using as assay the L chain-mediated cleavage of one (or two) SNARE proteins, employing as an internal standard another uncleaved SNARE protein. Such 'SNARE ratiometric method' provides quantitative data that clearly indicate that, in order to be productive, i.e. to conduce to SNARE cleavage, the toxin has to be bound to the membrane via two receptors during the low pH induced conformational change in order to be oriented correctly with respect to the bilayer and form a protein-conducting channel that is competent to deliver functional L chain. This extends the double receptor mode of interaction of clostridial neurotoxins with the presynaptic membrane (Montecucco, 1986; Montecucco *et al.*, 2004; Rummel *et al.*, 2007; Binz and Rummel, 2009) to the low pH induced membrane insertion and translocation of the L chain. It appears that the chemical nature of the receptor is not a major determinant as the Syt lumenal domain for BoNT/B, and the PSG for TeNT, BoNT/C and /D, similarly support intoxication. Recent results have indicated the possibility that SV2 may be the protein receptor of TeNT and BoNT/D (Yeh *et al.*, 2010;

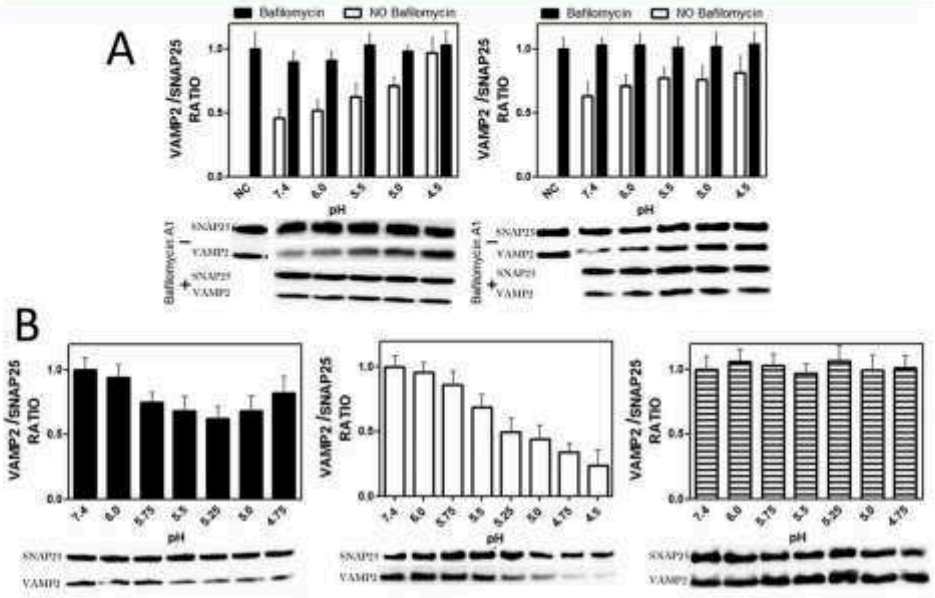

Fig. 5. BoNT/B-mediated cleavage of VAMP2 in cerebellar granular neurons and in NGF differentiated wild-type PC12 cells and in synaptotagmin transfected PC12 cells in the presence of bafilomycin A1.
A. The results of the BoNT/B induced cleavage of VAMP2 in cerebellar granular neurons (BoNT/B, 1 nM, left panel) and differentiated wild-type PC12 cells (BoNT/B 5 nM, right panel) exposed to external media of different pH values, not containing (empty bars) or containing 100 nm Bafilomycin A1 (filled bars), and analysed as in the legend of Fig. 2 using SNAP-25 as internal standard.
B. PC12 cells exposing the lumenal domain of synaptotagmin on their surface (PC12-N24Q) were incubated with BoNT/B (1 nM), and Bafilomycin A1 (100 nM) without (left panel) or with (central panel) a preliminary incubation with a mixture of gangliosides and then treated as in (A). The right panel of (B) shows that BoNT/B does not cleave VAMP2 in wild-type PC12 cells pre-incubated with a mixture of gangliosides. The lower part of the various panels show immunoblotting patterns obtained in representative experiments. SD values refer to three different experiments performed in duplicates.

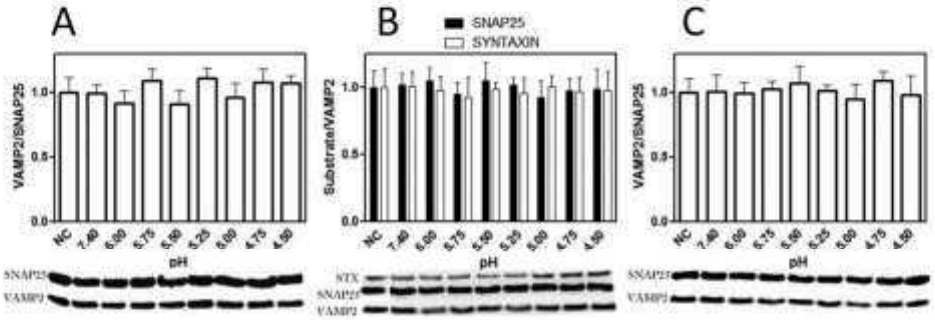

Fig. 6. Reduction of the inter-chain disulfide bond impairs the toxicity of TeNT and BoNT/C and BoNT/D in cerebellar granular neurons. The neurotoxins were reduced by treatment with DTT as indicated in the experimental procedures and then incubated with neuronal cultures as in Figs 2 and 3. SD values are derived from two different experiments run in duplicates.

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Peng *et al*., 2011), but this receptor does not appear to be relevant for their low pH-induced entry into CGN and PC12 cells as we found that exposure to acidic media was unable to induce the entry of BoNT/A, whose protein receptor is SV2 (Dong *et al*., 2006; Mahrhold *et al*., 2006).

Another strict requirement for a productive low pH-induced structural change of the clostridial neurotoxins is the integrity of the single inter-chain disulfide bond. If this is reduced, low pH does not lead to entry of the L chain into the cytosol. It was known before that reduced TeNT (Schiavo *et al*., 1990; Kistner *et al*., 1993), and reduced BoNT/A, /B, /C, /D, /E and /F (De Paiva *et al*., 1993; Simpson *et al*., 2004; Shi *et al*., 2009) were non-toxic, but it was not known which of the intoxication steps was affected. Experiments performed on excised patches of Neuro 2A cells indicated that this disulfide bond of BoNT/A and /E had to reach intact the cytosolic face of the membrane for a correct delivery of the L chain (Fischer and Montal, 2007b; Montal, 2010). The data presented here are fully consistent with these findings, but, in addition, they clearly indicate that the earlier step of the low pH-induced structural change of these neurotoxins does not take place in a correct way when the inter-chain disulfide bond is reduced. This suggests that the two chains of clostridial neurotoxins act in a concerted manner to perform the low pH induced toxin structural change and that the inter-chain disulfide bond and the subtended segments of the L and H chains play an essential role in membrane insertion (see below).

A third important result obtained here is that the conformational changes of TeNT and BoNT/B, /C and /D occur in the same slightly acidic range, and this intervals of pH values is higher than the pH values determined previously in model systems. This is not surprising as liposomes, planar lipid bilayers and monolayers represent crude models of the nerve plasma membrane. The present findings indicate that an appropriate toxin-neuronal membrane interaction is required for the structural change of the toxin to occur in a productive way. This conclusion is further supported by the fact that the range of pH values determined here includes the values estimated to be present in the lumen of SV (Miesenböck *et al*., 1998; Sankaranarayanan and Ryan, 2000).

The interval of pH values found here indicates that functional groups with pKa in the 4.5–6 range are implicated, but their identity is presently unknown. The fact that the interval is the same for four different clostridial neurotoxins suggests that the residues and segments involved in their low pH driven structural change are conserved. We have updated previous sequence comparisons by including recently determined sequences of clostridial neurotoxins (Fig. S1). At variance from diphtheria toxin, which enters the cytosol from the slightly acidic early endosomes upon protonation of three His residues (Collier, 2001; Perier *et al*., 2007; Rodnin *et al*., 2010), there are no conserved histidines in the HN domain, while there are several conserved carboxylate residues. The pKa values of the lateral chain of monomeric Asp and Glu in water are about 3.9 and 4.4, respectively, but it is known that the chemical environment of a residue may change its pKa considerably. To estimate the pKa values of conserved carboxylate residues, we have used the program PROPKA3.0 (Li *et al*., 2005; Bas *et al*., 2008; Olsson *et al*., 2011), which considers the positions of the residue in the crystallographic structure; our analysis was based on the structure of BoNT/B because this is available at higher resolution (PDB: 2NP0) (Chai *et al*., 2006). The pKa values, which are compatible or close to the pH interval determined here, are reported in Table S1 and the corresponding residues are marked in red in Fig. 7. It is noteworthy that: (i) there are no conserved carboxylate residues in the Hc-C binding domain, suggesting that it does not participate directly into the low pH driven structural change; (ii) only one (Asp-877) is present in Hc-N and it is very close to HN; (iii) there are three conserved carboxylate residues in the L chain and they are clustered in the N-terminal region, in agreement with the possibility that the N terminal segment detaches upon protonation and enters into the H channel, as suggested in the N to C direction of translocation postulated by Montal (2010); and (iv) the comparison of the pKa values of Table S1 suggests that the first residues to be protonated are Glu-653 and Glu-48. The neutralization of the latter residue may bring about a partial detachment of the N-terminus of the L chain which would be functional to its engagement in the H channel. Together with Glu-657, Glu-653 is part of a segment (marked in pink in Fig. 7) predicted to interact with the membrane (Eswaramoorthy *et al*., 2004), and, clearly, the protonation of these two residues would strongly enhance its hydrophobicity. It should be considered that what is important here is the relative values of the pKas and the fact that the initial protonations can then influence the pKa values of the other carboxylates. However, the most stricking aspect is that almost all the conserved carboxylate residues, with estimated pKa values close to or included in the interval determined here, lie on one side of the molecule (compare Fig. 7A with B), meaning that the protein surface marked by the presence of the hydrophobic disulfide bond is the one that becomes hydrophobic upon lowering the pH.

The present results are very relevant with respect to a set of important previous findings. These are: (i) low pH does not induce major structural changes in BoNT/B (Swaminathan and Eswaramoorthy, 2000; Eswaramoorthy *et al*., 2004); (ii) the high membrane interaction propensity estimated for segment 637–668 (pink in Fig. 7) (Eswaramoorthy *et al*., 2004); (iii) thioredoxin reduces the inter-chain disulfide bond of TeNT adsorbed on liposomes

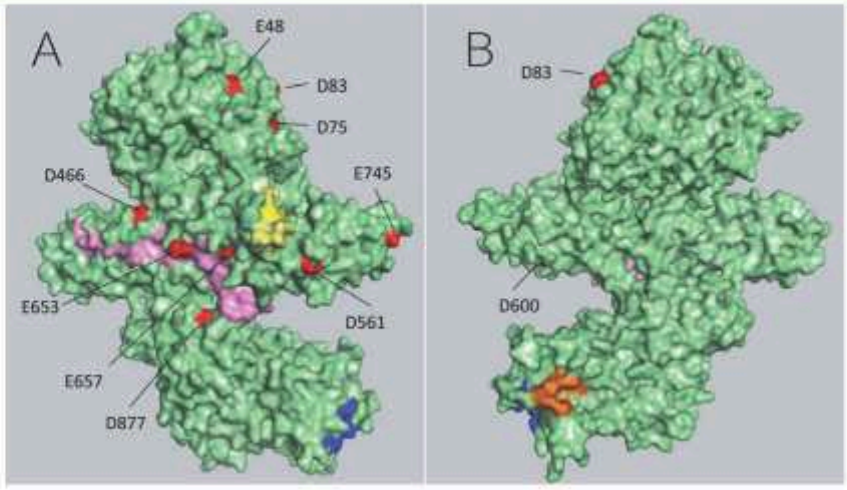

Fig. 7. Surface view of BoNT/B. (A) shows the inter-chain disulfide bridge-containing surface and panel (B) shows the opposite one. Conserved residues with predicted pKa between 4.0 and 7.0 are coloured in red. Ganglioside and synaptotagmin binding site are highlighted in orange and blue respectively. The inter-chain disulfide bond and the subtended two beta sheets are coloured in yellow and pale yellow respectively. The segment 637–668 is in pink.

of bovine brain lipids at neutral pH but not at pH 4.5 (Schiavo *et al*., 1990), and (iv) this disulfide bond must remain intact until complete translocation of the L chain (Fischer and Montal, 2007b). They can be combined with the present results in a stepwise molecular model for the insertion of the toxin in the membrane upon lowering pH.

The first step is the binding of the toxin to two membrane receptors with localization on the lumenal surface of SV (BoNTs at peripheral nerve terminals and TeNT at central synapses). As the lumen pH lowers following the activity of the v-ATPase, the disulfide bond containing surface of HN becomes hydrophobic by protonation of conserved carboxylate residues (a particular role is predicted to be played by Glu-653 and Glu-657). This event promotes the interaction of this part of the toxin molecule with the membrane surface and would bring the hydrophobic SS bond in contact with the membrane surface, thus limiting its accessibility to bulky reducing agents such as thioredoxin. It is then tempting to suggest that segment 637–668 and the β-hairpin subtended by the hydrophobic and polarizable disulfide inter-chain bond (in yellow in Fig. 7) are the first parts of the toxin molecule that penetrate into the lipid bilayer. This hairpin includes charged residues: the carboxylates can be neutralized by protonation, while the positively charged residues could be neutralized by interaction with the negatively charged head groups of acidic phospholipids, as it has been shown to occur with colicins (van der Goot *et al*., 1993). Considering the length of the two HN central helices and that a minimum of four transmembrane helices are needed to form a channel, it can be speculated that, once lying on the membrane surface, the two long α-helices break in the middle and bend towards the other side of the membrane, possibly together with part of segment 637–668 and with the accessory shorter α-helices. Some of the events of this sequence are highly hypothetical, but are amenable to experimental testing, and the cellular assay presented here will be extremely valuable in this respect.

Experimental procedures

Reagents

Escherichia coli XL1-Blue was from Stratagene and pEGFP-N3 was from Clontech. Anti-VAMP2 Mab, anti-Syntaxin Mab and Oyster 550-labelled anti-Synaptotagmin I (lumenal domain) Mab were from Synaptic System. Polyclonal anti-SNAP25 was produced by injecting in rabbit the C-terminal peptide ANQRATKM-LGSG of SNAP25 conjugated to keyhole lymphet haemocyanin. Restriction enzymes were from New England Biolabs. HiTrap Chelating HP, ECL Advanced were from GE Healthcare Life Science. TeNT, BoNT/A, /B, /C and /D were purified as previously described (Shone and Tranter, 1995). In some experiments, the neurotoxins were reduced by incubation with 15 mM DTT at 37°C for 30 min. The complete reduction of the inter-chain disulfide bond was assessed by SDS-PAGE under non-reducing conditions.

Cell cultures

PC12 were plated onto 100 mm Petri dishes and maintained in RPMI supplemented with 10% HS, 5% FBS, 2 mM L-alanyl-L-glutamine (GlutaMAX), 100 U ml^{-1} penicillin, 100 µg ml^{-1} streptomycin, 250 ng ml^{-1} amphotericin B at 37°C in a humid incubator. When stated, PC12 were differentiated in RPMI 1% horse serum, 2 mM GlutaMAX, 100 U ml^{-1} penicillin, 100 µg ml^{-1} streptomycin, 250 ng ml^{-1} amphotericin B with 50 ng ml^{-1} NGF (Alomone Laboratories, Israel) for 5 days.

Primary cultures of rat cerebellar granule neurons (CGNs) were prepared from 6- to 8-day-old rats (Rigoni *et al.*, 2004), and grown on poly-L-lysine (Sigma) coated plates. Experiments were performed after 6–8 days from plating.

PC12 cell line expressing EGFP-synaptotagmin I fragment 1–140

The DNA sequence encoding for rat synaptotagmin I fragment 1–140 was purchased from Blue Heron Biotechnology as a Xho1/BamH1 insert and subsequently subcloned into a pEGFP-N3 vector (Clontech). PC12 cells were transfected using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions and selected with 500 µg ml^{-1} Geneticin (Invitrogen). A PC12 cell line expressing cytosolic EGFP was generated as a control. Individual cells, chosen after fluorescence microscopy, were isolated and plated. Stable clones of PC12 expressing on their surface the chimeric synaptotagmin-EGFP protein (PC12-SYT N24Q) and EGFP in their cytosol (PC12-N3) were maintained in standard medium, supplemented with 50 µg ml^{-1} of Geneticin.

pH jump protocol

A total of 10^4 PC12 cells were seeded into 12-well plates and were differentiated with NGF for up to 5 days. When used in the undifferentiated form, wild-type PC12 or PC12-SYT N24Q were plated into 12-well plates at 10^5 cell per well 24 h before the experiment. CGNs were plated into 12-well plates at 5×10^5 cells per well and used at the 6–8 div. The day of the experiment, cells were incubated with toxin in ice-cooled MEM 10% FBS pH 7.4 and left at 4°C for 15 min. After washing twice with the same cold medium, pre-warmed medium A (123 mM NaCl, 6 mM KCl, 0.8 mM $MgCl_2$, 1.5 mM $CaCl_2$, 5 mM NaP_i, 5 mM citric acid, 5.6 mM glucose, 10 mM NH_4Cl), adjusted at different pH values with 1 M TRIS-base, was added and left for 5 min at 37°C. Cells where then washed twice with MEM and further incubated in MEM 10% FBS pH 7.4 containing 100 nM Bafilomycin A1 for the indicated times. The translocation of the L chains of the various neurotoxins was evaluated following their specific proteolytic activity (next section).

Toxin reduction

TeNT, BoNT/C and BoNT/D were treated with reducing buffer (150 mM NaCl, 10 mM NaH_2PO_4, 15 mM DTT pH 7.4) for 30 min at 37°C. Reduction was assessed by SDS-PAGE performed under non-reducing conditions. After reduction, the neurotoxins were added to cells and experiments of low pH jump were performed as described above.

Immunoblotting

Cells were lysed with 100 mM Tris-HCl, 1% SDS, pH 6.8 containing protease inhibitors (complete Mini EDTA-free, Roche). Protein concentration was determined with the BCA test (Pierce BCA protein assay, Thermo Scientific), and equal amounts were loaded onto a 4–12% NuPage gel (Invitrogen) and separated by electrophoresis in 1× MES buffer (Invitrogen). Proteins were transferred onto Protran nitrocellulose membrane (Whatman). The membranes were then saturated for 1 h in PBS-T (PBS 0.1% Tween 20) containing 5% non-fat milk, incubated with primary antibodies (specific for SNAP25, or VAMP2 or Syntaxin) for 2 h at room temperature or overnight at 4°C, washed three times with PBS-T, and incubated with secondary antibodies HRP-conjugated. After three washes with PBS-T, visualization was carried out using the ECL Plus Western Blotting Detection System (GE Healthcare). The amount of cleaved VAMP (TeNT, BoNT/B and /D) was determined as a ratio with respect to SNAP-25, while the opposite was done in the case of BoNT/A; in addition, in the case of BoNT/C, syntaxin was evaluated using as internal control VAMP2.

Bioinformatic analysis

Toxin sequences were retrieved from UniProtKB database or from GenBank database [accession numbers: P10845 (A), B1Q6N6 (A1), Q58GH1 (A2), Q3LRX9 (A3), ABA29018 (A4), C7BEA8 (A5), P10844 (B), B1INPS(B1), P18640(C), Q9LBS7 (CD), A5JGM8 (DC), P19321(D), Q00496 (E), P30996 (F), Q60393 (G), P04958 (T)]. Alignment have been performed using CLUSTALW2 and then manually refined. Bont/B lateral chain pKa prediction was obtained with PROPKA3.0 submitting the pdb structure 2NP0. Tridimensional visualization of BoNt/B and amminoacids mapping were performed using PyMOL software.

Acknowledgements

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. CNTs primary sequence alignment. Conserved acidic residues with predicted pKa between 4 and 7 are indicated by grey bars.
Table S1. Conserved residues with predicted pKa between 4 and 7. Residues are reported following the numbering of the BoNT/B primary structure.

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Time Course and Temperature Dependence of the Membrane Translocation of Tetanus and Botulinum Neurotoxins C and D in Neurons

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Time course and temperature dependence of the membrane translocation of tetanus and botulinum neurotoxins C and D in neurons

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ABSTRACT

Tetanus and botulinum neurotoxins act inside nerve terminals and, therefore, they have to translocate across a membrane to reach their targets. This translocation is driven by a pH gradient, acidic on the cis side and neutral on the cytosol. Recently, a protocol to induce translocation from the plasma membrane was established. Here, we have used this approach to study the temperature dependence and time course of the entry of the L chain of tetanus neurotoxin and of botulinum neurotoxins type C and D across the plasma membrane of cerebellar granular neurons. The time course of translocation of the L chain varies for the three neurotoxins, but it remains in the range of minutes at 37 °C, whilst it takes much longer at 20 °C. BoNT/C does not enter neurons at 20 °C. Translocation also depends on the dimension of the pH gradient. These data are discussed with respect to the contribution of the membrane translocation step to the total time to paralysis and to the low toxicity of these neurotoxins in cold-blood vertebrates.

1. Introduction

Tetanus is caused by the tetanus neurotoxin (TeNT) which is produced by *Clostridium tetani* [1]. TeNT binds to peripheral motoneurons and migrates retroaxonally to enter inhibitory interneurons of the spinal cord where it blocks neurotransmitter release causing a spastic paralysis [2]. At variance, the botulinum neurotoxins (seven serotypes with many variants: BoNT/A to /G) act mainly on peripheral cholinergic nerve terminals [3,4]. TeNT and BoNTs are made of two chains linked by a single inter-chain disulfide bond: the L chain (one domain of 50 kDa) and the H chain (100 kDa, three domains) [2,5–9]. The C-terminal half of the H chain (H_C) is responsible for neurospecific binding [10] and consists of two 25 kDa domains: the N-terminal one is highly conserved, and has been proposed to assist the correct positioning of the toxin on the membrane for the subsequent membrane insertion via binding PIPs [11,12]. The C-terminal 25 kDa domain of H_C of TeNT, BoNT/C and /D were recently shown to harbor two polysialoganglioside binding sites [13–19]. This is a unique feature among the clostridial neurotoxins [10,20] and it allows them to bind to the neuronal membrane in such a way that exposure to low pH is sufficient to induce their entry into the membrane with translocation of the L chain into the cytosol [21]. This is not the case of the other BoNTs which require the presence of their proteins receptors [10].

After binding, the BoNTs are endocytosed into an intracellular compartment whose luminal pH is acidified by a v-ATPase proton pump, which is specifically inhibited by bafilomycin A1 [22,23].

Low pH induces the N-terminal half of the H chain (H_N) to insert into the membrane with formation of a transmembrane ion channel [24–28]. There is evidence that H_N acts as a chaperone that assists the translocation of the L chain into the cytosol in a process dependent on the transmembrane pH gradient [9,29–33].

In the cytosol of nerve terminals, the L chains cleave one of the three SNARE proteins: VAMP/synaptobrevin (by TeNT, BoNT/B, /D, /F and /G), SNAP-25 (by BoNT/A, /C and /E) and syntaxin (by BoNT/C) [34–36].

The intoxication of nerve terminals by TeNT and BoNTs can be assayed in neurons [37–43], but the process of membrane translocation from the low pH intracellular compartment is not accessible to investigation. Recently, methods to bypass this entry process and induce the translocation of the L chain from the plasma membrane have been devised [21,28]. Using this approach, we have made an extensive analysis of the time course of the membrane translocation of the L chains of TeNT, BoNT/C and /D and have found that at 37 °C the translocation of the L chain from the cell exterior to the cytosol is very rapid, i.e. it occurs in minutes. Translocation is strongly temperature dependent and virtually no translocation takes place at 20 °C.

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2. Materials and methods

2.1. Toxins

TeNT was isolated and purified from supernatant of *C. tetani* as previously described [44]. BoNT/C was isolated and purified from supernatants of *Clostridium botulinum* strain NCTC 8264 as previously described [45], whilst BoNT/D (derived from strain 1873) was produced in *Escherichia coli* and purified as described [46].

2.2. Cell cultures

Primary cultures of rat cerebellar granule neurons (CGNs) were prepared from 6- to 8-days-old rats. Cerebella were mechanically disrupted and then treated with trypsin (Sigma, 800 μg/ml) in the presence of DNase I (Sigma, 100 μg/ml). Cells were collected and plated into 24 well plates coated with poly-L-lysine (50 μg/mL) at a cell density of 3×10^5 cells per well. Cultures were maintained at 37 °C, 5% CO_2, 95% humidity in BME supplemented with 10% fetal bovine serum, 25 mM KCl, 2 mM glutamine and 50 μg/mL gentamycin. To arrest growth of non-neuronal cells, cytosine arabinoside (Sigma, 10 μM) was added to the medium 18–24 h after plating.

2.3. Assay of SNARE cleavage in CGNs by tetanus and botulinum neurotoxins after transient low pH exposure

Cells were incubated with toxin in ice-cooled MEM 10% FBS pH 7.4 and left at 4 °C for 15 min. After washing twice with the same cold medium, pre-warmed medium A (123 mM NaCl, 6 mM KCl, 0.8 mM $MgCl_2$, 1.5 mM $CaCl_2$, 5 mM NaP_i, 5 mM citric acid, 5.6 mM glucose, 10 mM NH_4Cl), adjusted at pH 7.4 (positive control, PC), 5.5 or 4.5 with 1 M TRIS-base, was added and left for indicated times at the indicated temperature. Cells where then washed twice with MEM and further incubated at 37 °C in MEM 10% FBS pH 7.4 containing 100 nM Bafilomycin A1 (Sigma) for 12 h. The translocation of the L chains of the various neurotoxins was evaluated following their specific proteolytic activity by immunoblotting with anti-SNAREs antibodies.

2.4. Immunoblotting

Cells were lysed with 100 mM Tris–HCl, 1% SDS, pH 6.8, containing protease inhibitors (complete Mini EDTA-free, Roche). Protein concentration was determined with the BCA test (Pierce BCA protein assay, Thermo Scientific), and equal amounts were loaded onto a 4–12% NuPage gel (Invitrogen) and separated by electrophoresis in 1X MES buffer (Invitrogen). Proteins were then transferred onto Protran nitrocellulose membranes (Whatman) and then saturated for 1 h in PBS-T (PBS 0.1% Tween 20) containing 5% non-fat milk. Incubation with primary antibodies specific for VAMP2 (monoclonal, Synaptic System, Germany), for SNAP25 [21], and for Syntaxin 1A [47] was performed overnight at 4 °C. The membranes were washed three times with PBS-T and incubated with secondary antibodies HRP-conjugated. Finally membranes were washed several times with PBS-T and visualization was carried out using Luminata Crescendo (Merck Millipore). The amount of cleaved VAMP (TeNT, BoNT/D) was determined as a ratio with respect to SNAP25, whilst in the case of BoNT/C, SNAP25 and Syntaxin cleavage was reported as a ratio vs. VAMP.

3. Results

3.1. Translocation of the TeNT L chain into neurons

It is well established that TeNT and BoNTs enter into nerve terminals via endocytosis inside acidic compartments, but the molecular mechanism of membrane translocation of the L chain remains ill known. Recently, the L chain was induced to enter into nerve terminals from the plasma membrane with a protocol that bypasses endocytosis and involves the exposure of the cell surface bound toxin to a low pH medium [21,28]. In such a way several parameters of the process can be determined. Here, we have used this approach to study the temperature and time dependence of the membrane translocation of the TeNT, BoNT/C and /D into primary cultures of cerebellar granular neurons (CGN) which are highly sensitive to neurotoxins. These three neurotoxins were chosen because they bind to two polysialogangliosides and therefore can enter into cells at low pH without the requirement of cell culture manipulation to expose the synaptic vesicle receptor [28].

Fig. 1 shows that the L chain of TeNT enters the cytosol of CGN within minutes of exposure at 37 °C, as deduced by the fact that its metalloprotease L chain cleaves the same amount of VAMP as that cleaved by controls where the toxin was allowed to enter via its normal endocytic route which involves synaptic vesicles in neurons of the central nervous system [39,48]. In preliminary experiments we had established that an incubation time of 12 h at 37 °C, in the presence of bafilomycin A1 to block the normal endocytic route of entry, was sufficient to achieve maximal VAMP cleavage and therefore the read-out time was not limiting. The number of L chains which are translocated in the cytosol is slightly higher when the external acid pH was 4.5 than when it was 5.5. This is due to the fact that TeNT must be protonated to insert into the membrane and at the lower pH value a higher proportion of the protonatable groups are titrated.

Membrane translocation is much less efficient at 28 °C and it is prevented at 20 °C. This latter finding is important because it contributes to explain why cold-blood animals like reptiles and amphibians are poorly sensitive to TeNT [49–51].

3.2. Translocation of the BoNT/C L chain into neurons

BoNT/C is the only BoNT, which cleaves two SNARE proteins at the same time: it cleaves both SNAP-25 within its C-terminus and syntaxin close to the transmembrane segment [9,34,36]. The low pH driven translocation of BoNT/C into CGNs is different from that of TeNT (Fig. 2). In fact this neurotoxin shows a similar time course at 37 °C, but it is ineffective at 28 °C even when it was exposed to a medium of pH 4.5. This holds true for the assay of syntaxin cleavage as well as for that of SNAP-25 (left panels of Fig. 2). As the cleavage of a SNARE protein depends on both the number of L chain that reach the cytosol and on their metalloproteolytic turnover number, a strict comparison among different neurotoxins is not warranted. However, the extent of difference is remarkable and data obtained with the same toxin type remain comparable. BoNT/C has been associated with outbreaks of botulism in birds [3,4,52] and it is tempting to associate this finding to the fact that birds have a higher body temperature than mammals. BoNT/C could have been modified through evolution to act in animals endowed with a high body temperature.

3.3. Translocation of the BoNT/D L chain into neurons

BoNT/D is the most toxic of the BoNTs and this high toxicity is paralleled by its activity of neurons in culture. Fig. 3 shows that already at a concentration of 0.05 nM the large majority of VAMP2 is cleaved by the L chains that have translocated across the plasma membrane within one minute of exposure at 37 °C. Even at 20 °C half of the cell content of VAMP2 is proteolysed in the samples exposed to an external pH of 4.5 for 10 min. These results indicate that the membrane translocation of the L chain of BoNT/D is very rapid and has an apparent lower temperature dependence than those of TeNT and BoNT/C.

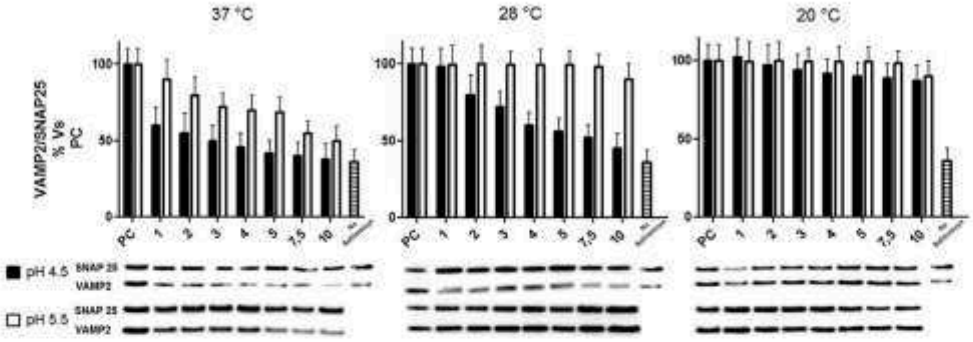

Fig. 1. Temperature and time dependence of TeNT mediated cleavage of VAMP/synaptobrevin in cerebellar neurons in culture. Cells were incubated with TeNT (1 nM) at 4 °C for 15 min, washed and incubated at the indicated temperature with buffers at different pH value (4.5, filled columns; 5.5 empty columns) for the indicated time in minutes (abscissa); after washing, the samples were incubated for 12 h with cell culture medium in the presence of bafilomycin A1 (100 nM), and their content of SNARE proteins was estimated by immunoblotting with specific antibodies. Values are reported as the ratio between the staining with the antibody specific for VAMP/synaptobrevin2 and the staining with the antibody specific for SNAP25, and normalized vs. the value obtained by incubating the neurons with the toxin for 10 min at pH 7.4 (PC), 37 °C, taken as 100%. Striped columns at the right of each panel are values obtained by treating neurons as PC without adding bafilomycin A1 during the 12 h incubation and gives the maximum SNARE cleavage that can be obtained with this toxin. SD values refer to three different experiments performed in triplicates. The lower part shows immunoblotting patterns obtained in a representative experiment.

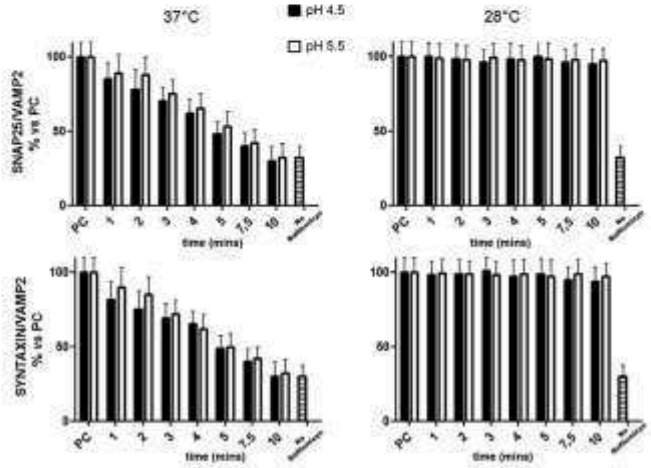

Fig. 2. Temperature and time dependence of BoNT/C mediated cleavage of SNAP25 and syntaxin A1 in cerebellar granular neurons in culture. Cells were incubated with BoNT/C (1 nM) at 4 °C for 15 min and treated as described in Fig. 1 legend. Values are reported as the ratio between the staining with the antibody specific for SNAP25 (upper panels) or syntaxin (lower panels) and the staining with the antibody specific for VAMP/synaptobrevin2, as described in Fig. 1. White striped columns are as in Fig. 1 legend and give the maximum of SNARE cleavage that can be obtained with this toxin. SD values refer to three different experiments performed in triplicates.

4. Discussion

The main general result presented here is that the membrane translocation of the L chain driven by a transmembrane pH gradient, acidic on the toxin side, is very rapid and it is completed within minutes at 37 °C. Given the large structural similarity among the known clostridial neurotoxins [7], this conclusion may be extended to the other neurotoxins, but this remains to be investigated. Tetanus and botulism develop over many hours/days from intoxication [1–4]. Also intoxication of the hemidiaphragm preparation or of neurons in culture require at least half an hour or two hours, respectively. The present results indicate that the long period of time between toxin application and blockade of neurotransmitter release caused by SNARE cleavage cannot be attributed to the membrane translocation step. Most likely, the time required to cleave a substantial fraction of the SNARE proteins takes the large part of the time to intoxication *in vitro* and *in vivo*.

The experimental system used here offers the possibility of dissecting the low pH driven membrane translocation step, but requires that the two toxin receptors are exposed on the surface [21]. Another aspect that has to be considered is that its read out, i.e. the extent of cleavage of the SNARE protein target, de-

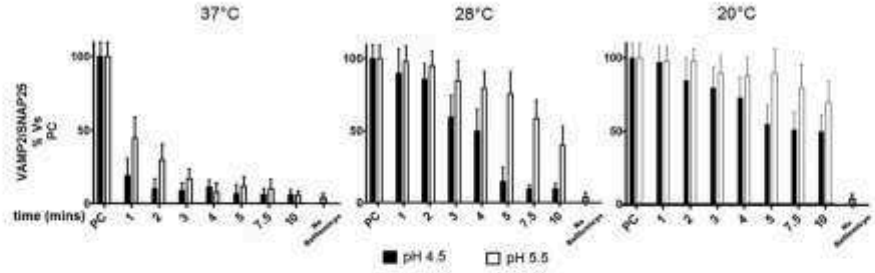

Fig. 3. Temperature and time dependence of BoNT/D mediated cleavage of VAMP/synaptobrevin2 in cerebellar granular neurons in culture. Cells were incubated with BoNT/D (50 picoMolar) at 4 °C for 15 min and treated as described in Fig. 1 legend. White striped columns are as in Fig. 1 legend and give the maximal cleavage of VAMP2 that can be obtained with this toxin. SD values refer to three different experiments performed in triplicates.

pends on both the number of L chain translocated into the cytosol and on their catalytic activity. The latter critical parameter has been analyzed in a scattered series of studies for only some BoNT types and always using artificial substrates *in vitro* [14,53–58], and never at the same time for the three toxins used here. Moreover, one does not know to what an extent the enzyme kinetics data obtained *in vitro* can be translated to the *in vivo* situation as the membrane environment and other parameters can change substantially the kinetics [59]. Therefore, the data obtained here for the same toxin at the different temperatures are homogeneous, but the comparison of those obtained with the different toxins is less stringent. Within this limitation, some differences of possible biological relevance have emerged. TeNT, BoNT/C and BoNT/D differ in the temperature dependence of the membrane translocation of their L chains. BoNT/C does not translocate at 28 °C and this property may be correlated with the fact that this BoNT serotype is associated to outbreaks of botulism in birds which have a body temperature of 42 °C. As a certain degree of L chain unfolding is believed to be involved in membrane translocation [9], it is possible that this toxin is the result of an evolution that has led to the long known higher resistance of BoNT/C to temperature [60]. On this line, the present finding that the action of TeNT is very limited if neurons are exposed to low pH at 20 °C fits in the well-documented temperature dependence of the TeNT sensitivity of amphibians and reptiles [49–51]. However, the outcome of *in vivo* toxicity experiments is the results of several steps, including tissue distribution, binding, endocytosis, membrane translocation and enzymatic cleavage of SNARE target, whilst the protocol employed here selects out only the membrane translocation step.

The extent of cleavage of VAMP2 by TeNT and BoNT/D at 28 °C (middle panels of Figs. 1 and 3) in the samples incubated for 10 min at pH 4.5 is similar to the one achieved under the optimal conditions of incubation without bafilomycin A1, whilst a significantly lower extent of cleavage was found at pH 5.5. This is consistent with the possibility that the pH of the lumen of synaptic vesicles is closer to 4.5 than to 5.5. This interesting possibility deserves to be studied with direct methods.

Further studies involving the other BoNT types are required to fully substantiate the relevant points that have emerged from the present study. But one conclusion that appears to be extendable to all the clostridial neurotoxins is that the membrane translocation step at 37 °C is very rapid and should not be considered in the attempt to explain the long lag phase existing between toxin exposure and blockade of neurotransmitter release with the development of a neuroparalysis.

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The thioredoxin reductase-thioredoxin system is involved in the entry of tetanus and botulinum neurotoxins in the cytosol of nerve terminals

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ABSTRACT

Tetanus and botulinum neurotoxins cause paralysis by cleaving SNARE proteins within the cytosol of nerve terminals. They are endocytosed inside acidic vesicles and the pH gradient across the membrane drives the translocation of their metalloprotease L domain in the cytosol. This domain is linked to the rest of the molecule by a single interchain disulfide bridge that has to be reduced on the cytosolic side of the membrane to free its enzymatic activity. By using specific inhibitors of the various cytosolic protein disulfides reducing systems, we show here that the NADPH-thioredoxin reductase-thioredoxin redox system is the main responsible for this disulfide reduction. In addition, we indicate auranofin, as a possible basis for the design of novel inhibitors of these neurotoxins.

1. Introduction

Bacteria of the genus *Clostridium* produce one tetanus neurotoxin (TeNT) which causes the spastic paralysis of tetanus and seven different botulinum neurotoxins (BoNT/A, /B, /C, /D, /E, /F, and /G) which cause the flaccid paralysis of botulism [1]. They differ for antigenic properties and for their intracellular targets [2,3]. They have a similar four-domains structure [4–7] which makes them capable of entering, via a four step mechanism, the cytosol of nerve terminals [7–9]. Only the N-terminal domain of 50 kDa, termed L chain, reaches the cytosol where it cleaves the three SNARE proteins which form the core of the neuroexocytosis apparatus thus blocking neurotransmitter release causing paralysis [10,11]. Tetanus has been a major killer in the past, but nowadays it is prevented by a very effective vaccine except for those countries not provided with an operative system of preventive medicine [12]. Botulinum neurotoxins are so toxic to humans (MLD_{50} is in the range of 0.2–1 ng/kg) as to be considered for potential use in bioterrorism [13], but, at the same time, they are currently used as therapeutics to treat a number of human syndromes characterized by hyperfunction of peripheral nerve terminals [14–16]. Human botulism is rare, and it follows the ingestion of BoNT contaminated food or of spores of neurotoxigenic Clostridia, contamination of wounds or excessive dosage during cosmetic treatments [1,17]. The duration of human botulism depends on the amount and type of BoNT, with BoNT/A and /C having the longest duration and BoNT/E the shortest [1,18,19]. This situation calls for the development of novel drugs that can counteract TeNT and BoNT action by affecting any of the four steps of the mechanism of intoxication: (a) binding; (b) endocytosis; (c) membrane translocation and (d) metalloprotease cleavage of SNAREs. Before their binding to the presynaptic membrane of neurons, the toxins are best neutralized by specific anti-toxin antibodies [20,21] which prevent the first step of the entry of TeNT and BoNT into their target neurons. Currently, several groups are attempting to block any of the three subsequent steps [22–30].

The present knowledge on the mode of membrane translocation of these neurotoxins is summarized in a model which envisages the insertion of the H_N domain into the membrane of the acidic toxin-containing vesicle to form a ion channel which acts as a chaperone assisting the unfolding and translocation of the L chain, which remains linked to the H_N domain via a single interchain

Abbreviations: AF, auranofin; ATO, arsenic trioxide; BoNT, botulinum neurotoxin; BSO, buthionine sulfoximine; CGN, cerebellar granule neurons; TeNT, tetanus neurotoxin

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disulfide bridge [7,31,32]. The L chain then refolds at the neutral pH of the cytosol and detaches following reduction of the interchain SS bond by an unknown reducing agent of the cytosol [32]. An intact SS bond is essential for neurotoxicity [33,34] and for translocation of the L chain and it must be reduced on the cytosolic side [32].

There are several disulphide bond reducing systems in the cytosol of cells [35,36] and, here, we have used a pharmacological approach to identify which of them is involved in the reduction of the interchain SS bond of TeNT and BoNTs. At the same time, this study has identified a molecule, which may lead to novel drugs effective for the treatment of tetanus and botulism patients.

2. Materials and methods

2.1. Reagents

Auranofin, (1-thio-β-D-glucopyranosatotriethylphosphine gold-2,3,4,6-tetraacetate) BSO (buthionine-sulfoximine), ATO (arsenic trioxide), $KAuCL_4$ (potassium tetrachloroaurate(III) hydrate), cDDP (cisplatin), cytosine arabinoside, DNAse I, poly-L-lysine, were purchased from Sigma Aldrich. Anti-VAMP2 Mab, anti-Syntaxin Mab were from Synaptic System. TeNT, BoNT/B, and /C were purified as previously described [37,38] whilst BoNT/D was produced in *Escherichia coli* via recombinant methods as described [39].

2.2. Cell cultures

Primary cultures of rat cerebellar granule neurons (CGNs) were prepared from 6- to 8-days-old rats [40]. Briefly, rat cerebella were firstly mechanically disrupted and then trypsinated in the presence of DNase I. Cells were collected and plated into 24 well plates, coated with poly-L-lysine (50 μg/ml), at cell density of 3×10^5 cells per well. Cultures were maintained at 37 °C, 5% CO_2, 95% humidity in BME supplemented with 10% fetal bovine serum, 25 mM KCl, 2 mM glutamine an 50 μg/ml gentamycin. To arrest growth of non-neuronal cells, cytosine arabinoside (10 μM) was added to the medium 18–24 h after plating.

2.3. Assay of inhibitors of the intoxication of CGNs with tetanus and botulinum neurotoxins

CGNs were used at 6–8 div. Cells were pre-incubated with AF or BSO or ATO or cisplatin or $KAuCl_4$ at increasing concentrations in BME 10% FBS, 25 mM KCl and left for 6 h at 37 °C and 5% CO_2. Then the indicated toxin was added and left for 15 min at 37 °C. Thereafter, the cells were washed with BME in order to remove the toxin and the normal culture medium was restored with the indicated concentration of inhibitor for 12 h at 37 °C. The translocation of the L chains of the various neurotoxins was evaluated following their specific proteolytic activity by immunoblotting with anti-SNAREs antibodies.

2.4. Immunoblotting

Cells were lysed with 100 mM Tris–HCl, 1% SDS, pH 6.8, containing protease inhibitors (complete Mini EDTA-free, Roche). Protein concentration was determined with the BCA test (Pierce BCA protein assay, Thermo Scientific), and equal amounts were loaded onto a 4–12% NuPage gel (Invitrogen) and separated by electrophoresis in 1× MES buffer (Invitrogen). Proteins were then transferred onto Protran nitrocellulose membranes (Whatman) and then saturated for 1 h in PBS-T (PBS 0.1% Tween 20) containing 5% non-fat milk. Incubation with primary antibodies (specific for VAMP2 and Syntaxin 1A) was performed overnight at 4 °C. The membranes were washed three times with PBS-T and incubated with secondary antibodies HRP-conjugated. Finally membranes were washed several times with PBS-T and visualization was carried out using the Luminata Crescendo (Merck Millipore). The amount of cleaved VAMP (TeNT, BoNT/B and /D) was determined as a ratio with respect to Syntaxin 1A, whilst the opposite was done in the case of BoNT/C.

2.5. In vitro proteolytic activity

TeNT and BoNT/D (2 μg) were treated with reducing buffer (150 mM NaCl, 10 mM NaH_2PO_4, 15 mM DTT pH 7.4) for 30 min at 37 °C. The activated toxins were split into different tubes and in half of them AF was added (1 μM final concentration). Five micrograms of recombinant GST-VAMP2 was added in each tube and the reaction was left for 2 h at 37 °C. SNARE cleavage was assessed by Comassie staining in SDS–PAGE.

2.6. Viability test

CGNs were seeded in a 96 wells plate at a cell density of 10^5 cells per well. After 6 div, different concentration of AF, ranging from 0 to 10 μM, were added and left for 24 h. Neurons were then washed and MTS assay (Promega) performed according to supplier indication. Absorbance was recorded at 490 nm using a Spectra Count™ plate reader (Canberra Industries, Meridien, USA) and cell viability has been reported as percentage vs. non-treated neurons.

2.7. BoNT/D LD_{50} assay

For in vivo experiments Swiss-Webster adult male CD1 mice weighing 25–30 g were used. Mice were housed under controlled light/dark conditions and food and water was provided ad libitum. All experiments were performed in accordance with the Italian guidelines, law n. 116/1992 and were approved by the Animal Ethical Committee of our University. AF was dissolved in a stock solution with ethanol. Mice were i.p. injected (injection volume 250 μl) with 12 mg of AF per kg of body weight or with the vehicle (0.9% NaCl). Animals were incubated for 6 h before the injection of the indicated dose of BoNT/D diluted in 0.9% NaCl (injection volume 250 μl). The animals were monitored every 3 h for 96 h, after which the experiment was considered ended.

2.8. Estimation of thioredoxins reductase activity

Thioredoxin reductase activity was assayed as previously described [41]. Briefly, 0.1 mg of cell extract was tested at room temperature in 0.2 M Na,K-phosphate buffer (pH 7.4), supplemented with 2 mM EDTA and 0.4 mM NADPH. Reactions were started adding 2 mM DTNB and its reduction was followed spectrophotometrically at 412 nm. Values after 1 min after start had been reported.

3. Results

The major protein disulfide reducing systems of the cell cytosol, in addition to the glutathione pool, are the NADPH-thioredoxin reductase-thioredoxin (Trx) system [35] and the glutathione-glutaredoxin system [36]. They are implicated in controlling a variety of cell functions altered in a number of human diseases [42]. Accordingly a large number of drugs have been developed to be evaluated as candidate drugs for clinical use [43,44]. We have taken here a pharmacological approach and used a set of inhibitors of these cell disulphide reducing system to identify which of them is implicated in the reduction of the interchain disulphide bond of clostridial neurotoxins, a reduction that takes place in the cytosol [32–34].

Buthionine sulfoximine (BSO) and arsenic trioxide (ATO) are well characterized for the depletion of the cytosolic pool of glutathione [43,44]. Under conditions in which residual GSH of cerebellar granular neurons was lowered by these drugs below 20% of the normal content, no effect on the VAMP2 cleavage induced by BoNT/D and TeNT was observed (Fig. S1 A and B), indicating that neither GSH nor the glutathione-glutaredoxin system play a relevant role in the process. This negative result left open the possibility that the Trx system might be implicated.

Several inhibitors of the Trx system are available, including curcumin and other flavonoids, cisplatin, $KAuCl_4$, but the most specific one is auranofin (AF) [45]. An attractive feature of AF is that it is currently used in the treatment of human rheumatoid arthritis [46] in addition to being considered for the treatment of different forms of cancers and HIV infection [45,47].

Fig. 1B shows that AF protects cerebellar granular neurons from the action of different doses of tetanus neurotoxin (TeNT) and that about 1 μM AF confers a full protection against TeNT intoxication (Fig. 1A), measured as cleavage of VAMP-2, while it does not affect cell vitality (Fig. S2).

The inhibitory effect of AF is not limited to TeNT as it protects CGN from the cleavage of VAMP-2 induced by BoNT/B (panel A of Fig. 2) and the cleavage of syntaxin 1A induced by BoNT/C (Fig. 2B). It is noteworthy that AF protects the intoxication of CGN from different neurotoxins in the same range of concentrations. This is consistent with an action of AF on the same step of the intoxication process. Auranofin is reported to be very specific for Trx [41,48,49], however we tested its ability to inhibit the Trx of CGN. Fig. S3 shows that the same holds true for the neurons used here. The different range of concentration values found for the inhibition of the activity of the neurotoxins and for the Trx inhibition could be attributed to the different permeability of the plasma membrane of AF in different cell lines (or to the different activity of Trx in different cell types) [41].

In addition to syntaxin, BoNT/C cleaves SNAP-25 [50]. However, we could not quantify the inhibitory activity of AF on the action of BoNT/C on its second substrate because AF interferes with the immunoblotting assay of SNAP-25 cleavage used here. As AF interacts with selenium and sulfur containing compounds, it might as well interact with the cysteine quartet of SNAP-25 after cell solubilization. In vivo, this does not occur as AF is not cytotoxic to neurons, and no AF neurological side effects in humans have been reported. This difference is likely to be due to the fact that the SNAP-25 cysteine quartet is inserted in the membrane and is therefore protected from the interaction with AF. However, this effect of AF prevented us to test its activity on BoNT/A and BoNT/E. We attempted to use two other Trx inhibitors that may overcome this problem: cisplatin and $KAuCl_4$. However, they were found to be very toxic to CGN in culture at concentrations close to their reported IC_{50} (not shown) [48].

In terms of toxicity in mice, the most powerful clostridial neurotoxin is BoNT/D (this preparation gave a LD_{50} value <0.4 nanogram/kg). Fig. 3 shows that AF also protects CGN from the cleavage of VAMP-2 induced by this neurotoxin type and again in the same range of concentrations.

The inhibitory activity of AF on the intoxication of neurons by TeNT and BoNT/B, /C and /D is not due to an effect on the fourth step of the process, i.e. their metalloprotease activity as control experiments performed in vitro with toxins and substrates alone did not reveal in any case an inhibition by AF (not shown).

These results prompted us to test the possible protective activity of AF in vivo in mice injected with BoNT/D, chosen because it is the most powerful clostridial neurotoxin in mice. However, AF used at the dose of 12 mg/kg, corresponding to about 175 μM considering the total body fluids as 10% of the mouse weight, did not protect mice from the flaccid paralysis induced by BoNT/D (Fig. 4). This dose was recently used to show that AF inhibits the activation of the Nlrp1b inflammasome [51].

4. Discussion

The main finding reported here is that auranofin (AF), a specific active site inhibitor of thioredoxin reductase, a member of one of the major cell protein disulfide reducing systems, inhibits the intracellular activity of TeNT and of BoNT/B, /C and /D. At the same time, AF does not inhibit the metalloproteolytic activity of the L

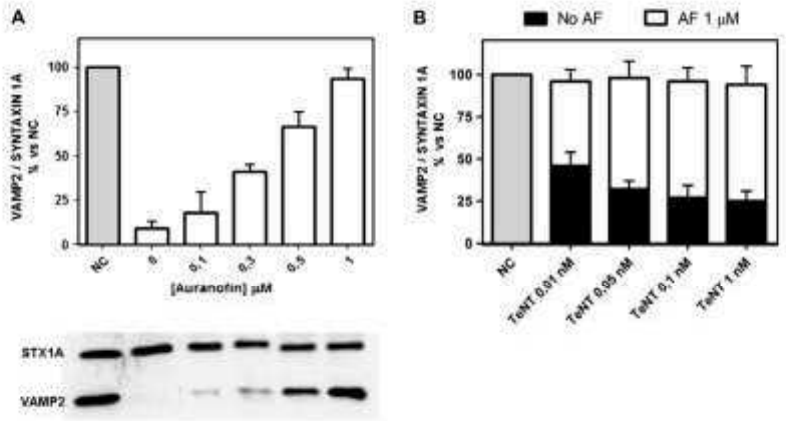

Fig. 1. TeNT cleavage of VAMP is inhibited by auranofin in cerebellar granular neurons. The neurons were pre-incubated for 6 h with the indicated concentrations (A) or with 1 μM of auranofin (B, empty bars) at 37 °C, washed with culture medium and incubated with 1 nM (A) or the indicated concentrations of TeNT (B) at 37 °C for 15 min in culture medium, washed and incubated for 12 h at 37 °C in the presence of the reported inhibitor concentration. In panel B, black bars represent the samples incubated without auranofin. Cells were lysed and the SNARE proteins were estimated by immunoblotting with specific antibodies (lower panel). Residual VAMP2 was quantified by densitometry and its percentage ratio with syntaxin 1A plotted, taking the value in non-treated cells (grey bar) as 100%. S.D. values derive from three independent experiments performed in triplicates. The lower panel shows a representative immunoblotting.

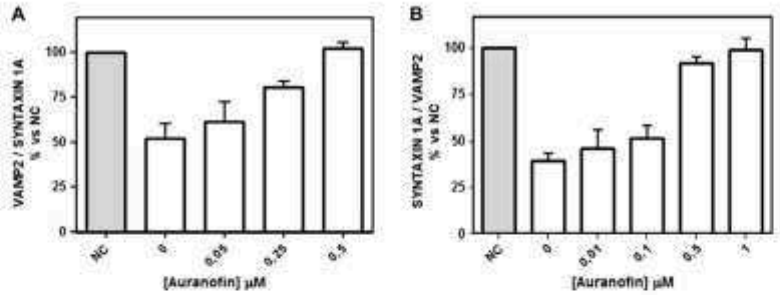

Fig. 2. BoNT/B and C cleavage of VAMP in cerebellar granular neurons is inhibited by auranofin. The neurons were pre-incubated for 6 h with the indicated concentrations of auranofin at 37 °C, washed with BME and incubated with 10 nM BoNT/B (A) or 1 nM BoNT/C (B) at 37 °C for 15 min in culture medium, washed and incubated for 12 h at 37 °C in the presence of the reported inhibitor concentration. Cells were lysed and the SNARE proteins were estimated by immunoblotting with specific antibodies. Residual VAMP2 (A) or residual syntaxin 1A was quantified by densitometric analysis and its percentage ratio with the respective non-substrate SNARE plotted, taking the value in non-treated cells (grey bar) as 100%. S.D. values derive from three independent experiments performed in triplicates.

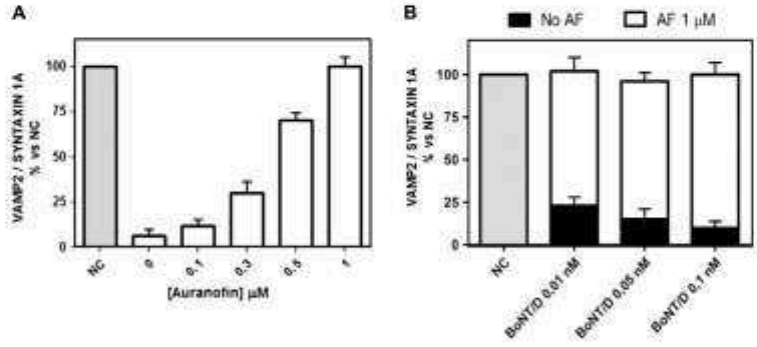

Fig. 3. Auranofin inhibits the VAMP cleavage by BoNT/D in cerebellar granular neurons. The neurons were pre-incubated for 6 h with the indicated concentrations (A) or with 1 μM auranofin (B, empty bars) at 37 °C, washed with medium and incubated for 15 min with 0.1 nM (A) or the indicated concentration (B) of BoNT/D at 37 °C in culture medium, washed again and incubated for 12 h at 37 °C in the presence of the reported inhibitor concentration. (B) black bars represent the samples incubated without auranofin. Cells were lysed and the SNARE proteins were estimated by immunoblotting with specific antibodies; residual VAMP was quantified as in Fig. 1. S.D. values derive from three independent experiments performed in triplicates.

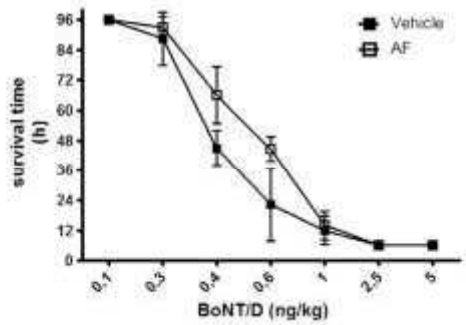

Fig. 4. Effect of auranofin on the BoNT/D MLD_{50} assay. Adult male CD1 mice were i.p. injected with 12 mg/kg of AF or with the vehicle 6 h before the i.p. injection of the indicated dose of BoNT/D. The animals were monitored every 3 h for 96 h. S.D. values derive from three different experiments performed with groups of three animals each.

chains of these neurotoxins. The NADPH-thioredoxin reductase-thioredoxin system very efficiently reduces in vitro the single interchain disulfide bond of TeNT [33] and of BoNTs (our unpublished results). The reduction of this SS bond in the cytosol is essential to free the SNARE cleaving activity of the L chain [31,32]. Taken together, these findings provide a strong indication that the thioredoxin system plays a major role, if not a unique one, in releasing the L chain of TeNT and BoNTs after their translocation across the membrane of the endocytic vesicle assisted by their respective H_N domains.

An attractive feature of thioredoxins is that they share structural similarities with group-I and group-II chaperonins [52,53] and indeed thioredoxins promote the folding of proteins in redox-independent reactions [53–55]. Thus, it is tempting to speculate that the thioredoxin system not only detaches the L chain from the H_N domain by severing the remaining disulfide link, but also acts before by assisting the refolding of the L chain as it emerges from H_N transmembrane channel.

The disappointing lack of protective activity of AF on the in vivo intoxication of mice with BoNT/D can be explained by the fact that AF does not cross the blood–brain barrier and it may as well be not capable of reaching the cytosol of the motor axon terminals

protected by the terminal Schwann cells on one side and the muscle fiber on the other side. However, the present data indicate that it is possible to use auranofin as a lead to design auranofin-based novel inhibitor of the clostridial neurotoxins capable of acting in vivo.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2012.11.007.

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Botulinum Neurotoxin type A is Internalized and Translocates from Small Synaptic Vesicles at the Neuromuscular Junction

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Abstract:	Botulinum neurotoxin type A is the most frequent cause of human botulism and at the same time is largely used in human therapy. Some evidence indicates that it enters inside nerve terminals via endocytosis of synaptic vesicles, though this has not been formally proven. The metalloprotease L chain of the neurotoxin then reaches the cytosol in a process driven by low pH, but the acidic compartment wherefrom it translocates has not been identified. Using immunoelectron microscopy, we show that BoNT/A does indeed enter inside synaptic vesicles and that each vesicle contains either one or two toxin molecules. This finding indicates that it is the BoNT/A protein receptor SV2, and not its polysialoganglioside receptor that determines the number of toxin molecules taken up by a single vesicle. In addition, by rapid quenching the vesicle transmembrane pH gradient, we show that translocation of the neurotoxin into the cytosol is a fast process. Taken together, these results strongly indicate that translocation of BoNT/A takes place from synaptic vesicles, and not from endosomal compartments, and that the translocation machinery is operated by one or two neurotoxin molecules.

Botulinum Neurotoxin type A is Internalized and Translocates from Small Synaptic Vesicles at the Neuromuscular Junction

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Abstract

Botulinum neurotoxin type A is the most frequent cause of human botulism and at the same time is largely used in human therapy. Some evidence indicates that it enters inside nerve terminals *via* endocytosis of synaptic vesicles, though this has not been formally proven. The metalloprotease L chain of the neurotoxin then reaches the cytosol in a process driven by low pH, but the acidic compartment wherefrom it translocates has not been identified. Using immunoelectron microscopy, we show that BoNT/A does indeed enter inside synaptic vesicles and that each vesicle contains either one or two toxin molecules. This finding indicates that it is the BoNT/A protein receptor SV2, and not its polysialoganglioside receptor that determines the number of toxin molecules taken up by a single vesicle. In addition, by rapid quenching the vesicle transmembrane pH gradient, we show that translocation of the neurotoxin into the cytosol is a fast process. Taken together, these results strongly indicate that translocation of BoNT/A takes place from synaptic vesicles, and not from endosomal compartments, and that the translocation machinery is operated by one or two neurotoxin molecules.

Keywords: Botulinum neurotoxin / electron microscopy /endocytosis/ membrane translocation/ neuromuscular junction/ synaptic vesicles

Introduction

The seven known different serotypes of botulinum neurotoxins (BoNT/A to /G) are produced by bacteria of the genus *Clostridium* and are the sole cause of the flaccid paralysis of botulism [1]. These neurotoxins are the most poisonous substances known so far, and their potential use in bioterrorism [2] is a matter of great concern. The BoNTs consists of three domains of 50 kDa each, which are termed L chain (the N-terminal one), HN (the intermediate one) and HC (the C-terminal domain) [3-5]. This structure is closely related to their mechanism of poisoning nerve terminals, which consists of four steps: a) binding to nerve terminals mediated by HC, b) endocytosis, c) a low pH-driven membrane translocation of the L chain mediated by HN, and d) L chain mediated cleavage and inactivation of the SNARE proteins with ensuing inhibition of neuroexocytosis [6-8]. The BoNTs are peculiar with respect to their presynaptic membrane binding which is mediated by a polysialoganglioside and by a protein receptor, which is the luminal portion of a synaptic vesicle membrane protein [8,9]. BoNT/A, /E and /F bind to SV2 [10-14], whilst BoNT/B and /G bind to synaptotagmin [15-18]; the protein receptor of the other BoNT serotypes has not been conclusively determined. While the fourth step of intoxication has been clarified long ago [19,20], endocytosis and membrane translocation remain largely unknown. The fact that tetanus neurotoxin is taken up by small synaptic vesicle in neurons of the central nervous tissue [21], but not at the neuromuscular junction (NMJ) where it is endocytosed inside coated vesicles [22], and the fact that the known BoNT protein receptors are in the lumenal domain of proteins of synaptic vesicles strongly suggests that BoNTs are endocytosed inside synaptic vesicles at peripheral nerve terminals. However, their actual presence inside vesicles, and the type of synaptic vesicles, has not been determined. Even less is known about the mode of membrane translocation, but biophysical studies have indicated that, at low pH, the HN of BoNT forms transmembrane channel conducing the L chain into the cytosol and involving from one to four neurotoxin molecules [8,23-28].

Here, we have studied the entry of BoNT/A into the nerve terminal of the mouse NMJ, because this serotype is the one responsible for the majority of cases of human botulism, and because BoNT/A is almost invariably used in the therapy of human diseases caused by hyper function of peripheral cholinergic nerve terminals [29-31]. We have generated a chimera consisting of a green fluorescent protein linked to the HC domain of BoNT/A (EGFP-BoNT/A-Hc) in order to visualize its distribution at the NMJ using fluorescence microscopy and immunoelectron microscopy with gold-labelled anti-GFP antibodies. This protocol avoids artefacts of binding deriving from the cross-linking of a number of neurotoxin molecules to the same gold particle.

We found that the injection of EGFP-BoNT/A-Hc into a mouse skeletal muscle leads to its specific binding to the presynaptic membrane at the active zones of the NMJ, and that one/two gold particles are present inside small clear synaptic vesicles. We also showed that the translocation of the L chain into the cytosol is a very rapid process, and we discuss the general implications of these findings.

Material and Methods

Preparation of the BoNT/A-Hc chimera

Polymerase chain reaction was performed using as a template the DNA of BoNT/A-Hc cloned in pGEX4T1-GST-HA (kind gift of Dr. G. Schiavo, Cancer Research, UK), and the DNA of EGFP cloned in N1. The following primers were used: for the HC of BoNT/A, residues 845-1295, AAACTCGAGAGTACAGATATACCTTTTCAGCTT and AAATTTAAGCTTTTACAGTGGCCTTTCTCCCCA; for EGFP AAAGGATCCATGGTGAGCAAGGGCGAG and AAACTCGAGCTTGTACAGCTCGTCCATGCC. The amplified sequence of Hc was digested with XhoI/HindIII and then cloned in the pRSETa vector to obtain pRSETa -BoNT/A-Hc; then the EGFP sequence digested BamHI/XhoI was ligated into the previous vector at the N-terminal domain in order to obtain finally pRSETa-EGFP-BoNT/A-Hc. DNA inserts were sequenced by CRIBI (Padova).

BL21 (DE3) pLysS *E. coli* strain transformed with pRSETa-EGFP-BoNT/A-Hc were grown in LB medium until the OD_{600} (optical density at 600 nm) reached 0.6. Cultures were induced with 1 mM IPTG (isopropyl β-D-1-thiogalactopyranoside) for 4 h at 30°C. Cells were pelleted, frozen in liquid nitrogen and kept at -80°C. Cells were then lysed by two passages of pre-cooled French Press. The lysate was centrifuged (17,000 x g, 20 min), and the supernatant was loaded on a HiTrap Chelating HP on a ÄKTAprime system (GE Healthcare) and eluted with a linear gradient from 0 to 500 mM Imidazole. Pooled fractions were dialyzed against 20 mM Tris, 200 mM NaCl, 0.1 mM DTT, 5% trehalose, 10% glycerol, pH 7.4. Protein purity was assessed by staining with SimplyBlue Safestain of a 12% SDS–PAGE gel. Protein identity was confirmed by Western blotting using an anti-Tag antibody.

In vivo mouse intramuscular injection of fusion proteins

Experiments were performed according to the Italian, French and European Community guidelines for laboratory animal handling. Young Swiss female mice were obtained from Charles River Breeding laboratories. EGFP-BoNT/A-Hc (25 μg) dissolved in phosphate-buffered saline (PBS), was injected into the sternocleidomastoid muscle of mice anesthetized with sodium pentobarbital (90 mg/Kg, i.p.). The animals were sacrificed by intracardiac perfusion of freshly prepared 4% paraformaldeyde (Electron Microscopy Sciences, Hatfield, PA, USA) 5-10 min after the injection of EGFP-BoNT/A-Hc.

Mouse sternocleidomastoid muscles

The muscles were dissected, their fibers were teased apart and stained for 30 min at 4 °C with AlexaFluor-594- or AlexaFluor680-conjugated α-bungarotoxin (2 µg/ml, A594-α-BTX, or A680-α-BTX; Molecular Probes Europe BV, Leiden, The Netherlands) in PBS and mounted with Vectashield antifade mounting medium (Vector Laboratories, Inc, Burlingame, Ca, USA), and neuromuscular junctions (NMJs) were analyzed using a Leica upright SP2 DM RXA2 confocal microscope through an oil-immersion objective x 63/1.32 NA, controlled by Leica Confocal Sotware version 2.61 Build 1537(Argon laser 488 nm for EGFP, HeNe laser 543 for A594 and HeNe laser 633 nm for A680). Intensity profiles analyses were performed on series of "look-through" projections of mean intensity.

Immunogold electron microscopy

After 4% paraformaldehyde perfusion, mouse EGFP-BoNT/A-Hc-injected sternoclaidomastoid muscles were dissected out, and postfixed for 1 h with a mixture of 4% paraformaldehyde, 0.1% glutaraldehyde (Electron Microscopy Sciences, Hatfield, PA, USA), 0.2% picric acid (Sigma-Aldrich Chimie, Saint Quentin, Fallavier, France) and 3% sucrose in PBS solution. Muscle regions containing NMJs were treated with 0.5% osmium tetroxide (Electron Microscopy Sciences) for 30 min in PBS, followed by dehydration in a graded ethanol series and embedded in LR-White resin (Electron Microsocopy Sciences). Silver-gray thin sections (65 nm) were treated on grids with 0.1% sodium borohydride in PBS for 15 min. After washing with PBS containing 0.1% acetylated BSA solution (solution A), non-specific labeling was prevented with a 30 min incubation with 5% BSA in 0.1% gelatine (Aurion, Wageningen, The Netherlands) / 5% normal goat serum (NGS, Sigma). Sections were incubated for 2 h with polyclonal rabbit anti-GFP antibody (diluted 1:100 in solution A, MBL, Watertown, USA) followed by 1 h incubation with goat anti-rabbit IgG conjugated with 10 nm-diameter colloidal gold (Aurion, ImmunoGold Reagents, The Netherlands) at 1:25 dilution in solution A. After washing, the sections were fixed with 2.5% glutaraldehyde in PBS. Finally, the grids were counterstained with 2.5% uranyl acetate and 1% lead citrate solutions and examined using a Jeol 1400 transmission electron microscope.

Cell cultures

Rat embryonic spinal cord motoneurons (MNs) were prepared essentially as described [32]. Briefly, spinal cords were collected from fetal rats at gestation day 14, and first mechanically disrupted and then cells were dissociated by mild trypsination in the presence of DNAse I. Finally, isolated cells were plated on 24 well-plates, pretreated with poly-ornithine (1.5 µg/ml overnight) and then with laminin (10 µg/ml for 2 h). Cultures were maintained at 37°C and 5% of CO_2 before starting the assay at DIV 15. The culture medium consisted of Neurobasal (Gibco) supplemented with 2% heat-inactivated horse serum, 0.5 mM L-glutamine, 25 mM 2-mercaptoethanol, 25 mM L-glutamic acid, B27 supplement, 10 ng/ml CNTF, 100 pg/ml GDNF and 5 mg/ml penicillin/streptomycin.

Rat cerebellar granular neurons (CGNs) were prepared as previously described [33]. Rat pups were sacrificed 6 days after birth, cerebella was solated and cells dissociated by trypsination in the presence of DNAse I. Collected cells were plated in 24 well-plates, pretreated with poly-L-lysine (50 µg/mL overnight),

at cell density of $3 \cdot 10^5$ cells per well. Cultures were maintained at 37 °C, 5% CO_2, in BME supplemented with 10% fetal bovine serum, 25 mM KCl, 2 mM glutamine and 50 μg/mL gentamycin. To arrest growth of non-neuronal cells, cytosine arabinoside (10 μM) was added to the medium 18–24 h after plating. Experiments were performed on neurons that were 6-/ DIV.

Neutralization of the acidic compartment on BoNT/A entry into cultured neurons

MNs or CGNs were treated with BoNT/A (10 nM) in high-potassium buffer (85 mM NaCl, 60 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 5.5 mM glucose, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.4) for 5 min. Unbound neurotoxin was washed out using low-K^+ buffer (140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 5.5 mM glucose, 10 mM HEPES, pH 7.4) and normal culture medium was restored. Before, together or at the indicated time points after neurotoxin addition, Bafilomycin A1 (baf A1, 100 nM, Sigma-Aldrich) alone, or in combination with monensin (Sigma-Aldrich, 100 nM) or NH_4Cl (50 mM) was added to the culture media. After 15 min, the medium was removed and replaced with one containing only baf A1 and the neurons were further incubated for 12 h, and cells were lysed directly in Laemmli buffer. BoNT/A entry was evaluated by determining the extent of SNAP25 cleavage by immunoblot analysis using a polyclonal antibody which specifically recognizes the C-terminal part of SNAP25 [34] A monoclonal antibody (Mab) against VAMP2 (Synaptic Systems, Göttingen Germany) was used as an internal loading control. Cleavage was reported as the percentage ratio of whole SNAP25 with respect to VAMP2, considering data collected from three independent experiments made in triplicate. Values are expressed as the mean ± SD.

Results

A characteristic of BoNT/A, which is essential for its clinical use, is its rapid and specific binding to the presynaptic membrane of the vertebrate NMJ with limited diffusion near the site of injection [35]. Fig. 1 A shows that this property is conserved by the EGFP-BoNT/A-Hc chimera, since it labels only motor nerve terminals within the boundaries defined by the post-synaptic nicotinic acetylcholine receptor visualized here by the very specific binding of fluorescent α-BTX. The neuromuscular branches side view presented in row B of Fig. 1 clearly show that the chimera binding is restricted to the presynaptic membrane. However, the level of resolution of this analysis does not allow one to distinguish toxin bound to the nerve terminal surface from internalized neurotoxin.

To visualize the distribution of the binding domain of BoNT/A at higher resolution we performed immunoelectron microscopy, using as a read out a GFP specific antibody, which was then revealed with a gold-labelled secondary antibody. This procedure is somewhat more laborious than the direct coupling of BoNT/A-Hc to gold particles, but was chosen to avoid possible artefacts owing to possible multi-receptor binding of the several BoNT/A-Hc molecules cross-linked to the same gold particle. On the other hand, the present procedure can introduce some unspecific gold deposition due to increased possibility of antibody

cross-reaction. Fig. 2 shows that the majority of the neurotoxin molecules are inside small clear synaptic vesicles (SSV) in muscle samples fixed 5 or 10 min after injection (arrowheads), while few are still bound to the presynaptic membrane, mainly at active zones (long arrows); only occasionally gold particles are found in the cytosol, possibly owing to antibody cross-reaction. This clearly indicates that binding and endocytosis *in vivo* is rapid and that the toxin ends up inside SSV.

Many motor nerve terminals were analysed, and Table 1 reports the statistics of the gold particle distribution. This distribution is rather similar in samples analysed 5 or 10 min after neurotoxin injection, as expected from the fact that only the Hc presynaptic membrane binding domain of BoNT/A is present here and therefore translocation in the cytosol cannot take place. The average number of gold particles, and therefore of BoNT/A-Hc molecules, present inside SSV is around 1.5 molecule per vesicle (see table 1). This method may over-estimate the number of toxin molecules present in the lumen of a single SSV owing to the use of a secondary antibody, to possible antibody cross reactivity, and to accidental deposition of a gold particle. In addition, the simultaneous binding of one gold labeled secondary antibody to more than one anti-GFP antibodies cannot be excluded. Remarkably, the average figure of 1.5 correlates well with the number of BoNT/A protein-receptor-SV2 molecules present in a single SSV, estimated to be between 1 and 2 [36].

To provide a functional estimation of the time course of the translocation process of the BoNT/A-L chain, we used different agents able to quench the transmembrane pH gradient existing across the SSV membrane at defined time points: Bafilomycin A1 (bafA1) which inhibits the SSV ATPase proton pump, monensin which is a very rapid exchanger of H^+ for monovalent cations, and ammonium which buffers the lumen pH. The effect of their addition at different time points before and after a bacterial toxin, whose entry into the cytosol occurs via an acidic compartment, provides a reliable estimation of the time course of the entry of the toxin active chain entry into the cytosol [37,38]. When a rapid quenching is needed these agents can be combined [37]. Two different primary neuronal cultures were used to obtain information of more general relevance: cerebellar granular neurons and spinal cord motoneurons. Fig. 3 shows that whatever pH gradient quenching method was used (bafA1, bafA1 plus monensin, bafA1 plus ammonium) the neutralization of the lumenal pH of SSV prevented the low pH driven membrane translocation of the L chain of BoNT/A only for a few minutes after BoNT/A addition indicating that the entry of its L chain in the cytosol takes place early after endocytosis. These data compare well with previous findings obtained with concanamycin A which is another inhibitor of the V-ATPase [39,40] and indicate that the quenching of the transmembrane pH gradient of SSV is sufficiently rapid also with this slower inhibitor [37].

Discussion

The most important information delivered by the present work are: i) BoNT/A is internalised into the lumen of small synaptic vesicles, ii) it then translocates very rapidly its L chain into the cytosol, and iii) the L chain translocating channel is made of one, or at the most, two HN domains. These conclusions derive from a quantitative visualization of the toxin molecule within the lumen of small synaptic vesicles and from a time

resolved quenching of the effector of the translocation, which is the lumenal low pH. This latter experiment was performed with two different types of neurons and three different transmembrane pH gradient quenchings in order to obtain results of more general value. The present data fit very well with the estimation that a population of small synaptic vesicles contain an average of one to two SV2 molecules per small synaptic vesicle [36]. At the same time, this finding indicates that it is the SV2 protein receptor and not the polysialoganglioside receptor that determines the number of toxin molecules loaded within one SSV.

These considerations are not limited to BoNT/A, but can be extended with large confidence to BoNT/E, which differs structurally from BoNT/A [3,5] but enters the cytosol of synaptic terminals within few minutes [39,40] as BoNT/A does. The similar time-course and the fact that BoNT/E shares with BoNT/A the SV2 protein receptor speaks strongly in favour of the possibility that also the L chain of BoNT/E enters the cytosol shortly after neurotoxin endocytosis in the SSV lumen by crossing the SSV membrane.

The present study has not found evidence for an internalization of BoNT/A inside other type of vesicles nor does it support the possibility that the membrane translocation of L chains of BoNT/A and BoNT/E takes place at the level of endosomes of synaptic terminals after fusion of the SSV with such compartments.

The conclusion that the protein conducing channel made by the HN domain in the SSV membrane involves no more than two molecules has to be confronted with previous data obtained with model systems that the low pH driven membrane inserted BoNT/A is a tetramer [23,24]. However, these data were obtained using very different conditions that may explain the different figures obtained. In one case segment 659-681 of BoNT/A was used in a planar lipid bilayer [23], whilst the other one used an artificial liposomal system [24]. More recently, using atomic force microscopy of BoNT/B interacting with polysialoganglioside containing lipid bilayers at pH 4.4, a significant amount of trimers of the toxin were detected and it was suggested that the L chain conducing channel of this neurotoxin is composed of three HN domains [28]. In a more recent study, the formation of oligomers of BoNT/E was not detected [40], and though negative, this finding is in agreement with the suggestion advanced here.

In conclusion, the present work visualizes for the first time BoNT/A inside small clear synaptic vesicles present within the main target of this neurotoxin, i.e. the motor nerve terminal of the NMJ. The present data were obtained with a method that avoids the possibility of receptor cross-linking, and provide a reliable estimate of the number of neurotoxin molecules present within a vesicle. The figure obtained in this way is comprised within one and two suggesting that membrane translocation occur at the level of the membrane of small clear synaptic vesicles via a protomer consisting of no more than two neurotoxin molecules.

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Conflict of interest

The authors declare that they have no conflict of interest.

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FIGURE LEGENDS

Fig. 1 The Botulinum neurotoxin type A HC binding domain (BoNT/A-Hc) binds to axon terminals of the mouse sternocleidomastoid neuromuscular junction (NMJ) shown by confocal fluorescent microscopy. Panel A: Top view of two NMJs, revealed by the staining of the postsynaptic acetylcholine receptors with Alexa 594-labelled α-bungarotoxin (α-BTx), showing that the EGFP fluorescence of BoNT/A-Hc toxin (EGFP-BoNT/A-Hc) is distributed along the motor nerve terminal and restricted to the NMJ defined by the α-BTx staining (Merge). Panel B: Side view of a NMJ branches clearly shows that EGFP-BoNT/A-Hc label is mainly found at the synaptic side of the nerve terminal (around the presynaptic membrane) and does not co-localize with the facing Alexa 680-labelled α-BTx (Merge). Scale bars: Panel A= 20 µm ; panel B= 10µm.

Fig. 2 Immunogold electron micrographs showing the ultrastructural localization of the binding domain of BoNT/A in the mouse sternocleidomastoid neuromuscular junction (NMJ). A fluorescent derivative of the binding domain of BoNT/A (EGFP-BoNT/A-Hc) was intradermically injected at the sternocleidomastoid muscle and immunocytochemically processed with a anti-EGFP following a postembedding procedure using a secondary 10 nm gold-labelled antibody. The electron micrographs show that toxin is rapidly internalized and is localized mainly within the lumen of small synaptic vesicles (SSVs; arrowheads), with some molecules still bound the presynaptic membrane mainly at the active zones (AZs; long arrows) either at 5 (A and B) or 10 min (C) after toxin injection. Scale bars: A-C= 150 nm.

Fig. 3 Time course of the entry of the L chain of BoNT/A in cerebellar granular neurons (A) and in spinal cord motoneurons (B) . Cells were incubated with BoNT/A (10 nM) at 37 °C and, at the times indicated, bafilomycin A1 (100 nM) alone (black bars), in combination with monensin (100nM, striped bars) or with NH_4Cl (50 mM, empty bars) was added. The incubation was prolonged for 12 h and the content of SNARE proteins was estimated by immunoblotting with specific antibodies. Values are reported as the ratio between the staining with the antibody specific for SNAP25 and the staining with the antibody specific for VAMP/synaptobrevin2 expressed as the control value taken as 100%. SD values refer to three different experiments performed in triplicate.

Table
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Table 1. Estimates of the number of SSVs labelled with EGFP-BoNT/A-Hc, revealed with a 10 nm colloidal gold labeled anti-EGFP specific antibody, in motor nerve terminals 5 and 10 min after a subcutaneous toxin injection in the mouse sternocleidomastoid muscle.

Time after BoNT/A-Hc-EGFP injection	5 min [a]	10 min [b]
Number of SSVs counted	57.64 ± 0.76 [c]	54.03 ± 0.59
% SSVs labeled	37.52 ± 0.12	40.34 ± 0.15
Number of grains/SSV counted	1.46 ± 0.18	1.51 ± 0.25

[a] Data obtained from 26 different motor nerve terminals examined.

[b] Data obtained from 24 different motor nerve terminals.

[c] Data represent the mean ± S.E.M.

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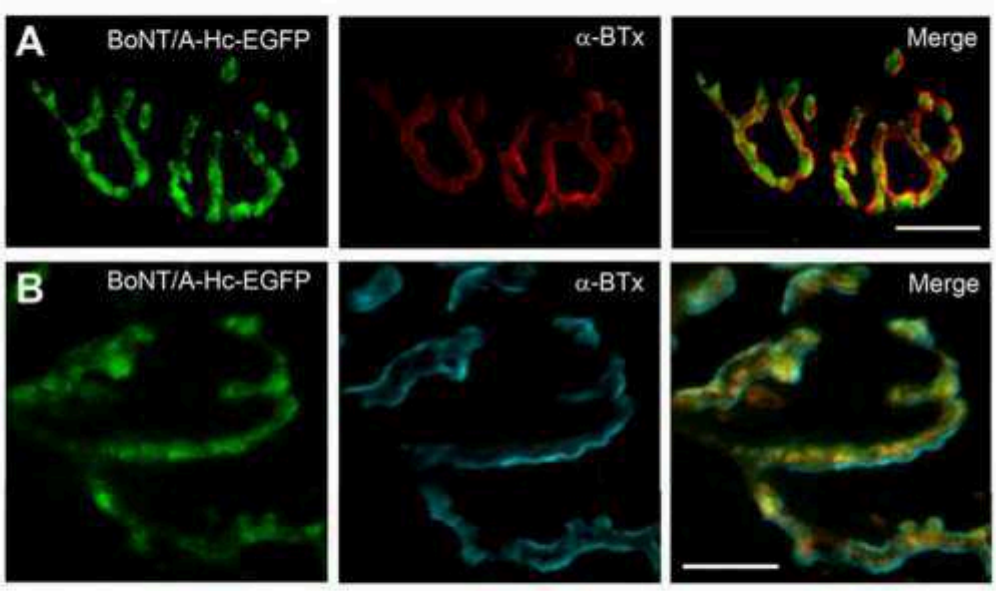

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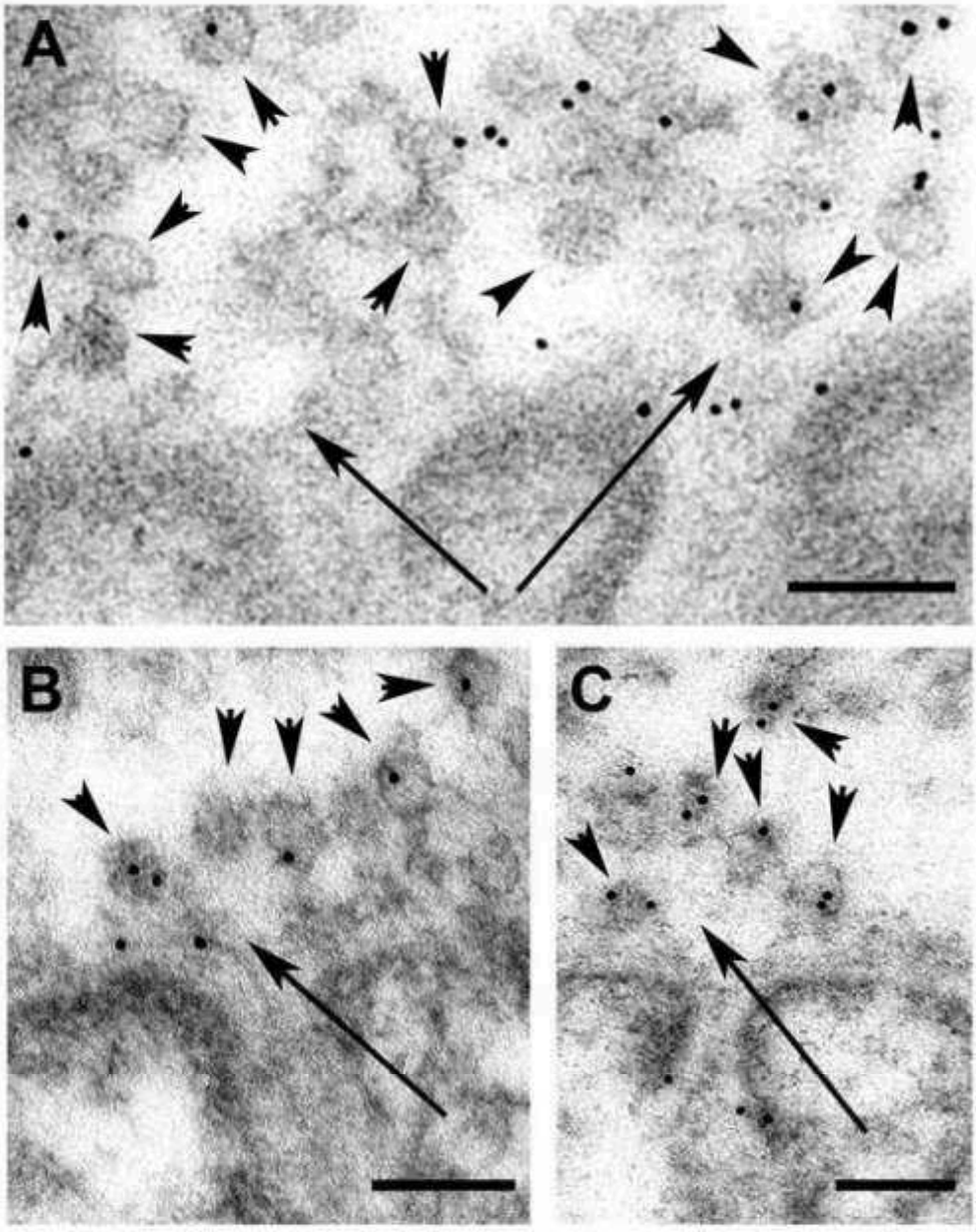

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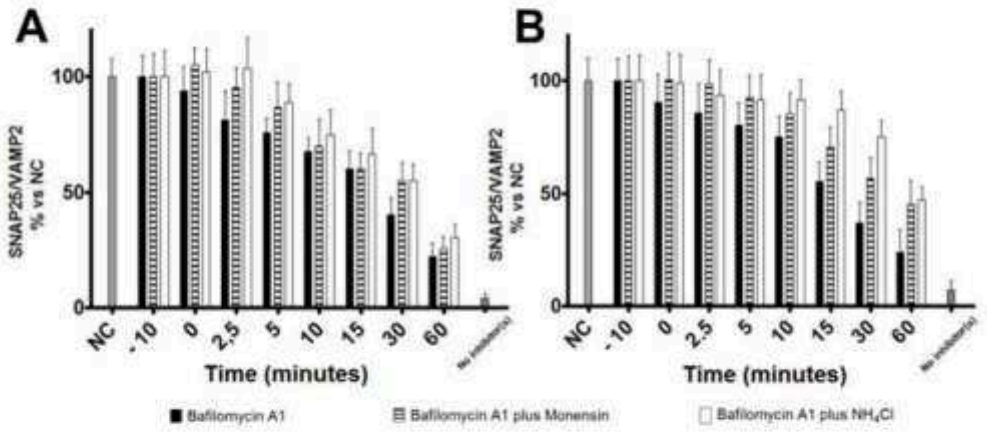

Botulinum neurotoxin serotype D is poorly effective in humans: an in vivo electrophysiological study

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Botulinum neurotoxin serotype D is poorly effective in humans: An *in vivo* electrophysiological study

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HIGHLIGHTS

- The effect of botulinum toxin type D has been tested for the first time in humans *in vivo*.
- In injected human EDB muscle, the compound muscle action potential was measured.
- Botulinum toxin type D is very effective in mice but poorly effective in humans.

ABSTRACT

Objective: Botulinum neurotoxins act on nerve endings and block neurotransmitter release. Their potency is due to their enzymatic activity and high affinity binding to neurons. Botulinum toxin type A is used in the treatment of human diseases characterized by hyperactivity of peripheral cholinergic nerve terminals, but some patients are or become resistant to it. This can be overcome by using other botulinum toxins, and studies have been performed with different toxin serotypes. Botulinum neurotoxin type D has never been tested in humans in vivo, and, therefore, we investigated the action of this toxin in mouse and human muscles.
Methods: Botulinum toxin type D potency was determined on mouse hemidiaphragm and on rat neuronal cultures. From these experiments, doses to be injected in human volunteers were decided. The compound muscle action potential of toxin-injected Extensor Digitorum Brevis muscle was measured at different times points after injection in human volunteers.
Results: Botulinum toxin type D is poorly effective in inducing human skeletal muscle paralysis.
Conclusions: Botulinum toxin type D is very potent in mice and almost ineffective in humans in vivo.
Significance: The results shed new light on the mechanism of toxin type D binding to the neuronal surface receptors.

1. Introduction

Toxigenic bacteria of the genus *Clostridium* produce seven different serotypes of botulinum neurotoxin (abbreviated as BoNT/A to BoNT/G), which is the sole responsible of all the symptoms of botulism (Rossetto et al., 2006; Johnson and Botulism, 2008); in addition several isoforms of serotypes were identified (Moriishi et al., 1996; Arndt et al., 2006; Kalb et al., 2012). The BoNTs bind and enter peripheral nerve terminals, and cause a flaccid paralysis with autonomic symptoms (Rossetto et al., 2006; Johnson and Botulism, 2008). These neurotoxins consist of a light chain (L, 50 kDa) and a heavy chain (H, 100 kDa) linked by a single inter-chain disulfide bond (Lacy and Stevens, 1999; Swaminathan and Eswaramoorthy, 2000; Kumaran et al., 2009; Montal, 2010). Their process of nerve terminal intoxication can be conveniently divided into four steps (Montecucco et al., 1994): (a) binding to a sialoganglioside molecule of the presynaptic membrane, mediated by the C-terminal part of H; (b) endocytosis inside synaptic vesicle mediated by the binding of H to a receptor located on the lumen of the vesicle; (c) membrane translocation driven by the acidification of the lumen of the vesicle (Pirazzini et al., 2011; Sun et al., 2012); (d) cleavage of a SNARE protein via the metalloproteolytic activity of the L chain (Schiavo et al., 1994).

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It has long been known that animal species vary extensively in terms of sensitivity to the different BoNT serotypes (Payling-Wright, 1955; Gill, 1982; Smith and Sugiyama, 1988). Such differences could be due either to different membrane binding and/or to differences in the SNARE proteolytic substrates; other aspects such as inefficient membrane translocation appear to be less likely as basic membrane structure is preserved among different animal species (Montal, 2010; Pirazzini et al., in preparation). Indeed, insects are BoNT-insensitive because they do not possess polysialogangliosides (PSG), though may have SNARE proteins cleavable by the BoNT L chains. In contrast, rats bind tetanus neurotoxin (TeNT) very efficiently, but are highly resistant to tetanus because the rat spinal cord SNARE protein isoform VAMP-1 carries a Gln-Val mutation at the cleavage site of the TeNT L chain (Patarnello et al., 1993).

Of particular importance is the determination of the susceptibility of humans to the various toxin serotypes and isoforms of serotypes, and of their kinetics of action, because of the large employment of BoNT/A in the therapy of a large variety of human diseases (Hallett, 2000; Jankovic, 2004; Montecucco and Molgo, 2005; Dressler and Hallett, 2006). A largely unexplored, but potentially important area of investigation is that of the improvement achievable in the treatment of different human diseases with different BoNT serotypes. We were the first to report the great potential of the use of BoNT/C injection in humans by using an electrophysiological testing (Eleopra et al., 1997b) and have afterwards compared several serotypes in human muscles (Eleopra et al., 2004). In these works it was erroneously reported that BoNT/C was from Wako Chemicals, whilst we had actually used BoNT/C purified from strain NCTC 8264 (Shone and Tranter, 1995).

BoNT/D is a very powerful toxin, which inhibits neurotransmitter release (Molgó et al., 1989) by cleaving VAMP (Schiavo et al., 1993; Yamasaki et al., 1994) and has been reported not to be active on surgically excised human pyramidalis muscle (Coffield et al., 1997). In a recent review, however, Anderson et al., 2009 mentioned their unpublished studies in which human intercostal muscles were found to be BoNT/D sensitive. This neurotoxin is very active in mice (Nakamura et al., 2012). Very recent investigations have revealed that the C-terminal part of the H chain of BoNT/C and /D binds two molecules of PSG at two different sites (Karalewitz et al., 2010; Strotmeier et al., 2010, 2011; Zhang et al., 2011). In addition, it was reported that the endocytosis of BoNT/D is mediated by the lumenal domain of the synaptic vesicle SV2 (Peng et al., 2011). As it is well established that PSG do exist on the presynaptic membrane of the neuromuscular junctions (NMJ) of both mice and humans (Cochran et al., 1982; Wiegandt, 1985; Ledeen et al., 1986; Chiba et al., 1992; Willison et al., 1994; Lehmann et al., 2007), and given the conflicting reports on the sensitivity of human muscles to BoNT/D, we decided to investigate the action of BoNT/D in mouse and in human muscles *in vivo* by the electrophysiological evaluation of the compound muscle action potential amplitude, which is a quantitative neurophysiological testing of the neuromuscular paralysis.

2. Methods

2.1. Toxins preparation

BoNT/A used for mouse study was prepared as described (Shone and Tranter, 1995). BoNT/D was from WAKO Chemicals, Japan (strain CB-16) and purified as described previously (Schiavo et al., 1993; Schiavo and Montecucco, 1995). The toxins were dissolved in 10 mM Hepes, 150 mM NaCl, pH 7.4 containing 2% defatted human or mouse albumin, sterilized by passage through 0.22 μm microfilters and stored at −80 °C after freezing in liquid nitrogen.

2.2. Antibodies

Anti-VAMP monoclonal antibody was from Synaptic System, Germany (cat. n. SYSY104211). Polyclonal anti-SNAP25 was produced by injecting in rabbit the C-terminal peptide AN-QRATKMLGSG of SNAP25 conjugated to keyhole lympet emocyanin.

2.3. Animals

Swiss-Webster adult male CD1 mice weighing 25–30 g were used. Mice were housed under controlled light/dark conditions and had free access to food and water was provided ad libitum. All experiments were performed in accordance with the Italian guidelines, law n. 116/1992 and were approved by the Animal Ethical Committee of our University.

2.4. Mouse lethality assay

The LD_{50} of each neurotoxin was determined following i.p. injection into mice (Pearce et al., 1994). Five doses were used to determine the LD_{50}, and 6 animals were used per dose. Mice were evaluated at 12 h intervals after administration.

2.5. Phrenic nerve hemidiaphragm

Mouse phrenic nerve-hemidiaphragm preparations were used as previously described (Rigoni et al., 2004). BoNT/A or BoNT/D were added at a final concentration of 0.1 nM and incubated in physiological medium at 20 °C for 30 min without nerve stimulation. At the end of the incubation, tissues were washed and transferred to 37 °C in a bath without toxin and phrenic nerve stimulation was applied via two ring platinum electrodes by supramaximal stimuli of 3–6 V amplitude and 0.1 ms pulse duration with a frequency of 0.1 Hz. Toxin-induced paralysis was measured as a 90% reduction in muscle twitch response to neurogenic stimulation. The time required to decrease the amplitude to 50% of the starting value (paralytic half-time) was also determined.

2.6. Primary neuronal cultures and Western blot

Rat cerebellar granule neurons (CGNs) were prepared from 6 days neonatal Wistar rats as previously described (Levi et al., 1984; Rigoni et al., 2004). Briefly, fresh cerebella were disrupted mechanically in presence of trypsin and DNase I and the cells plated onto 24 well culture plates functionalized with poly-L-lysine (10 μg/mL). Cultures were maintained at 37 °C, 5% CO_2, 95% humidity in BME supplemented with 10% fetal bovine serum, 25 mM KCl, 2 mM glutamine an 50 μg/mL gentamycin. To arrest growth of non-neuronal cells, cytosine arabinoside (10 μM) was added to the medium 18–24 h after plating.

Primary rat spinal cord motoneurons (MNs) were isolated from Sprague-Dawley rat embryos (embryonic day 14) and cultured following previously described protocols (Arce et al., 1999).

After 5–8 days of neuronal differentiation *in vitro*, CGNs and MNs were incubated for 24 h at 37 °C with different doses of either BoNT/D or BoNT/A diluted in Basal Medium Eagle (BME) supplemented with 10% FBS and 25 mM KCl for CGNs or in Neurobasal medium supplemented with 10% horse serum for MNs. In some experiments CGNs were incubated with BoNT/D in ice-cooled MEM 10% FBS pH 7.4 and left at 4 °C for 15 min. After washing twice with the same cold medium, pre-warmed medium A (123 mM NaCl, 6 mM KCl, 0.8 mM $MgCl_2$, 1.5 mM $CaCl_2$, 5 mM

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NaP_i, 5 mM citric acid, 5.6 mM glucose, 10 mM NH_4Cl), adjusted at different pH values with 1 M TRIS-base, was added and left for 5 min at 37 °C. Cells were then washed twice with MEM and further incubated for 12 h in MEM, 10% FBS pH 7.4 containing 100 nM bafilomycin A1 in order to induce entry of the toxin from the cell surface which bypasses endocytosis (Pirazzini et al., 2011).

After incubation with the toxins, cells were directly lysed in the wells with Laemmli buffer containing protease inhibitors (complete Mini EDTA-free, Roche) and separated by SDS–PAGE electrophoresis for Western immunoblotting. Primary antibodies against SNAP25 and VAMP/synaptobrevin were incubated overnight at 4 °C, washed three times with PBS-Tween, and incubated with secondary antibodies conjugated to horseradish peroxidase. After three washes with PBS-Tween and one with PBS, visualization was carried out using enhanced chemiluminescence kit (ECL, GE Healthcare). SNAP-25 and VAMP bands were quantified by densitometric analysis and the amount of cleaved substrate was determined as a ratio with respect to the uncleaved SNARE protein.

2.7. Effect of BoNT/D injection in human muscles

A group of ten healthy human volunteers was gathered among the members of our laboratories, including some of the authors of the present paper; all volunteers were known to be sensitive to BoNT/A. A written consent was obtained from each of them after a detailed explanation of the procedure to be used and of the biochemical, cellular and tissue procedures of characterization of the BoNT/D preparation to be injected in the Extensor Digitorum Brevis (EDB) muscle of the feet. In five subjects (10 EDB muscles) a dose of 3 IU of BoNT/D was injected and its effect was determined. After collection of these results, five subjects (10 EDB muscles) were injected with a higher dosage of BoNT/D (10 IU). The effect in the EDB injected with the toxin was quantified by the electrophysiologic evaluation of the compound muscle action potential (CMAP) amplitude, elicited by supramaximal electrical stimulation of the peroneal nerve at the ankle, before and after the treatment (at the 1, 2, 3, 4, 6 and 8 weeks). Electrical stimulation was performed by using a single shock of 0.5 ms of duration, delivered in a random pattern at low frequency. The recording electrodes placements were the same for each patient, using similar environmental conditions, at the same time of the day and after checking the skin temperature. Before the study, the long-term stability of the CMAP in 20 normal EDBs was checked by testing in various subjects this technique in different days and obtaining a CMAP percentage trend variation (test–retest amplitude variability) within 20%. Therefore, the peak-to-peak amplitude of the evoked CMAP was measured and compared in percentage with the baseline's value.

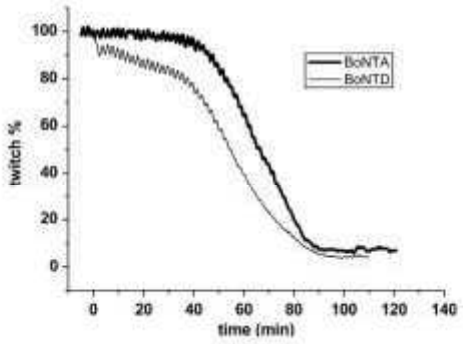

Fig. 1. Twitch tension assay on mouse phrenic nerve hemidiaphragm. BoNT/A (bold trace) or BoNT/D (thin trace) was added at 20 °C for 30 min in resting condition at a final concentration of 0.1 nM. The tissue was then washed twice, the temperature reset at 37 °C and the nerve stimulated with a frequency of 0.1 Hz. Toxins effect was evaluated as twitch reduction upon neurogenic stimulation over the time.

3. Results

3.1. Comparative toxicity of BoNT/D and BoNT/A in mice in vivo

To determine the toxicity of BoNT/D and BoNT/A used in this study, we estimated their biologic activity using the mouse lethality assay. BoNTs were injected i.p.in mice at doses ranging between 0.2 ng/Kg and 20 ng/Kg and percent of lethality was determined at each doses over four days. The resulting specific activity was 0.4×10^8 LD_{50}/mg for BoNT/A and 0.6×10^8 LD_{50}/mg for BoNT/D.

For the injections in human volunteers, the LD_{50} was expressed in International Units (IU) with the resulting specific activity of 0.016 ng/IU for BoNT/D and 0.025 ng/IU for BoNT/A.

3.2. Comparative toxicity of BoNT/D and BoNT/A in the mouse hemidiaphragm preparation

The mouse phrenic nerve-hemidiaphragm preparation is a well-established method of testing the biologic activity of a BoNT and, because of its reliability, the time course of paralysis is taken as a standard by several health agencies and production laboratories (Dressler et al., 2005; Rasetti-Escargueil et al., 2009). Fig. 1 shows the development of the muscle paralysis induced by the progressive silencing of the NMJ by BoNT/A and BoNT/D: BoNT doses of comparable efficacy in causing mouse death exhibit different kinetics of action. BoNT/D acts faster than BoNT/A. In principle these kinetics differences can be attributed to any of the four steps of their process of nerve entry and intoxication. It is unlikely that binding is a major determinant as the interaction of a protein ligand with a membrane receptor, not involving proteolysis or other chemical modifications, is usually very rapid. Available evidence indicate that the endocytosis of all BoNTs is mediated by acidic compartments of the nerve terminals, most likely synaptic vesicles (Montecucco and Schiavo 1995), and therefore this second step cannot account for the different kinetics. Therefore, the different time courses of paralysis of the two BoNTs have to be attributed to the third and/or the fourth steps of cellular intoxication process.

3.3. BoNT/D cleaves VAMP/synaptobrevin in cultured rat neurons

To complete the characterization of the action of the BoNT/D preparation to be used in human volunteers, we tested its VAMP cleavage activity with rat cerebellar granular neurons and spinal cord motoneurons, using the traditional procedure of incubation of the toxin in the cell culture medium (Fig. 2) or the low pH jump induced entry of the toxin from the cell surface which by-passes endocytosis (not shown) (Pirazzini et al., 2011). Fig. 2 shows that the present preparation of BoNT/D is very effective in cleaving VAMP/synaptobrevin in both neuronal cultures. This result is very important in the light of the fact that the predominant VAMP isoform in the central nervous system (CNS) is isoform 2, whilst VAMP-1 predominates in spinal cord and peripheral motoneurons (Elferink et al., 1989; Patarnello et al., 1993; Li et al., 1996; Jacobsson et al., 1998) and that in human and rat VAMP-1 the essential Met46 of mouse (Pellizzari et al., 1998) is replaced by a Ile residue which renders VAMP-1 less sensitive to the cleavage by BoNT/D *in vitro* (Yamasaki et al., 1994; Nakamura et al., 2012). The present findings indicate that despite the Met/Ile replacement rat VAMP-1 remains sensitive to BoNT/D in

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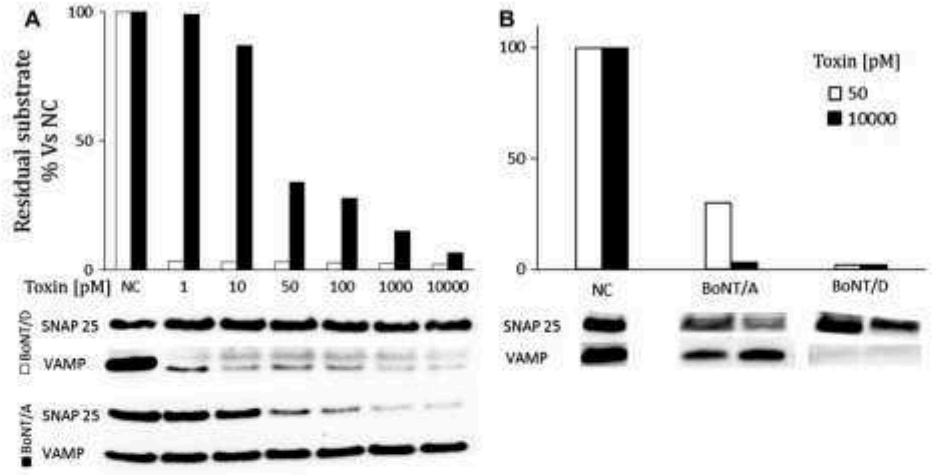

Fig. 2. BoNT/A and BoNT/D cleavage of their SNARE target proteins in rat cerebellar granular neurons (A) and in rat spinal cord motoneurons (B). Cells were incubated for 24 h at 37 °C with indicated concentration of BoNT/A or BoNT/D diluted in the respective culture medium. Cells were then lysed and their SNARE proteins were estimated by immunoblotting with specific antibodies (lower panels); SNAP-25 and VAMP bands were quantified by densitometric analysis (upper panels), and their ratios percentage plotted, taking the value in non-treated cells as 100%; VAMP which is not cleaved by BoNT/A is taken as an internal standard for BoNT/A treated cells and vice versa in BoNT/D treated cells. The lower panels show the immunoblotting obtained in a representative experiment.

motoneurons. It should also be considered that much longer times are implicated in human intoxication (botulism) or therapeutic injection and this renders the time course of cleavage of the BoNTs substrates a less stringent parameter.

3.4. Effect of the injection of BoNT/D in the human EDB muscle in comparison with those caused by BoNT/A

BoNT/D was injected into the EDB muscles of five human volunteers using a low dosage (3 IU of BoNT/D, 10 EDB muscles tested). The temporal profile of the mean CMAP amplitude percentage variation in EDB revealed no sign of paralysis when BoNT/D was injected (after 1, 2, 3, 4, 6 and 8 weeks) (Fig. 3A and Supplementary Fig. S1A). On the contrary, BoNT/A, injected 6 months before in the same subjects, was very effective and showed a profile of action in terms of percentage of inhibition and time course of recovery identical to those determined previously by our and by other laboratories (Eleopra et al., 1997a, 1998, 2002, 2004; Aoki, 2001; Rosales et al., 2006).

On the basis of the negative results of Fig. 3A, we performed another set of injections using a higher dose (10 IU of BoNT/D, 10 EDB muscles tested). The results are shown in Fig. 3B and in Supplementary Fig. S1B. Here, a slight paralytic effect can be noticed at the first week after injection, which returns within control value already at the second week as the CMAP amplitude becomes statistically indistinguishable from values obtained at later time points and from controls. However this effect is really minor and it appears that much larger doses have to be injected to detect an appreciable effect.

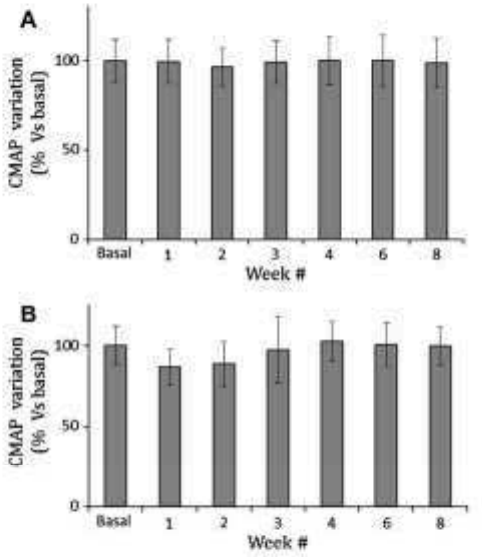

Fig. 3. Percentage trend variation of the compound muscular action potential (CMAP) over time. The values are expressed as mean and standard deviation of the percentage CMAP obtained in voluntary subjects treated with 3 IU of BoNT/D (A) and with 10 IU of BoNT/D (B).

4. Discussion

Experimental and epidemiological studies have identified differences in epidemiology of botulism in humans compared to many other animal species. Few reports citing human outbreaks of type C and D are available. Lamanna (1959) mentions two human type C outbreaks and one type D outbreak but provides

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no reference for these cases and no recent reports of human type C and D botulism is present in literature but for only one report of type C poisoning in a single infant (Oguma et al., 1990). Lack of human susceptibility to BoNT/C and /D intoxication could be attributed either to poor absorption of these specific toxins from the human gastrointestinal tract or to resistance of human cholinergic nerve terminal to the activity of this toxin. For BoNT/C the former possibility is the most probable since BoNT/C has been extensively demonstrated to be active on injected human neuromuscular junction and it has been proposed as a valid therapeutic alternative to BoNT/A (Eleopra et al., 1997b, 2002, 2004). Human susceptibility to type D neurotoxin remains unclear, with contrasting reports (Coffield et al., 1997; Anderson et al., 2009) and therefore we decided to tackle this issue by comparing the toxin activity in mice and rats *in vitro, ex vivo* and *in vivo* and in humans *in vivo*.

The major finding described here is that a preparation of BoNT/D, which is very active in mice and rats, is poorly effective in inducing human skeletal muscle paralysis. This work performed *in vivo* extends the work of Coffield et al. (1997) carried out with *ex vivo* preparations of human pyramidalis muscles and establishes that BoNT/D has a minimal effect on an *in vivo* human compound muscle action potential which is used as a standard for testing the susceptibility of human patients to BoNT/A (Eleopra et al., 1997a). The present work also explains the statement that BoNT/D is effective on human muscles (Anderson et al., 2009) because the slight electrophysiological CMAP inhibition found after one week from the injection of 10 IU into the EDB muscle indicates that much higher doses may produce paralysis.

The present "negative" result is very significant in light of the recent efforts to characterize the presynaptic receptors of BoNT/D. BoNT/D has been well documented to bind *in vitro* two polysialoganglioside molecules and their binding sites in the C-terminal domain of the H chain have been defined by site-directed mutagenesis and X-rays crystallography (Strotmeier et al., 2010; Zhang et al., 2011). These studies led to the suggestion that PSGs are responsible for the binding and entry of BoNT/D inside peripheral nerve terminals. On the contrary, another laboratory reported evidence obtained *in vitro* that the synaptic vesicle protein SV2 may be a co-receptor of BoNT/D in addition to a PSG molecule, as it is of BoNT/A and BoNT/E (Peng et al., 2011). The present finding that humans and mice have a very large difference in sensitivity to BoNT/D, whilst are similarly sensitive to BoNT/A indicates that, if indeed SV2 is the protein co-receptor of BoNT/D it must bind to a segment different from those bound by BoNT/A and BoNT/E.

It is well known that mice and human skeletal NMJ possess a similar array of PSG (Cochran et al., 1982; Wiegandt, 1985; Ledeen et al., 1986; Lehmann et al., 2007) and PSG which are bound by BoNT/D are also bound by anti-PSG specific antibodies that in the presence of complement do damage both the mice and human nerve terminal (Chiba et al., 1992; Willison et al., 1994; Willison and Yuki, 2002; Halstead et al., 2004). Therefore, PSG cannot be the sole functional receptors that bind BoNT/D and drive it inside intracellular acidic compartments. Clearly the biological activity of BoNT/D depends on the expression of complex PSG as demonstrated in phrenic nerve-hemidiaphragm preparations derived from mice only expressing either GM3 or the a-series gangliosides (GM3, GM2, GM1 and GD1a) (Strotmeier et al., 2010) but they cannot be the sole functional receptors of BoNT/D.

On the other hand, the work where SV2 was proposed as the protein co-receptor of BoNT/D (Peng et al., 2011) has not reported the identification of the segment of protein involved in BoNT/D binding. At the present stage, a way to reconcile the available results is to assume that BoNT/D interacts with the oligosaccharide lateral chain of SV2 (or of another nerve terminal vesicular membrane protein) and this opens a novel and relevant possibility to be experimentally tested.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.clinph.2012.11.004.

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3. DISCUSSION

Membrane translocation is the less understood event along CNTs intoxication process. Actually, it is very hard to study such event because it takes place inside endocytic compartments of neurons which are poorly amenable to manipulation. We therefore decided to avoid this limitation by making the process occur at the plasma membrane level, taking example from an experiment first performed with diphtheria toxin[143-145]. This assay permits a tight control of translocation environment which can directly influence toxins behavior. We firstly attempted with BoNT/A and BoNT/B, because they are the most investigated and are used in therapy. Unfortunately, the experiment performed with them clearly shows the lack of translocation, but, interestingly, TeNT, BoNT/C and BoNT/D displayed the ability to translocate across the plasma membrane. Given that CNTs are very conserved from the structural point of view but maintain proper features which make them unique, we ascribed such difference to the diverse binding modalities. It has been discussed above that BoNT/A and BoNT/B interact with only one exposed molecules on neuronal membranes, while TeNT, BoNT/C and BoNT/D have at least two anchorage point. We therefore speculated that, once bound, CNTs must limit their rotational degrees of freedom in order to properly predispose the structural rearrangement for translocation. Accordingly, providing BoNT/B secondary binding partner on the plasma membrane, it recovers the ability to translocate in our experimental conditions. This is an interesting result because it extends the double receptor concept to the low pH induced membrane insertion and translocation of the L chain and demonstrates that, providing the correct binding configuration, CNTs can be inserted also into cells which normally

are refractory. This in turn gives the possibility to use CNTs as an interesting tool to study the role of SNARE proteins in secretion in non–neuronal cells.

Interestingly, all the toxins tested have similar pH dependence for the induced membrane translocation. This suggests that the machinery which governs translocation is probably maintained among different serotypes. Accordingly, a bioinformatic analysis has shown that a pool of aminoacids is strategically conserved in one face of the translocation domain. Interestingly, they are acid residues. Their properties can therefore compensate for histidines absence on the toxin–solvent interface, other feature shared among CNTs, and provides a mode to the toxins to sense pH variation.

More importantly, we found that our low pH induced translocation assay is a reliable method to study other parameters which generally influence molecular dynamics. Accordingly, we adjusted our protocol by changing the time and the temperature along the low pH period. The main general result was that, at 37 °C, translocation is very rapid for BoNT/C, BoNT/D and TeNT and is completed within minutes. On the other hand we also found that temperature strongly affects the translocation. BoNT/C is completely blocked at 28 °C. It is possible that this toxin is the result of an evolution that has led to the long known higher resistance of BoNT/C to temperature. Accordingly, this could be correlated with the fact that these serotypes mainly affects birds, which have a higher body temperature. Similarly, the finding that TeNT action is very limited when translocation is induced at 20 °C, fits very well with the low sensitivity of cold-blood animals such as amphibians and reptiles to tetanus. BoNT/D seems to be less sensitive to temperature decrease.

At the same time, our assay has shed light on the importance of interchain disulphide. Some evidences had already demonstrated that disulphide was important for CNTs toxicity[149-153], but it was not known which of the four main steps was affected. Here it has been shown that the disulphide is fundamental for the structural rearrangement and it is possible to speculate that probably it provides a third anchorage point in the membrane to help HN insertion during pH drop.

Besides its role along translocation, when L has passed the vesicular membrane, the disulphide must be reduced to allow the release from a membrane bound compartment into the cytosol. To date little is known about such event and it has been poorly investigated. Interestingly, given its importance, it could be considered as a new target to block CNTs entry in neurons. We decided to exploits the vast knowledge available about cytosolic reducing system and focused our investigation on the main two: the glutathione pool and the NADPH-thioredoxin reductase-thioredoxin system. Employing a pharmacological approach we found that thioredoxin reductase is involved in CNTs entry into neurons because its inhibition results in the block of TeNT, BoNT/B, BoNT/C and BoNT/D toxicity. Given that thioredoxin reductase in not directly involved in disulphide reduction/isomerization of cytosolic proteins, we guess that the directly involved reducing agent is thioredoxin. Interestingly, it has been reported that thioredoxin shares structural similarities with group-I and group-II chaperons[154, 155] and indeed thioredoxin can help the folding of proteins in redox-independent reactions[156, 157]. Thus, it is tempting to speculate that the thioredoxin system not only detaches the L chain from the HN domain, but also assists the refolding of the L chain which must necessary unfold in order to pass through the HN transmembrane channel. It remains unclear whether thioredoxin alone can accomplish such process or specialized chaperon proteins rather must assist it. Interestingly a well documented system of SV chaperons assists SNARE proteins folding turnover necessary for SNARE complex assembly and disassembly[158, 159].

Here it has been investigated the mechanism of internalization of BoNT/A. Electron microscopy experiments allowed us to visualize the internalization of BoNT/A into the lumen of small synaptic vesicles of NMJ *ex vivo* preparation. Moreover, by screening many replicates, we have reported that, along intoxication, there is an average value of 1.5 toxin molecules per vesicles. This fits very well with the estimation that a population of small synaptic vesicles contain an average of 1.7 SV2 molecules per each vesicle[37] and underlines that it is the proteic receptor, rather than the polysialogangliosides, that induce the molecule internalization and trafficking. In addition, this finding supports the idea that L chain translocating channel is made of one, or at the most, two H_N domains. Such evidence reinforces the concept of BoNT/A as a nanomachine[4],

which provides a model where each molecule is sufficient for the correct translocation of the own catalytic subunit. These considerations could be extended also to other toxins which exploits SV2 as proteic receptor. Recently, it has been reported by Sun and colleagues that BoNT/B and BoNT/E form a trimer and that the translocating channel is composed of three H_N molecules[160, 161]. Oligomerization is of course possible in the case of BoNT/B which interacts with synaptotagmin I/II, present inside synaptic vesicles in many copies (average 15.2)[37]. In the case of BoNT/E, the authors reported that oligomerization takes place on the plasma membrane, suggesting that, even if the number of SV2 copies per vesicle is lower than three [37], the interaction of one molecule of the trimer with the luminal portion of SV2 is sufficient for the internalization of the neurotoxin–receptor supercomplex. On the other hand, this cannot be the case of BoNT/A, since just one-two gold dots per vesicles are present. Moreover, the extreme potency of CNTs push the option of a non-cooperative translocation.
We also demonstrated that the achievement of translocation competent environment is very rapid, with a kinetic compatible only with the rapid acidification which occurs in small synaptic vesicles[162, 163]. Therefore it can be stated that translocation occurs from SV and, accordingly it can be excluded that, as many bacterial toxins do, BoNT/A exploits other cytosolic acid compartments like those found along the endosomes pathway.

The employment in human therapy remains one of the main goal concerning BoNTs research. Even if BoNT/A remains the first-choice toxin for human diseases treatment, sometimes intrinsic resistance as well as the development of antitoxin antibodies hamper its use. It is therefore important to explore the potential of other serotypes. The lack of reports citing human outbreaks of type C and D botulism is consistent with the fact that these serotypes are mainly correlated with animal botulism. BoNT/C and /D scarce susceptibility could be attributed either to poor absorption of these specific toxins from the human gastrointestinal tract or to resistance of human cholinergic nerve terminal to the activity of these toxins. Nevertheless, the latter hypothesis is for sure wrong, at least for BoNT/C, which has been found to be a good alternative to BoNT/A[142, 146, 147, 164]. On the other hand, little is known about BoNT/D human susceptibility. We therefore decided to increase the knowledge in this field, by testing, for the first

time, BoNT/D in human muscles through the electrophysiological evaluation of the compound muscular action potential (CMAP) amplitude. We demonstrated that this toxin serotype is very effective in mice either *in vitro, ex vivo* and *in vivo* but is poorly effective in in inducing human skeletal muscle paralysis.

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5. Extras

5.1 Publications list

Salmaso S, Bersani S, Pirazzini M, Caliceti P: pH-sensitive PEG-based micelles for tumor targeting. J Drug Target. 2011, 19:303-313.

Ferrari E, Maywood ES, Restani L, Caleo M, Pirazzini M, Rossetto O, Hastings MH, Niranjan D, Schiavo G, Davletov B: Re-Assembled Botulinum Neurotoxin Inhibits CNS Functions without Systemic Toxicity. Toxins (Basel). 2011, 3:345-355.

Pirazzini M, Rossetto O, Bolognese P, Shone CC, 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, 13:1731-1743.

Pirazzini M, Bordin F, Rossetto O, Shone C.C., Binz T., Montecucco C, The thioredoxin reductase-thioredoxin system is involved in the entry of tetanus and botulinum neurotoxins in the cytosol of nerve terminals, FEBS Letters, (Available online 21-NOV-2012)

Pirazzini M., Rossetto O.,Bertasio C., Bordin F., Shone C.C., Binz T. and Montecucco C. : Time Course and Temperature Dependence of the Membrane Translocation of Tetanus and Botulinum Neurotoxins C and D in Neurons, Biochemical and Biophysical Research Communications, accepted 6-NOV2012

Eleopra R, Montecucco C., Devigili G., Lettieri C, Rinaldo S, Verriello L., Pirazzini M., Caccin P., and Rossetto O.: Botulinum neurotoxin serotype D is poorly effective in humans: an in vivo electrophysiological study, Clinical Neurophysiology, accepted 7-NOV-2012

Colasante C., Rossetto O., Morbiato L., Pirazzini M., Molgò J. and Montecucco C.: Botulinum Neurotoxin type A is Internalized and Translocates from Small Synaptic Vesicles at the Neuromuscular Junction, submitted to *Molecular Neurobiology, currently under minor revision*

5.2 Congresses attended

25-28 May 2011: 1st French-Italian Joint Meeting on Subcellular Trafficking (Padua, Italy)
Abstract with poster presented: Tetanus and botulinum neurotoxins need two feet to jump into neurons

2-5 October 2011: 7th international conference on basic and therapeutic aspects of botulinum and tetanus toxins (Santa Fe, New Mexico, USA)
Abstract with poster presented: Tetanus and botulinum toxins need double anchorage to the membrane and intact disulfide bond for low pH induced entry into neurons

30 November 2011: Giornata di presentazione dell'attività di ricerca dei dottorandi del XXV Ciclo (Padua, Italy)
Abstract and oral presentation: Tetanus and botulinum neurotoxins need two feet to jump into neurons

20-21 April 2012: Membrane Trafficking and Organelle Biogenesis meeting (MTOB) (Bertinoro, Italy)
Abstract with poster presented: Tetanus and botulinum neurotoxins need two feet to jump into neurons