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NOVEL INSIGHTS INTO BOTULINUM NEUROTOXINS MECHANISM OF ACTION AND THE DISCOVERY OF PROPHYLACTIC INHIBITORS AGAINST BOTULISM

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SUMMARY

Botulinum neurotoxins (BoNTs), the most poisonous substances identified so far, are protein toxins that cause botulism, a severe neuroparalytic disease. They are produced by different species of neurotoxigenic Clostridia and can be grouped into seven serotypes (BoNT/A to /G). Using genomic and proteomic approach, many novel BoNTs have been recently identified and are classified as subtypes, though they cannot be completely neutralized by currently available immunological methods. However, all BoNTs have a similar molecular architecture which reflects a conserved mechanism of action. Therefore this situation can be tackled by developing inhibitors targeting the BoNT intracellular intoxication process.

The BoNTs consist of two main chains linked by a unique inter-chain disulfide bond: the heavy chain (H, 100 kDa) and the catalytic light chain (L, 50 kDa). The C-terminal part of H (HC) is responsible for the neurospecific binding and the internalization within an endocytic compartment, whilst the N-terminal part (HN) is involved in the translocation of L across the endosome membrane. L is a Zn^{2+} dependent metalloprotease that targets specifically the SNARE proteins, the three proteins constituting the core of neuroexocytosis. This cleavage results in a prolonged inactivation of neurotransmitter release and causes the flaccid paralysis typical of botulism. To penetrate into neurons, BoNTs exploit synaptic vesicles (SV) recycling and their lumen acidification induces the HN-mediated membrane translocation of L. It has been demonstrated that, once on the cytosolic side, the L metalloprotease remains connected to H via the interchain disulphide bridge and the reduction of this bond is necessary to release the protease in the cytosol and enable their catalytic activity.

Using a series of well characterized inhibitors of Thioredoxin Reductase (TrxR)/Thioredoxin (Trx) system, we found that this redox system is involved in the cytosolic reduction of the interchain disulphide bond of BoNTs. In neuronal cultures, these molecules prevent the metalloproteolytic activity of all toxin serotypes without significantly affecting cell viability. Moreover, such compounds are very effective *in vivo*, lowering the severity and the duration of paralysis caused by a local BoNT injection. More importantly, one of these drugs elicits a remarkable protection in mice systemically injected with lethal doses of different serotypes. These results entail that the reduction of the interchain disulphide bond is a strict prerequisite for the activity of BoNTs and that this class of inhibitors can prevent the neurotoxicity regardless of their different immunogenicity. Intriguingly, we also found that the TrxR/Trx system is bound to the cytosolic side of the SV membrane and that it is enriched

in those SV that are docked to active zones. We speculated that this redox system may play a role in maintaining SV protein function by controlling the redox state of the different SV protein disulfides.

Another step in BoNTs mechanism of action that might offer a good template for drug design is their trafficking. Recently, an inhibitor of different pathogens that require a passage through acidic endosomes to invade cells has been identified and dubbed EGA. We tested the effect of this molecule in neurons treated with BoNTs as also their neurotoxicity is strictly dependent on the passage through an intracellular acidic compartment. We focused our investigation on BoNT/A and BoNT/B, the two serotypes mainly associated with human botulism and used in therapy, and BoNT/D, that scarcely affects humans, but frequently causes botulism in animals. We found that EGA inhibits BoNTs activity on neuronal cultures, without interfering with any of the main steps characterising their cellular mechanism of intoxication. We speculated that, rather than having a direct effect on BoNTs, this compound impinges on an intracellular target which is responsible for their trafficking. Importantly, we found that EGA is not toxic *per se in vivo*, and is particularly efficacious in preventing botulism induced by BoNT/B and BoNT/D. Instead, in the case of BoNT/A the lethality was not reduced, but botulism symptoms developed later. We argued that the trafficking of the different BoNT types might be differently impacted by EGA and this compound may be used as a new tool for studying different intracellular routes exploited by BoNTs.

On the basis of the present knowledge about BoNTs mechanism of action, it is clear that once the LC has been released in the cytosol, the inhibitors tested here are no longer effective. Therefore, these drugs are to be considered as prophylactics. However, if given soon after diagnosis, these compounds could reduce symptoms severity by preventing the entry into neurons of circulating BoNTs, thus reducing the severity of poisoning and shortening the period of hospitalization that is related to the high costs of intensive care. Moreover, these molecules may be administered without knowing the BoNT serotype and subtype, therefore saving the time needed for toxin characterization.

SOMMARIO

Le neurotossine botuliniche (BoNTs) sono le esotossine più potenti attualmente conosciute nonché gli agenti eziologici di una grave malattia neuroparalitica, il botulismo. Storicamente classificate in 7 sierotipi (A-B-C-D-E-F-G), perché antigenicamente differenti, il loro numero risulta in rapida crescita poiché ogni sierotipo esiste in più sottotipi, la cui presenza sta progressivamente palesandosi grazie all'introduzione delle moderne tecniche di *next generation sequencing* (NGS). Sebbene il botulismo rappresenti un problema sanitario minore, la scoperta di nuovi inibitori contro le BoNTs è di assoluto rilievo dal punto di vista socio-economico visti i limitati trattamenti ad oggi disponibili e il possibile utilizzo delle tossine botuliniche come potenziali agenti di bioterrorismo.

Dal punto di vista strutturale, tutti i diversi sierotipi sono costituiti da due catene polipeptidiche unite covalentemente da un unico ponte disolfuro: una catena pesante (H, 100 kDa) e una leggera (L, 50 kDa). Dal punto di vista funzionale, la stessa struttura può essere invece suddivisa in tre principali domini con un determinato ruolo nel processo di intossicazione: 1) HC, definito anche dominio di legame, media l'adsorbimento specifico della tossina alla membrana plasmatica del motoneurone, 2) HN, denominato anche dominio di traslocazione, costituisce un canale di permeazione attraverso cui 3) L, riconosciuto essere il dominio catalitico, viene traslocato nel citoplasma. Qui, la catena leggera (L) viene liberata attraverso la riduzione del legame disolfuro intercatena ed è quindi pronta ad esercitare la sua funzione enzimatica. In dettaglio, le BoNTs sono metalloproteasi zinco-dipendenti capaci di idrolizzare in maniera specifica le proteine SNARE. Ogni sierotipo presenta un preciso bersaglio molecolare: BoNT/A e /E idrolizzano un diverso legame peptidico della proteina SNAP-25, BoNT/B, /D, /F e /G proteolizzano la proteina VAMP2, mentre, BoNT/C è in grado di idrolizzare due substrati differenti, sintaxina e SNAP-25. Le proteine SNARE costituisco il cuore del macchinario biochimico che permette il riconoscimento e la fusione delle vescicole sinaptiche con la membrana presinaptica del terminale nervoso a livello della giunzione neuromuscolare, pertanto, la loro idrolisi porta al blocco del rilascio di acetilcolina, inducendo neuroparalisi di tipo flaccido, conseguenza tipica del botulismo.

In una prima parte del lavoro, utilizzando un approccio farmacologico, si è dimostrato come il sistema ossido-riduttivo Tioredossina (Trx)-Tioredossina Reduttasi (TrxR) dell'ospite abbia un ruolo chiave nella riduzione citosolica del ponte disolfuro intercatena di tutti i sierotipi di BoNTs. In dettaglio, utilizzando colture neuronali primarie, si è potuto dimostrare come tali

molecole siano in grado di proteggere la coltura modello dall'intossicazione. Inoltre, i dati ottenuti *in vitro* sono stati confermati *in vivo*: la somministrazione dei differenti inibitori, in un modello murino, porta ad una diminuzione della severità e della durata della paralisi flaccida nonché ad una sostanziale protezione in topi trattati con dosi letali di tossina. Infine, si è riusciti ad identificare il sistema della TrxR/Trx a livello delle vescicole sinaptiche. In particolare, si è compreso come entrambi le proteine si arricchiscano a livello delle vescicole sinaptiche *docked*, ossia quelle legate alla membrana presinaptica pronte a rilasciare il neurotrasmettitore in esse contenuto. Questa evidenza è di particolare importanza se si prende in considerazione un possibile ruolo di tali proteine nel processo di neuroesocitosi.

In un successivo lavoro, si è dimostrato come l'endocitosi all'interno del terminale nervoso, può essere considerato un altro passaggio chiave nel meccanismo d'azione delle tossine botuliniche, da prendere in considerazione nello sviluppo di nuovi inibitori. In dettaglio, un gruppo americano nel 2014 ha dimostrato come una piccola molecola, chiamata EGA, sia capace di bloccare l'azione di diverse tossine batteriche e virus che utilizzano gli endosomi come "cavallo di Troia" per il loro ingresso nelle cellule. Sebbene il target intracellulare risulta ancora non noto, si è deciso di sintetizzare e testare tale inibitore per capire se anche nel caso delle tossine botuliniche sia in grado di inibire il loro ingresso *in vitro* e *in vivo*. I risultati ottenuti evidenziano come EGA sia capace di inibire *in vitro* l'azione di molteplici sierotipi di BoNTs: A e B, comunemente associati a casi di botulismo umano e utilizzati in terapia, e D, coinvolto in casi di botulismo animale. Inoltre, questa molecola risulta efficace nel prevenire la paralisi *in vivo* dovuta ai sierotipi B e D e ritarda quella dovuta al sierotipo A. Di conseguenza, i nostri risultati suggeriscono come questa molecola possa essere presa in considerazione come *lead* farmacologico per lo sviluppo di nuovi antidoti.

L'identificazione di questi inibitori potrà avere importanti implicazioni applicative volte a compensare il *gap* attualmente presente nel campo della prevenzione/terapia del botulismo. Infatti, il grande numero di sottotipi (>70) e la potenziale (probabile) esistenza di varianti non ancora identificate, è notevolmente limitante al controllo della loro azione patogena con il solo utilizzo di strumenti immunologici, quali antisieri e vaccinazione. In questa tesi verrà discusso come il nostro approccio risulta essere, invece, indipendente dal sierotipo di BoNTs coinvolta nell'intossicazione, dunque indipendente dall'antigenicità delle diverse tossine.

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1. INTRODUCTION

1.1 BOTULINUM NEUROTOXINS AND BOTULISM

Botulinum neurotoxins (BoNTs) are produced by neurotoxigenic strains of Clostridia and are the most potent toxins yet known, with an estimated lethal dose for humans around 1 ng/Kg of body weight[1]. BoNTs are A-B protein toxins, where protomer A is the enzymatically active part and protomer B mediates binding to a specific receptor on the host cell membrane and the delivery of A into the cytosol. The potency of botulinum neurotoxins is the result of an elaborate and efficient molecular mechanism of action, that impairs an essential physiological function for vertebrates life: the neurotransmission at peripheral nerve terminals[2]. Indeed, BoNTs are the etiologic agents of botulism, a severe neuroparalytic syndrome that is the pathological consequence of their action on nerve endings on both the skeletal and autonomous nerve systems[3].

BoNTs have been classified into seven different serotypes and indicated with the alphabetical letters (BoNT/A through /G), based on the fact that a serum raised against one toxin was not able to neutralise the others[4]. In details, more than a century ago, serological methods were introduced to distinguish botulinum neurotoxins. In 1910, Leuchs demonstrated that BoNTs produced by two European strains of *Clostridium botulinum* were antigenically different, with antitoxin raised against one neurotoxin not cross-neutralising neurotoxin formed by the opposite strain. Ten years later, Burke recognised two antigenically distinct BoNTs, and designed these as serotypes A and B. This work established, for the first time, the use of serological strategies based on type-specific antitoxin to differentiate BoNTs serotypes, and has currently led to the recognition of seven confirmed botulinum neurotoxins serotypes, types A-G[4,5]. However their number is much higher as each serotype exists in many variants (called subtypes), with difference in their amino acid sequence and tight antigenic relation to the parental serotype, as well as possibly individual biological properties[6,7]. In the last few years, this has led to a debate on what might constitute a botulinum neurotoxin type or subtype.

This complexity becomes greater if it consider the different vertebrates host of different BoNTs serotypes. Generally speaking, serotypes A, B, and E are those often related with human botulism, with fewer cases being cause by BoNT/F. Almost exclusively associated with botulism among birds is BoNT/C, whilst BoNT/D cause botulism in different animals species

but not in humans. BoNT/E is more frequently associated with botulism of marine vertebrates and fish eating birds[2,7].

The main and life threating outcome arising from BoNTs action in vertebrates is the blockage of neurotransmitter release at the neuromuscular junction, which results in the impossibility of stimulating voluntary muscles and therefore in the typical flaccid paralysis of botulism[8]. In the adults, botulism is generally caused by an intoxication through the ingestion of the mature toxin contained in contaminated food. It is not an infection, since Clostridia colonization of the intestinal tract is quite difficult. This can happen in infants because ingested spores can germinate in the absence of competing resident microbiota[7]. In this latter case BoNTs are produced and released in the intestines for prolonged periods of time causing infant botulism[9,10].

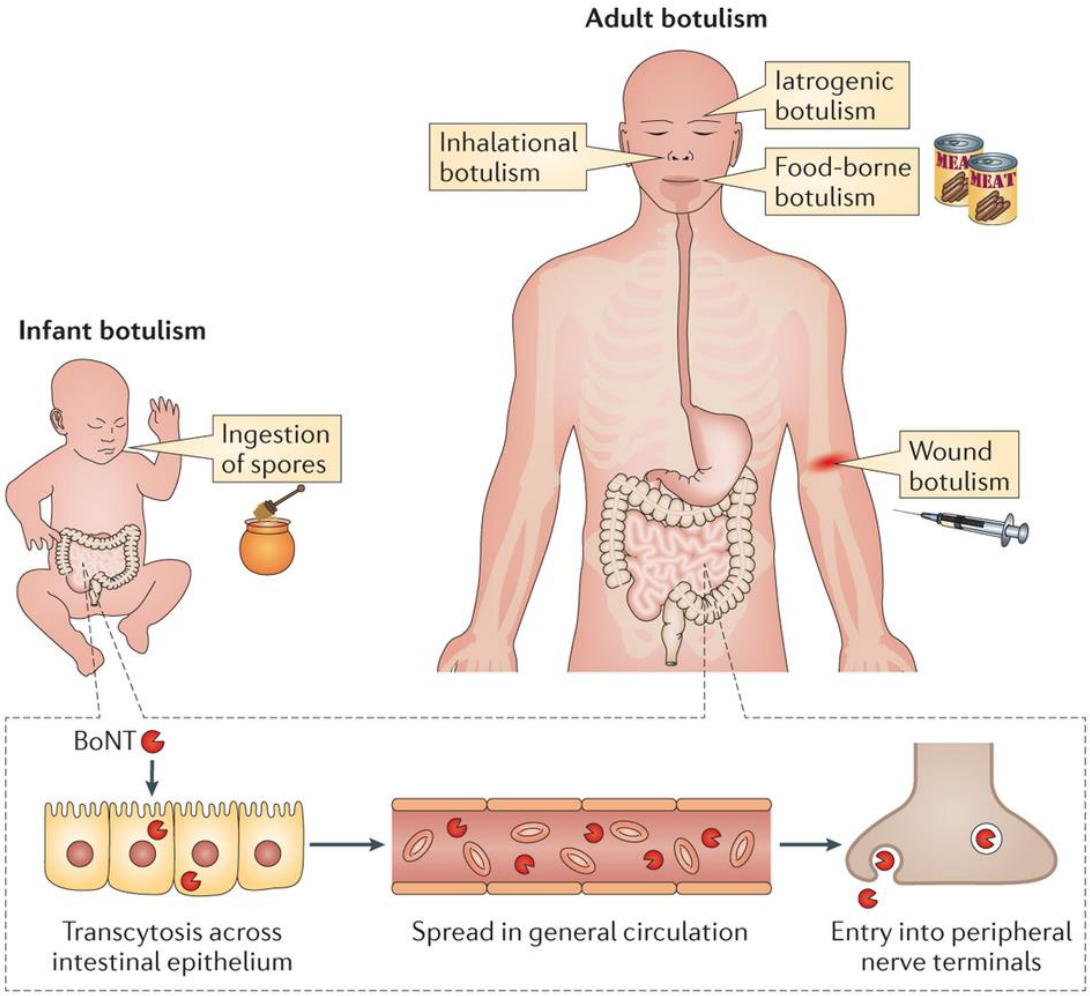

Fig. 1. Different forms of human botulism. Until now, it has been characterized five forms of human botulism. The two most common forms are food-borne botulism, which occurs following the ingestion of BoNT-containing foods, and infant botulism, that is caused by the ingestion of food contaminated with spores that

germinate in the gastrointestinal tract as a consequence of the lack of a mature microbiome. The other three forms are much rarer and include inhalational botulism, iatrogenic botulism and, wound botulism. Following transcytosis across the intestinal epithelium and consequent entry into the general circulation, the toxin eventually enters peripheral cholinergic nerve terminals, causing the flaccid paralysis of botulism. From Rossetto, Pirazzini *et al.*, 2014[7].

Other three forms of botulism exist, though rare (Figure 1): wound botulism results from tissue contamination with spores and is almost exclusively associated with drug users[11], iatrogenic botulism is due to the inappropriate administration or the abuse of the toxin for cosmetic or therapeutic purpose[12], instead, inhalational botulism, owing to inhalation of BoNT-containing aerosols, is manly associated to a possible use of BoNTs as bioweapon[13]. Despite the different forms, the symptoms of the disease are very similar. The facial and throat innervations are the first affected causing diplopia, ptosis and dysphagia. The paralysis continues its descent to the trunk[14,15] and when respiratory muscles are involved, breathing is compromised and death comes through respiratory failure. However, since intoxicated nerves remain intact and do not degenerate, if mechanical ventilation is timely performed, patients survive fully recovering from the neuroparalysis, in a time window which depends on the amount of toxin poisoning nerve terminals and on the BoNT serotype involved[7]. The current therapy is aimed to neutralize circulating toxin using anti-BoNT serum and keep alive patients using artificial ventilation[7]. Unfortunately, no countermeasures against BoNTs are available once entered in target cells.

BoNTs can be defined Janus toxins, as they are at the same time the most deadly exotoxins known to humans and one of the safest drugs used in several human pathologies[7]. Indeed, considering their relative ease of production and extremely potency, BoNTs are considered by the Center for Disease Control and Prevention (CDC) as category A agents[15], i.e. toxins that can be potentially used as biological weapons, but, at the same time, for their neurospecificity and reversibility, they have become very useful therapeutics for a growing and heterogeneous number of human disease characterized by peripheral nerve terminals hyperactivity[8,13,15-21]. In addition, thanks to the comprehension of their molecular mechanism of action, BoNTs have become useful tools for the study of neuronal physiology[3].

1.2 VARIABILITY OF BOTULINUM NEUROTOXINS

Clostridium is a genus gathering different sporulating and anaerobic Gram-positive bacteria including more than 150 species, widely distributed in the environment[7]. Typically, they are found as spores, that can resist for long time but under proper conditions (such as anaerobiosis, nutrients and low pH) they germinate into vegetative cells[8,22]. During this state, not solely the foremost *Clostridium botulinum*, but alternative species, such as *Clostridium butyricum* and *Clostridium baratii*, are able to produce BoNTs and become neurotoxigenic[7]. Interestingly, the capability of different species to produce the same neurotoxin indicates that, throughout the evolution, the toxin gene cluster has moved into different bacteria through recombination, such as horizontal gene transfer events[6].

Historically, Emilie van Ermengem was the first to isolate a *Clostridium botulinum* strain producing BoNT/A in 1895[23]. In 1910, Leuchs demonstrated that BoNTs produced by two European strains of *C. botulinum* were antigenically different, with antitoxin raised against one neurotoxin not cross-neutralising neurotoxin formed by the opposite strain. Ten years later, Burke recognised two antigenically distinct BoNTs, and designed these as serotypes A and B. This work established, for the first time, the use of serological strategies based on type-specific antitoxin to differentiate BoNTs serotypes[4,5]. Few years later, Landmann isolated a strain producing BoNT/B from an outbreak in Germany and within the following fifty years were discovered other serotypes: in chronological order BoNT/C, BoNT/D, BoNT/E, BoNT/F and BoNT/G[4]. The serotype designation was challenged once polyclonal antibodies generated against BoNT/D of strain South Africa (BoNT/D-SA) detected with the same efficiency also BoNT/C of strain Stockholm; seven years later, cloning associated with molecular characterization revealed that this cross reactivity is caused by an exchange of domains between different serotypes[24]. In details, this was the first example of BoNT mosaics: BoNT/C of strain Stockholm was renamed BoNT/DC, structurally composed by the active part of BoNT/D and the binding part of BoNT/C[24,25]. Recently, another BoNT mosaics was discovered and called BoNT/FA[26].

In the recent years, the development of next generation sequencing and mass spectrometry analysis, has permitted the analysis of humans and animals clinical cases accumulated over the time[2]. As a result, it has become rapidly clear that neurotoxigenic Clostridia have extensive genetic heterogeneity in terms of genome organization, toxin gene clusters, and most importantly, toxin sequences variability[6,27-29]. Consequently, many variants have been

discovered (more than 40) and these findings led to the introduction of the term subtype, a genetic BoNT variant with at least 2.5% difference in the amino acid sequence. The subtypes are given the toxin letter designation followed by a number (indicated as BoNT/A1, BoNT/A2, etc.)[6,7,27,30,31] (Figure 2).

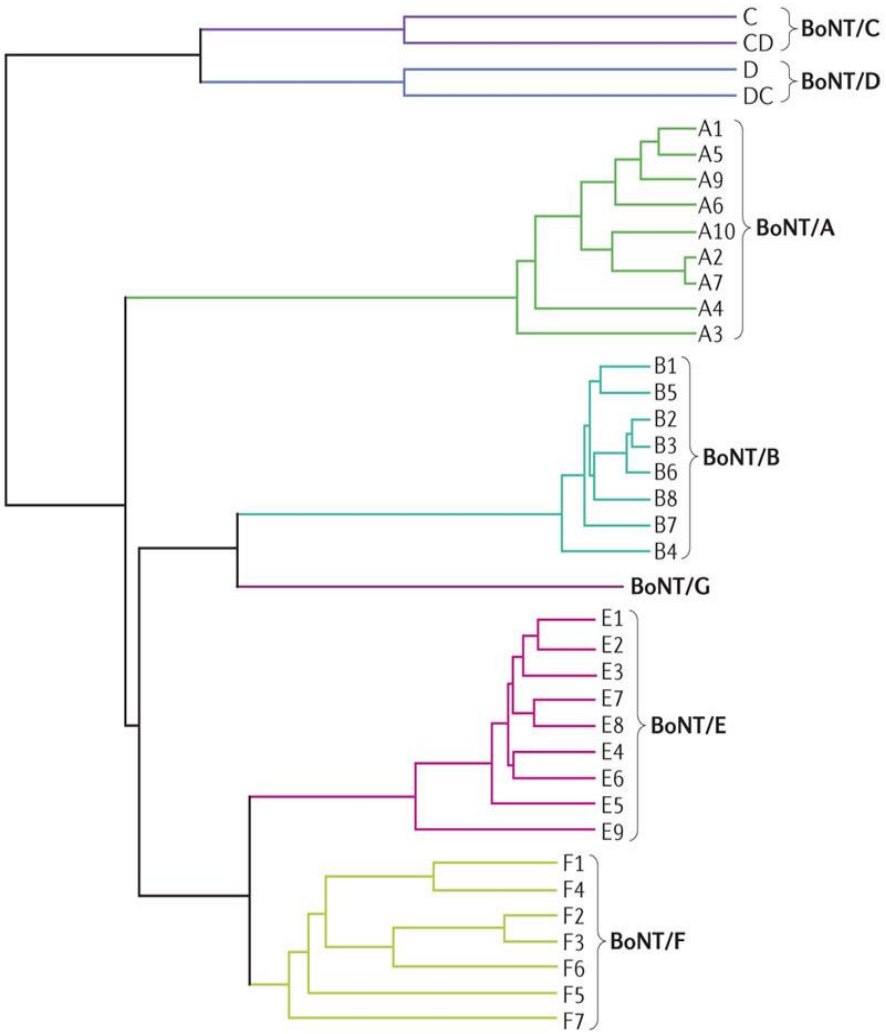

Fig. 2. Different serotypes and subtypes are produced by several neurotoxigenic species of Clostridium. Six phylogenetically distinct Clostridia (*Clostridium botulinum* groups I-IV and some strains of *Clostridium butyricum* and *Clostridium baratii*) produce seven botulinum neurotoxins antigenic different (BoNTs serotypes A-G). Each toxin serotype is grouped into various subtypes on the basis of their amino acid sequences (such as BoNT/A1-A10). BoNTs serotypes C and D are phylogenetically related to each other, as are serotypes B and G, and E and F. Even if most strains of *C. botulinum* produce a single toxin serotype, some isolates produce more than one serotype, and other it able to synthetize mosaic toxins, such as BoNT/DC, /CD or /FA. From Rossetto, Pirazzini *et al.*, 2014[7].

However, this has led to a debate on what might constitute a botulinum neurotoxin type or subtype, and, in which way this difference can be better highlighted: by serological methods or by protein sequence?[5] Importantly, apart from antigenicity, additional functional differences between the major serotypes with respect the binding capability, the catalytic activity, neurotoxicity and pharmacological properties like duration of action have been demonstrated[32-35]. To this end, it has been proposed that newly discovered neurotoxin gene variants should be named as such, only once it has been established that they display different neurotoxin biology such as toxicity, antigenicity or substrate cleavage site[5,36].

1.3 BOTULINUM NEUROTOXINS STRUCTURE AND MECHANISM OF ACTION

The available crystallographic structures of BoNTs (Figure 3) show an overall highly preserved molecular architecture, which is purposeful to their mechanism of action[37-39].

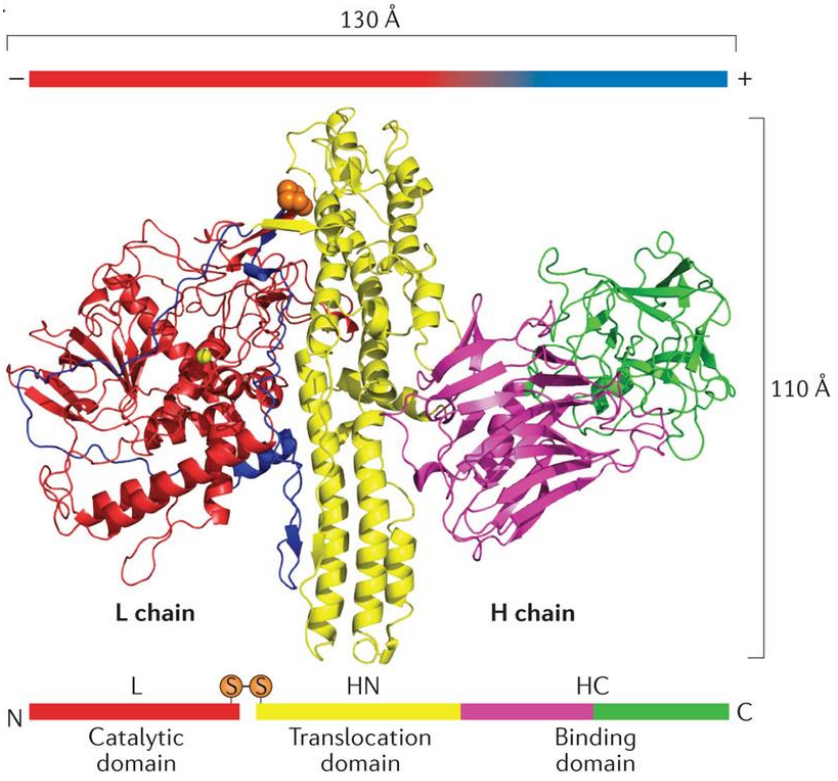

Fig. 3. Crystallographic structure of isolated BoNT/A1. Crystal structure of botulinum neurotoxin A1 (BoNT/A1), showing its associated electrical dipole and the organization of individual toxin domains, every of that encompasses a specific role in cell mechanism of intoxication: the HC domain binds specifically to nerve terminals; the HN domain translocate the L chain into the nerve terminal cytosol; and L chain is a metalloprotease that cleaves and inactivates specific SNARE proteins that are involved in neurotransmitter

release, thereby causing neuroparalysis. A peptide belt (showing in dark blue), that surrounds the L domain and the inter-chain disulfide bond (orange), links the L chain to the HN domain. From Rossetto, Pirazzini *et al.*, 2014[7].

The structure is constituted by two main chains: a light chain (L, 50 KDa) and a heavy chain (H, 100 KDa) held together by a strictly conserved inter-chain disulfide bond and non-covalent interactions[4,7]. This two chains, based on their functionality properties, can be divided into four domain (Figure 3): (I) HC-C (25 kDa, in green) is involved in nerve terminal binding and internalization[40-43]; (ii) HC-N (25 kDa, in purple), as not yet a well-defined role, but there is evidence that it may contribute to binding by interacting with membrane lipids[44-46]; (iii) HN (50 kDa, in yellow) assists the translocation of the catalytic part of the toxin from the internal part of an intracellular acidic compartment into the cytosol[47-49], (iv) the L catalytic domain (50 kDa, in red) is a metalloprotease that cleaves the SNARE proteins interfering with the release of neurotransmitters that result in a reversible neuroparalysis[3]. Despite the amino acid sequence variability among all BoNT variants, the structure organization is however maintained, as it mechanism of nerve intoxication[7].

BoNTs are very precise nanomachines, evolved to exploit different physiological features of vertebrates, conserved during their evolution: (i) functional binding to polysialogangliosides (PSG), highly enriched in the neuromuscular junction (NMJ), the *in vivo* site of action of these toxins, and to the lumenal domain of an integral membrane proteins of synaptic vesicles (SV), organelles unique to vertebrates; (ii) membrane translocation across the SV membrane driven by a pH lowering, which is physiologically necessary for the neurotransmitter refilling of these compartments; (iii) metalloproteolytic activity specific for VAMP, SNAP-25, or syntaxin, three proteins high specialized and conserved in terms of structure and function in different vertebrates[2]. Within the following sections, it will reviewed recent advances that have provided relevant insight concerning BoNTs mechanism of action.

1.3.1 The "double receptor model": neuronal surface accumulation and synaptic vesicles internalization

To account their specific neurotoxicity and high selectivity of binding have been proposed for BoNTs a double receptor model based on a first binding to a polysialoganglioside molecule followed by a second interaction with a protein receptor[40]. Later works has proven the

validity of this model. Indeed, BoNTs have evolved the ability to bind neurons via two receptors, one with low affinity, a polysialogangliosides (PSG), that increases toxin density on the target membrane[41,42], and a secondary one the luminal part of an integral membrane protein of synaptic vesicles (SV), which triggers the internalization into the endocytic pathway[50] (Figure 4). BoNT could have evolved this distinctive binding mode to simultaneously overcome many physiological obstacles, like the low toxin concentration in circulating fluids, the high-speed of movement of extracellular fluids around cells as well as the reduced surface area of peripheral nerve terminals[7].

In detail, gangliosides are a large family of glycolips (glycosphingolipids) present on the external plasma membrane of all animal cells which are involved in many pathways like cell signalling, protein sorting and they are very important for membrane domain formation and organization. Gangliosides are particularly enriched in neurons membrane, especially in axons and dendrites where they govern membrane curvature[51,52]. Indeed, this first binding of BoNTs to the negatively charged sialic acids of PSG is very efficient because these neurotoxins are dipoles, with their positively charged end situated near to the binding site on PSG. This effect could contribute to the rapid binding of BoNTs to the nerve terminals *in vivo*, as well as, their reorientation that allow the interaction with the second receptor[7]. Several groups have contributed in defining the role of PSG as critical molecules for the specific binding of all BoNTs, reviewed in[4,41], and their reciprocal interaction has been characterized intimately. In any case the PSG binding site is located on the HC-C domain and in BoNT/A, /B, /E, /F and /G, is outlined by the conserved motif E(or D or Q)...H(or K or G)...SXWY...G (where X is any amino acid and "…" denotes a variable number of residues)[39,53-56]. The PSG-binding site of BoNT/C, BoNT/DC and BoNT/D is found in a similar position, but the binding motif is different[57-61].

Despite the moderate affinity achieved by BoNTs throughout the contact with PSG, this interaction does not account for the functional uptake into neurons, which is instead mediated by the consecutive binding to the lumenal domain of a synaptic vesicle protein[7]. BoNT/B, BoNT/G and BoNT/DC bind to segment 40-60 of the lumenal domain of synaptotagmin-I/II (Syt-I, Syt-II) via a binding site within the HC-C domain that is close to the PSG-binding site[62-72]. Interestingly, due to a single point mutation, L51 in human and chimpanzee Syt-II versus the homologous F54 in Syt-II of mouse, rat and other species, Syt I/II do not act any longer as high affinity receptors for the aforementioned BoNTs, explaining

why elevated dosages of BoNT/B are necessary to achieve the same effects of BoNT/A in human treatment[66]. By contrast, BoNT/A and BoNT/E bind specifically to two different segments of the fourth lumenal loop of the synaptic vesicle transmembrane protein SV2[73-75]. SV2C appears to be the main receptor involved in BoNT/A binding, while SV2A e SV2B, but not SV2C, mediate BoNT/E entry. However all three isoforms are expressed in motoneurons[64,75]. The protein receptors of other BoNTs have not been yet fully characterized, albeit SV2A-C seems to play an important role in the uptake mechanism of BoNT/D and BoNT/F[4,55,76,77].

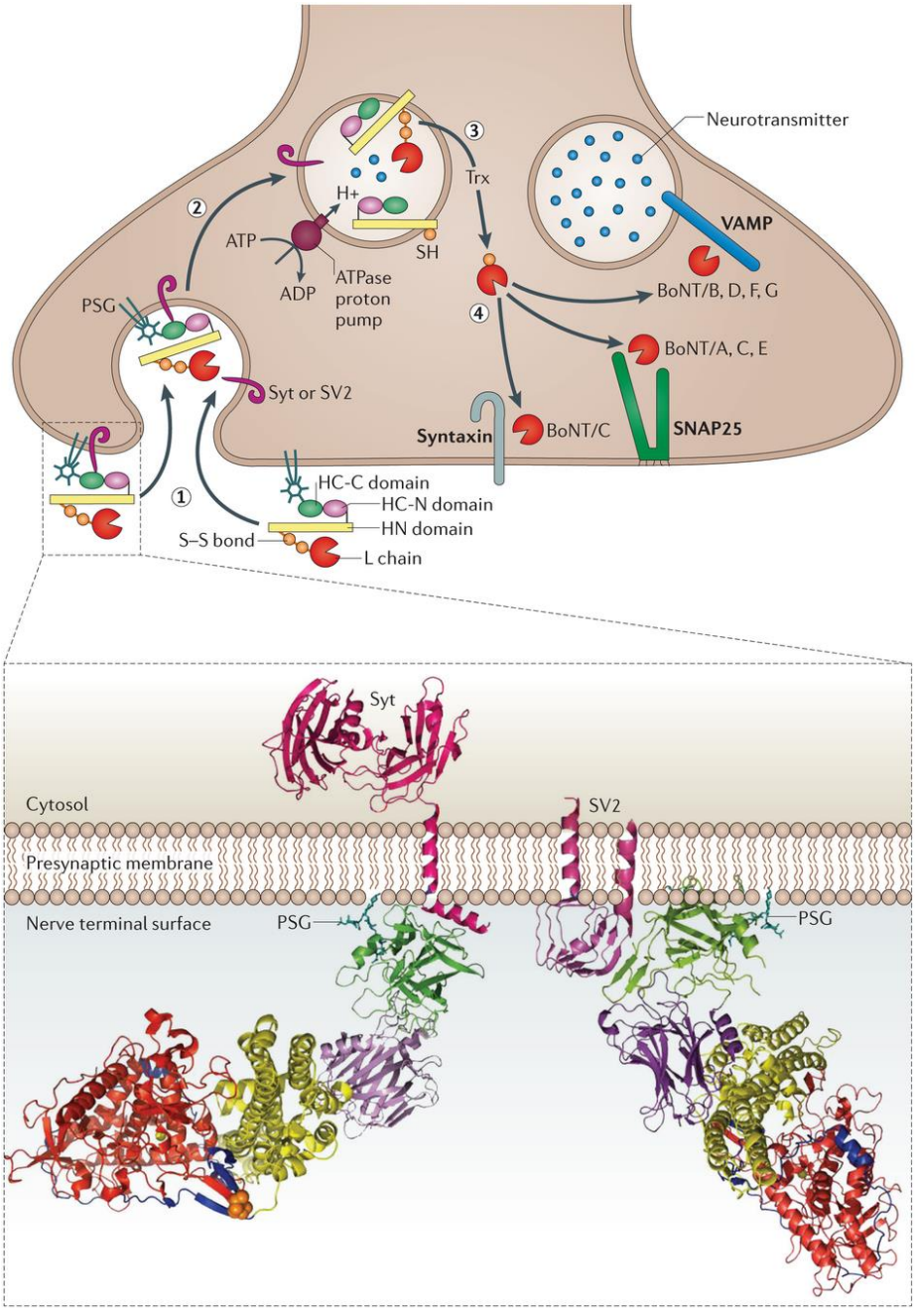

Fig. 4. Botulinum neurotoxins mechanism of action within peripheral nerve terminals. The until now known BoNT mechanism of action envisages a primary interaction between the carboxy-terminal end of HC domain (the HC-C domain) and a polysialogangliosides (PSG), that mediate the toxin accumulation to the plasma membrane. Subsequent lateral movements make possible the encounter of the toxin with a protein receptor which is the lumenal domain of a synaptic vesicle protein (step 1). The protein receptor has been identified as synaptotagmin I and II for BoNT/B, /DC and /G (crystal structure shown on the lower left-hand side), and SV2 for BoNT/A, /E and /F (crystal structure shown on the right left-hand side); the protein receptor for the remaining serotypes remains to be clarified. This latter binding is preliminary to the internalization of the toxin-receptors complex inside an acidic intracellular compartment (step 2) whose nature has been identified as SV for BoNT/A1. Little is known on the nature of endocytic compartments employed by the other serotypes, however considerable evidence show that the acidification of its lumen triggers a structural modification of L and HN together with membrane lipids which ultimately results in the translocation of the L chain within the cytosol (step 3). This process ultimate with the reduction of the inter-chain disulfide bond performed by the thioredoxin reductase-thioredoxin system. The free L metalloprotease specifically cleaves one of the three SNARE proteins (step 4) thereby preventing Ca^{2+} elicited release of the neurotransmitter contained insides SV. From Rossetto, Pirazzini *et al.*, 2014[7].

It is worth to note that BoNTs choose as receptors synaptic vesicles proteins which are highly conserved because fundamental for nerve physiology: synaptotagmins are the Ca^{2+} sensors regulating the synchronous neurotransmitter release, instead, the SV2 function is likely linked to SV priming or rendering primed vesicle fully Ca^{2+} responsive[78-80]. However, each of them are integral membrane proteins of SV and expose their BoNT-binding sites to the synaptic vesicle lumen; indeed in contrast to PSG, these protein receptors are not exposed on the surface of nerve terminal and are not accessible of BoNT. Nonetheless, they become available after the fusion of the SV with the presynaptic membrane, which exposes the synaptic vesicle lumen to the extracellular environment. Consequently, BoNT binding to protein receptors occurs solely after fusion of the synaptic vesicle to the presynaptic membrane, and this seems to facilitate the following step of intoxication, which requires the endocytosis of BoNTs[7].

In both cultured neurons and *in vivo*, BoNT/A rapidly enters the synaptic vesicle lumen[81,82]. The mechanism of internalization of other BoNTs remain to be established. By contrast in cultured neuron and probably also *in vivo*, alternative vesicles and trafficking route may contribute to their entry[83]. Indeed, information concerning the nature of the endocytic vesicle involved in the uptake of the other serotypes is still lacking. In this thesis, we will discuss about the possible distinctive trafficking exploit by different serotypes of BoNTs *in*

vitro as well as *in vivo*. Indeed, we found that a single compound, called EGA, is able to discriminate between different serotypes internalization[84].

1.3.2 Translocation across the membrane of acidic intracellular compartments

Considering the several steps of BoNTs mechanism of intoxication in nerve terminals, the translocation of the L chain from intracellular acidic compartments into the cytosol is the least understood in terms of molecular mechanism[49]. However, as in the case of binding and internalization, also to translocate the L chain into the cytosol, BoNTs have evolved to use a relevant physiological feature of nerve terminals. Indeed these toxins exploit the acidification of the synaptic vesicles (SV) lumen carried out by the v-ATPase, a proton pump present on their membrane[80], that lowers the lumenal pH to generate the pH gradient driving the re-uptake of neurotransmitters from the cytoplasm into SV[7,49]. The importance of the v-ATPase in BoNTs mechanism of action, is extensively demonstrated by the fact that specific inhibitors block BoNTs neurotoxicity[85,86].

Despite the exact mechanism is still under debate[49], it is well known that the translocation process comprises a concerted structural reconfiguration of the entire molecule, but in particular of the HN and L chain couple, which is promoted by low pH. Indeed it is long known that at acidic pH BoNTs forms ion conducting channels, and that this channel mediates the translocation of the L chain into the cytosol[47,48,87-89]. Montal and colleagues gave the major contribution with the patch clamp technique, studying the process in cell membranes and with single molecule resolution, conditions mimicking those found *in vivo*. The outcomes of these experiments were interpreted with the model reported in Figure 5[48,88], in which the HN domain of BoNT/A (in yellow), by lowering the pH on the *cis* side of the patched membrane (corresponding of the SV lumen), and by applying a negative membrane potential, forms a transmembrane channel that chaperons the passage of the L chain (in red) on the *trans* side (corresponding to cell cytosol). This mechanism was deduced following the current across the membrane: it begins with low values (~ 10 pS) corresponding to the phase during which the L chain occupies the channel to pass on the other side, and raises within 10 minutes to ~ 65 pS or ~ 110 pS, in PC12 cell line (Figure 5A)[85,90,91], corresponding to the full conductance of a transmembrane channel[47,48,88,92,93]. Notably, such channel permits the passage of only α-helices but not tertiary structure

elements, indicating that the L chain has to unfold, at least partially, to fit within the narrow cavity (15-20 Å in diameter) (Figure 5B)[40,48,94,95].

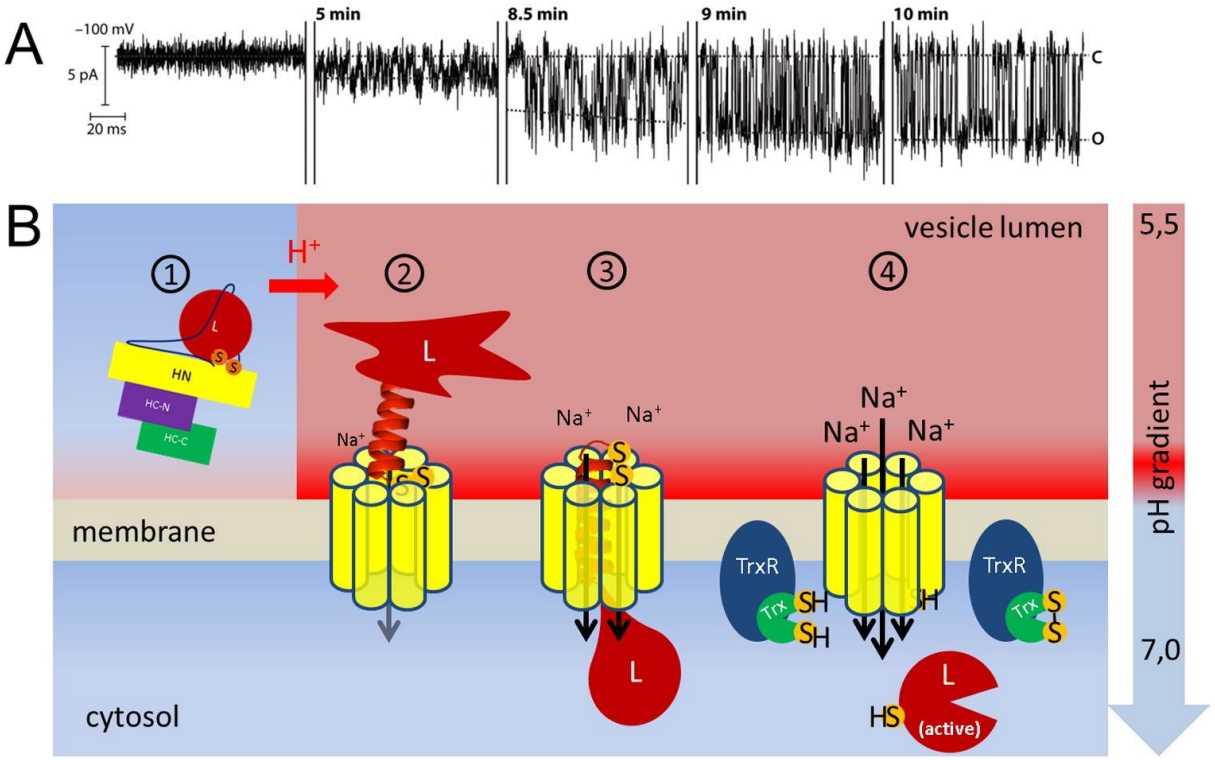

Fig. 5. Membrane translocation of BoNTs across the membrane of endocytic compartments. (A) The upper panel shows the elicited by BoNT/A increment of conductance at low pH in Neuro2A cell line. (B) The lower panel show the proposed steps involved in the membrane translocation of the L chain: 1) schematic structure of the toxin; 2) at acidic pH, the HN domain inserted into the membrane forming a transmembrane channel, hypothetically made of a six α –helices; 3) higher conductance accompanied the translocation of L chain from the acidic lumen into the neutral cytosol; 4) the L chain is completely translocated within the cytosol where it refolds and the inter-chain disulfide bond is reduced by the Thioredoxin Reductase-Thioredoxin system. From Pirazzini *et al.*, 2015[49].

Importantly, for a productive translocation, the interchain disulfide bond must remain intact during the preliminary phase of the process, and must be reduced only once it reaches the trans side of the membrane. This data are in keeping with the fact that pre-reduced BoNT does not form channels and that the reduction at any stage before reaching the cytosolic side aborts channel formation and L chain translocation[88,93]. Thus the model in figure 5 posits that upon acidification the BoNT molecules change their structure, HN penetrates the membrane and the L chain unfold maintaining only secondary structure elements. The initial low conductance state is taken as indication of the passage of the partially unfolded L chain

along the transmembrane channel to the cytosolic side of the SV membrane. Thereafter L chain remains connected to the SV till the inter-chain disulfide bond is reduced, that is the concluding step that ends the process leaving the HN channel to its full conductance (Figure 5A and Figure 5B).

Nevertheless, such a model does not take in account of other relevant data present in literature: i) the crystal structure of BoNT/B, and the L chain and HN domain of BoNT/A, do not change at low pH in solution[89,96], while they undergo conformational change in the presence of PSG or lipids[89,91,97-99]; ii) employing an experimental approaches that bypasses the internalization step and induces the translocation of the L chain directly from the cell surface, it had been found that BoNTs should be attached to the membrane by two receptors and that the translocation step occurs within few minutes at 37 °C in the pH range 4.5-6, which is consistent with the pH within the synaptic vesicle, and has a strong temperature dependence, with very little translocation taking place at 20 °C[100]; iii) several carboxylate amino acids, conserved among the different BoNTs, the inter-chain disulfide bond and a segment that possess high propensity for membrane insertion are all present in one face of the BoNTs molecule[49]; iv) replacement of three of the carboxylate residues with the corresponding amides in BoNT/B eliminates their protonation and causes the L chain to enter the cytoplasm quicker, increasing toxicity[101].

These data suggest that there is no single pH sensor in BoNTs, but several carboxylates that have high pKa values and have a role in the low pH-driven release of the L chain into the cytosol. By considering these data, an update model for BoNT translocation has been presented (Figure 6A and Figure 6B)[49]. BoNT initially binds to its two receptors within the SV lumen, which has a neutral pH, immediately after endocytosis. The v-ATPase then pumps protons and SV lumen becomes progressively more acidic. There is no a single pH sensor in BoNTs, but the conserved carboxylates predicted to have higher pKa, get protonated, and drive the partially protonated BoNT, which has a net positive charge, toward the membrane surface involving the disulphide-containing face of the toxin. Here the pH is even more acidic with respect to the lumen[102] allowing the further protonation of other carboxylates, while the positive charges make ionic couples with the anionic membrane, favouring the conformational change of the molecule. The subsequent molecular events are presently unknown, but on the basis of earlier studies, it can be speculated that the L chain becomes a “molten globule”, a protein state variant that retains native secondary structure and

increased hydrophobicity, enabling its insertion and passage across the membrane[7,49,101]. It has been proposed that the long α-helices of HN may break generating amphipathic helices with the length of 20-24 residues that, together with the other amphipathic helices of the HN domain, form a laterally opened transmembrane channel partially surrounding the L molten globule, whilst the remaining surface of L interacts with lipids. In this way, hydrophilic and hydrophobic surfaces and interactions are matched. The arc-shaped membrane inserted HN may act as chaperone for the translocation of the L chain, as suggested by Koriazova and Montal[48,87,88]. Facing the neutral pH of the cytosol, the L chain deprotonates and refolds into the metalloprotease domain whilst the membrane inserted HN closes laterally to form a stable ion channel. The process is terminated by the reduction of the disulfide bridge which releases the L chain and its protease activity, attaining the HN channel for its full conductance.

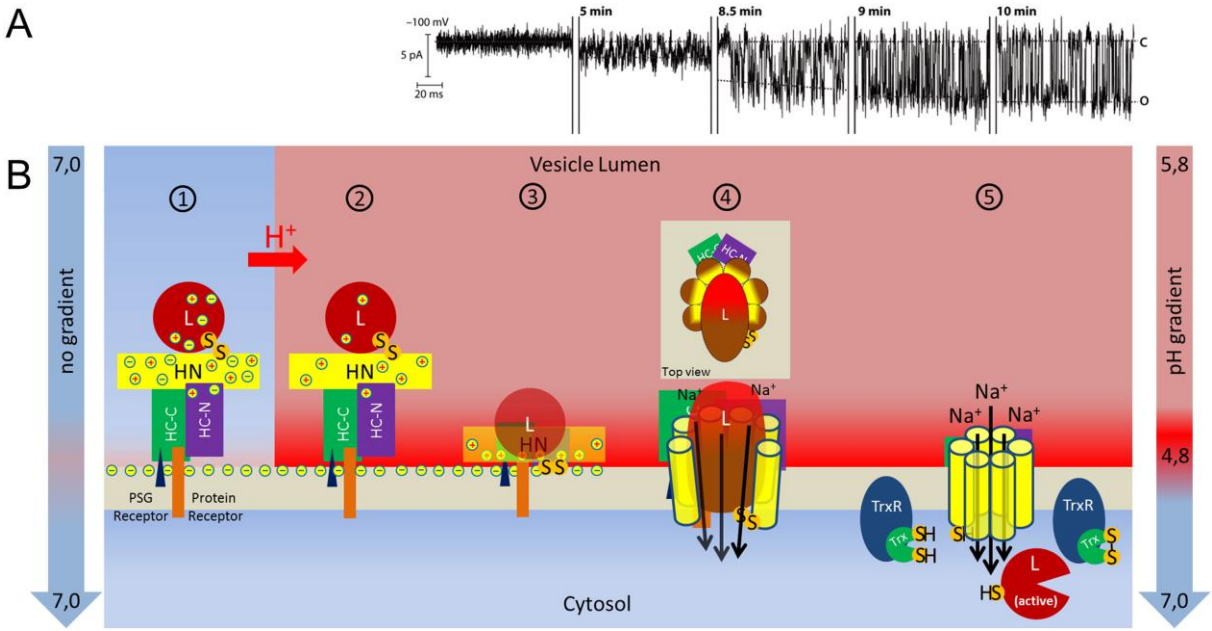

Fig. 6. A novel model might clarify the translocation step in botulinum mechanism of action . 1) The four domain of the toxin are schematically represented in different colours and some preserved charged amino acids residues are highlighted: L (red), HN (yellow), HC-H (purple) and HC-C (green). This latter domain, in this cartoon, binds its two receptors: the polysialoganglioside (blue triangle) and the lumenal part of SV protein (SV2 or synaptotagmin, orange rectangle). 2) Following the action of the v-ATPase proton pump, the vesicle lumen acidified and the high pKa carboxylate residues are protonated. 4) This face of the protein acquires a net positive charge and can eventually fall down on the anionic membrane surface; low pH and lipid interaction cause a combined and sequential structural change involving at the same time the L and HN domains and lipids.

5) L moves to the cytosolic side, refolds and is released upon reduction of the inter-chain disulfide bridge. From Pirazzini et *al.*, 2015[49].

However, whether this actually occurs is currently unknown, and clearly, additional studies are need to clarify this essential step of the BoNT intoxication process.

1.3.3 The inter-chain disulphide bond reduction

A part the structural role, the interchain disulphide bond plays a functional role in BoNTs toxicity. The first evidence was highlighted by the lack of toxicity *in vivo* of a previously reduced neurotoxin[103]. Later, Fischer and Montal demonstrated that for an effective translocation, the L chain needs to remain linked to H via the inter-chain SS bridge and that its reduction is the concluding event, that which frees the L chain enabling the metalloprotease activity[93,104]. Consistently, the premature reduction of this bond, at any stage before its exposure to the cytoplasm, abort the L chain translocation, indicating that it plays a fundamental role within the intoxication process and that it has to reach intact the cytosolic side of the membrane for a productive L chain delivery[7,100]. Consequently, it was proposed that the positioning, the size and the high hydrophobicity of the two sulphur atoms, is important in initiating the translocation event[101]. These data indicated that the reduction of the interchain disulfide bond within nerve terminal cytosol may be a "*conditio sine qua non*" to free the metalloprotease activity of botulinum neurotoxins, and thus represents a rational target for the development of mechanism-based antitoxins[7,105].

In details, the SV lumen of most intracellular organelle is oxidant, while the cell cytosol has a reducing potential, which is kept by a large number of redox couples[106-109]. The reduction of protein disulfides is catalysed in the cell by different enzymatic systems. The majors one are the glutathione-glutaredoxin system and the NADPH-Thioredoxin reductase (TrxR)-Thioredoxin (Trx). The first evidence that the TrxR may be involved in the reduction of the interchain disulfide bond of BoNTs, was the finding that Auranofin, the most potent TrxR inhibitor identified so far, prevented the toxicity of BoNT/B, /C and /D[110]. On the other hand, buthione sulfoximine, a compound capable to substantially reduce glutathione intracellular levels, had no inhibitory activity, indicating that the glutathione-glutaredoxin system is not involved in the entry of BoNTs in the cytoplasm[110]. In this thesis, we will discuss how a pharmacological approach led us to the identification of the entire NADPH-Thioredoxin

reductase (TrxR)-Thioredoxin (Trx) system being responsible for the cytosolic release of L in the cytosol and this has led to the discovery that both TrxR and Trx are extrinsic proteins of the cytosolic side of SV. Consequently, several inhibitor of this redox system were found to prevent the L chain of BoNTs from displaying their metalloprotease activity versus the SNARE proteins in cultured neurons and *in vivo*[111,112]. Interestingly, the TrxR-Trx redox system is also present on the endosomal membrane wherefrom diphtheria toxin enters the cytosol, and it is responsible for the inter-chain disulfide bond reduction of this toxin, which was previously shown to be the rate limiting step of the entire cell intoxication process[113]. Together, these data suggest that the couple TrxR-Trx is the reducing system involved in the release of the catalytic part of all A-SS-B toxins in the cytosol.

1.3.4 The L chains are metalloproteases specific for the SNARE proteins

Upon reduction of the disulfide bond, the free in the cytosol L chain functions as a Zn^{2+} dependent endopeptidase that exclusively hydrolyses distinct peptide bonds of neuronal SNARE proteins: VAMP (vesicle-associated membrane protein; also called synaptobrevin), SNAP-25 (synaptosomal-associated protein of 25 kDa) or syntaxin which are cleaved at single sites. In details, BoNT/A, and BoNT/E cleave SNAP25[114,115], BoNT/B, BoNT/D, BoNT/F and BoNT/G cleave VAMP[116-118], instead, BoNT/C is unique because cleave both SNAP25 and syntaxin[3]. The fact that inactivation of any of these three proteins inhibits neurotransmitter release is the strongest evidence that the three SNARE proteins form the core of the neuroexocytosis nanomachine. SNAREs form a heterotrimeric complex and their proteolysis impairs its assembly and/or function[3,119-121]. The SNARE family of proteins includes several isoforms that are differentially expressed in many non-neuronal cells and tissues. Even if many of those isoforms can be cleaved by BoNTs, these substrates are not accessible *in vivo*, as non-neuronal cells lack proper receptors for the toxin. With the exception of BoNT/A and BoNT/C, all BoNTs cleave SNARE proteins by removing large cytosolic segments, which prevents the formation of the SNARE complex. BoNT/A and BoNT/C remove only a few residues from the C-terminal of SNAP-25 and, this SNAP-25 truncated form are still able to form a stable SNARE complex; thus, the molecular mechanism of BoNT/A and BoNT/C-induced neuroparalysis remains to be elucidated. Nevertheless, no other substrates are presently known and this is related to the unique model of recognition of VAMP, SNAP-25 or

syntaxin by the L domain, which is based on an extended interaction including the cleavage site as well as exosites dispersed along the substrate sequence[122-127]. As a result of this in-depth interaction of the L chain with their substrates, the seven BoNTs serotypes exhibit exclusive specificities with respect to the different isoforms of the three SNARE proteins present within different neurons and within different animal species. Therefore, BoNTs can be used as simple tools to determine the effect of knocking-out specific SNAREs in cell physiology[3].

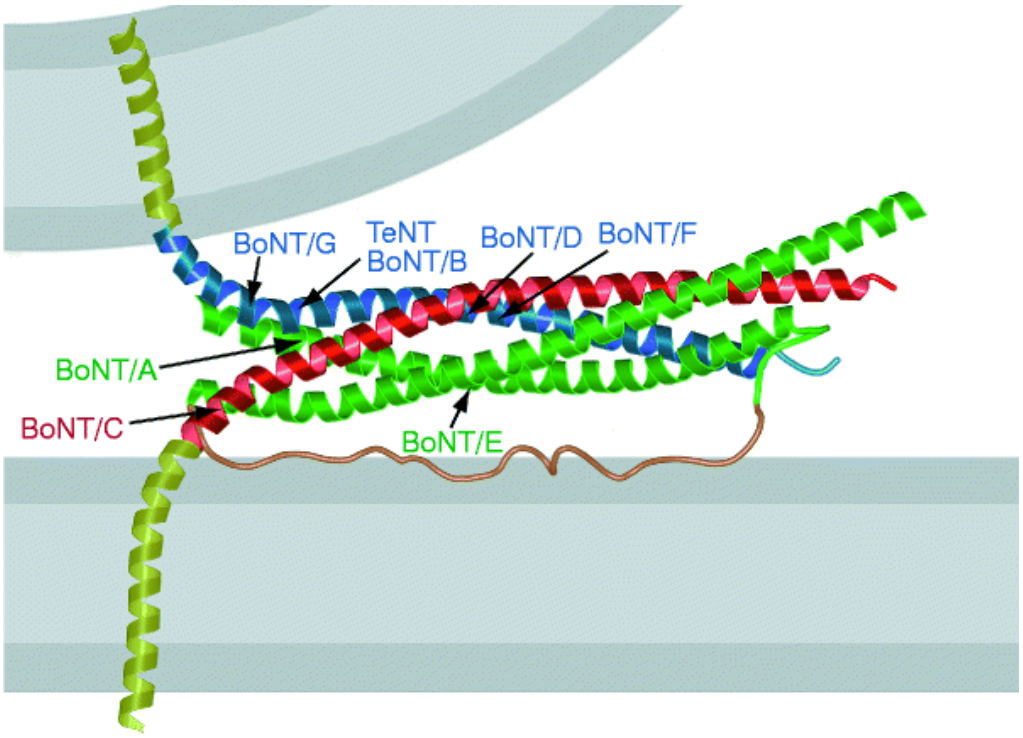

Fig. 7. Hypothetical model of synaptic vesicles fusion and BoNTs cleavage sites . The VAMP neurotoxin mediated cleavage site for BoNT/B is between Gln 76 and Phe 77; for BoNT/F, between Gln 58 and Lys 59; for BoNT/G, between Ala 81 and Ala 82; and for BoNT/D, between Lys 59 and Leu 60. The syntaxin BoNT/C cleavage site is between Lys 253 and Ala 254. Cleavage sites in SNAP25 are between Asp 193 and Glu 194 for BoNT/E, and between Arg 176 and Gln 177 for BoNT/A. From Sutton et *al.*, 1998.

2. AIM OF THE THESIS

The most potent poisons know to human are the botulinum neurotoxins (BoNTs), which are neurospecific metalloproteases acting within peripheral nerve terminals where they interrupt the release of neurotransmitter acetylcholine, causing the severe neuroparalytic syndrome of botulism[7]. BoNTs are produced by different species of Clostridia and have been classified in seven serotypes (BoNT/A to /G) based on their immunological properties. However, with the introduction of Next-Generation Sequencing (NGS) technique, several novel BoNTs have been recently identified, and many other are likely going to be discovered[6]. Human botulism is rare but remains a severe paralytic illness, and considering that the solely available therapy is based on immunological methods, the existence of so many variants represents a major safety problem, especially in the case of a possible use of BoNTs as bioweapons [4,7]. Indeed, from a clinical point of view, such variations in antigenicity would possibly cause a medical aid failure and largely preclude the possibility to develop a pan-vaccine[30,128,129]. This calls for implementing more studies aiming at the discovery of new drugs capable of block BoNTs regardless their antigenic difference.

The aim of the present thesis was unravelling novel aspects of the cellular and molecular mechanism of action of BoNTs and, consequently, identifying new approaches to prevent their neurotoxicity, starting from the evidence that, despite the amino acid sequence variability, all BoNT variants are structurally similar and display a likewise similar mechanism of action at nerve endings. This offered the rational bases to design new strategies able of inhibiting BoNTs, independently from their antigenic variability.

It is worth to mention that, even if the study of BoNTs in general seemed to have reached a sort of steady state, this type of investigation can yet shed light on novel fascinating details about BoNTs mechanism of action as wells as new features of neuron physiology .

3. RESULTS

Thioredoxin and Its Reductase Are Present on Synaptic Vesicles, and Their Inhibition Prevents the Paralysis Induced by Botulinum Neurotoxins

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Article

Cell Reports

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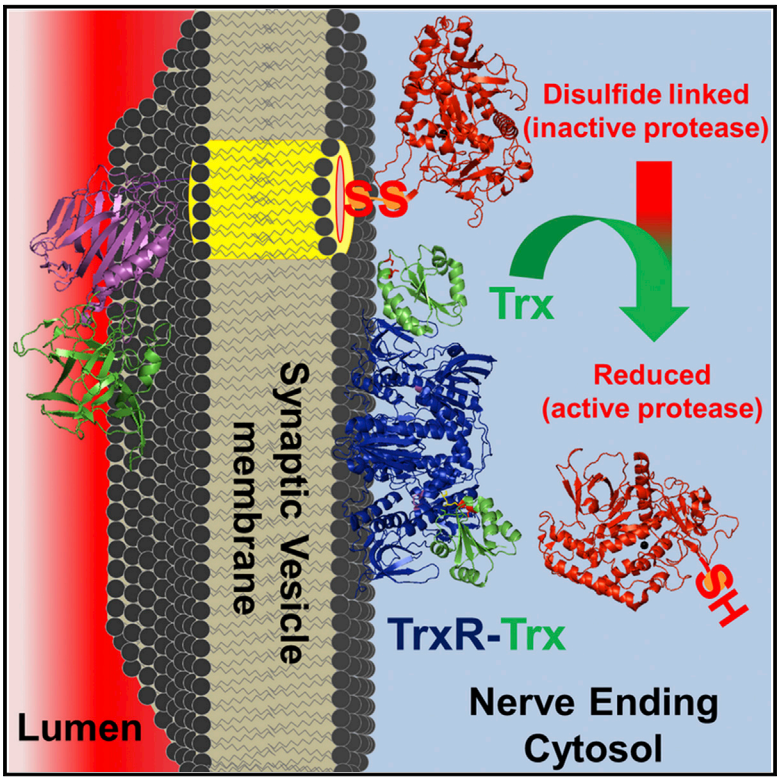
Graphical Abstract



Highlights

Synaptic vesicles possess an active thioredoxin reductase-thioredoxin system

The two proteins are on the cytosolic side of the synaptic vesicle membrane

This system reduces the interchain disulfide bond of botulinum neurotoxins

Specific inhibitors prevent the neuroparalysis induced by botulinum neurotoxins

Authors

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In Brief

About 40 botulinum neurotoxins have been recently discovered, highlighting the need for chemical inhibitors that target these potent toxins. Pirazzini et al. now find that synaptic vesicles possess the thioredoxin reductase-thioredoxin system and show that it is responsible for the selective cleavage of a key toxin disulfide bond, a step required for the entry of all such neurotoxins into neurons. The authors thus uncover a class of inhibitors capable of acting in vivo.

Pirazzini et al., 2014, Cell Reports 8, 1870–1878
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Thioredoxin and Its Reductase Are Present on Synaptic Vesicles, and Their Inhibition Prevents the Paralysis Induced by Botulinum Neurotoxins

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SUMMARY

Botulinum neurotoxins consist of a metalloprotease linked via a conserved interchain disulfide bond to a heavy chain responsible for neurospecific binding and translocation of the enzymatic domain in the nerve terminal cytosol. The metalloprotease activity is enabled upon disulfide reduction and causes neuroparalysis by cleaving the SNARE proteins. Here, we show that the thioredoxin reductase-thioredoxin protein disulfide-reducing system is present on synaptic vesicles and that it is functional and responsible for the reduction of the interchain disulfide of botulinum neurotoxin serotypes A, C, and E. Specific inhibitors of thioredoxin reductase or thioredoxin prevent intoxication of cultured neurons in a dose-dependent manner and are also very effective inhibitors of the paralysis of the neuromuscular junction. We found that this group of inhibitors of botulinum neurotoxins is very effective in vivo. Most of them are nontoxic and are good candidates as preventive and therapeutic drugs for human botulism.

INTRODUCTION

The botulinum neurotoxins (BoNTs) are released by different species of Clostridia in dozens of different isoforms that are grouped into seven different serotypes (BoNT/A–BoNT/G) (Hill and Smith, 2013; Rossetto et al., 2014). They inhibit peripheral cholinergic nerve terminals and cause the flaccid paralysis and autonomic dysfunctions of botulism (Johnson and Montecucco, 2008). BoNTs are so toxic to humans as to be considered for potential use in bioterrorism (CDC, 2012). At the same time, their neurospecificity and reversibility of action makes them excellent therapeutics for a growing and heterogeneous number of human diseases that are characterized by a hyperactivity of peripheral nerve terminals (Davletov et al., 2005; Dressler, 2012; Masuyer et al., 2014; Montecucco and Molgó, 2005).

BoNTs consist of a metalloprotease light chain (L; 50 kDa) and a heavy chain (H; 100 kDa) linked by a strictly conserved interchain disulfide bond. BoNTs bind specifically to the presynaptic membrane of peripheral nerve terminals (Dolly et al., 1984) and enter into the cytosol, where they block neurotransmitter release by the L-mediated cleavage of the essential SNARE proteins (Binz and Rummel, 2009; Pantano and Montecucco, 2014). The seven BoNT serotypes exhibit exclusive specificities with respect to the different SNARE proteins and therefore can be used as simple tools to determine the effect of knocking out specific SNAREs in cell physiology (Pantano and Montecucco, 2014). To penetrate into neurons, BoNTs exploit the endocytosis of synaptic vesicles (SVs) (Colasante et al., 2013), and the acidification of the SV lumen induces the H-mediated membrane translocation of L (Fischer and Montal, 2013; Montal, 2010). It has been demonstrated that, once on the cytosolic side, the L metalloprotease remains attached to H via the interchain SS bridge and the reduction of this bond releases the L metalloprotease activity, unblocking at the same time the ion channel formed by H in the membrane (Fischer and Montal, 2007). Here, we show that the thioredoxin reductase (TrxR)-thioredoxin (Trx) redox system is highly expressed in the motor neurons nerve terminals and that it is present on the SV cytosolic surface. This redox system is shown here to be functional, as inhibitors of TrxR or Trx effectively prevent the cleavage of SNAP25 by the L chains of BoNT/A, BoNT/C, and BoNT/E within neurons in culture and largely reduce the neuroparalysis of these neurotoxins in mice. Such a high inhibition of BoNTs by small-molecule drugs in vivo strongly suggests that these drugs may be useful to prevent and treat botulism.

RESULTS

Thioredoxin Reductase and Thioredoxin Are Present on the Cytosolic Surface of SVs

The recent finding that auranofin, a TrxR inhibitor, prevented the action of tetanus neurotoxin in cultured neurons (Pirazzini

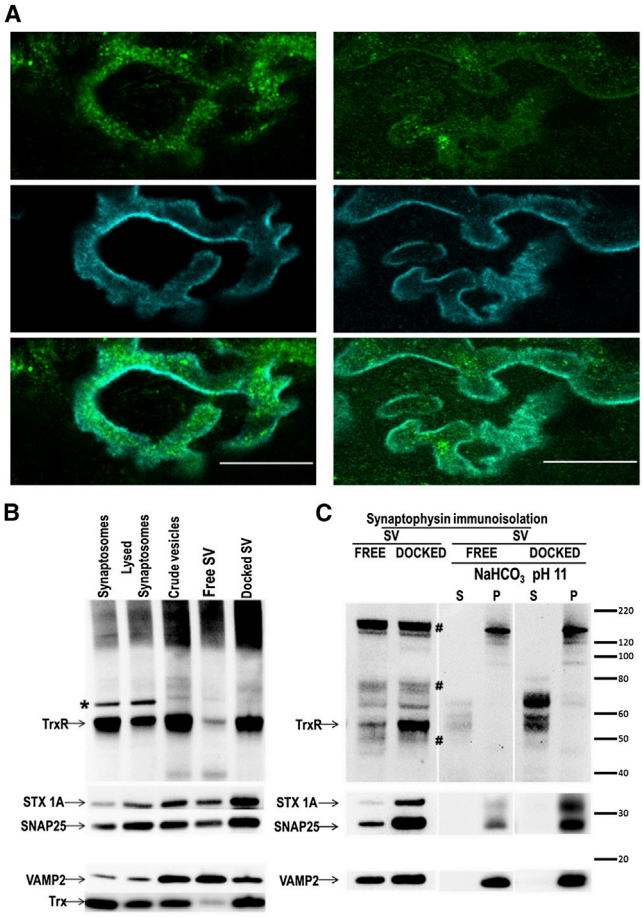

Figure 1. Thioredoxin Reductase and Thioredoxin Are Present in Nerve Terminals and Are Loosely Bound to the Surface of Synaptic Vesicles

(A) The left panels show representative confocal images of the *levator aureus longus* mouse neuromuscular junction stained with primary antibodies specific for thioredoxin reductase (TrxR, green) and α-bungarotoxin (cyan); similarly, the right panels refer to thioredoxin (Trx, green) and α-bungarotoxin (cyan). As expected, both proteins appear to be present also in muscle fibers; scale bar, 10 μm. See also Figure S1A.

(B) The immunoblot staining of different preparations from the rat brain (indicated on the top of the lanes; 10 μg of total lysate proteins per lane) after SDS-PAGE are shown. TrxR, anti-thioredoxin reductase; Trx, anti-thioredoxin; STX 1A, anti-syntaxin; SNAP25, anti-SNAP25; VAMP2, anti-VAMP2. The electrophoretic mobility corresponding to the different molecular weight markers is indicated. The asterisk indicates an immunoreactive band relative to an alternative splicing form of TrxR of 66 kDa (UniProt database).

(C) This panel shows immunoblottings with different specific antibodies of free and active zone-docked synaptic vesicles immunoisolated with an antibody specific for synaptophysin and probed for TrxR presence. The four lanes on the right part of the panel show that TrxR is detached from SVs upon treatment with bicarbonate/carbonate pH 11 buffer (S, supernatant; P, pellet). In both panels, membranes were stripped and restained for SNARE proteins. Hashtags indicate antibody bands.

et al., 2013) prompted us to investigate the presence of the TrxR-Trx system within nerve terminals and on synaptic vesicles, which are the Trojan horses used by BoNT/A to deliver its L chain in the cytosol (Colasante et al., 2013; Harper et al., 2011). Figure 1A shows that the neuromuscular junction, which is the major site of action of BoNTs, highly expresses both TrxR and Trx, as do primary cultures of neurons (Figure S1A). This is in agreement with previous work, where it was shown that both TrxR and Trx are transported from the cell body to axon terminals (Rozell et al., 1985; Stemme et al., 1985). Figure 1B demonstrates that both TrxR and Trx are present in synaptosomes purified from rat brain as well as in a crude preparation of SVs extracted from the same synaptosomes (Figure 1B). Further purification of SV indicates that this enzymatic redox system is indeed associated with SVs and that it is highly enriched in docked SVs (Figure 1B); i.e., SVs that are bound to the active zones in the presynaptic nerve terminal (Boyken et al., 2013; Morciano et al., 2005, 2009), and includes portions of the presynaptic plasma membrane, as disclosed by the presence of plasma membrane markers (Figure S1B, Ca-ATPase and Na/K-ATPase pumps); at the same time, all the fractions display the typical relative abundance of presynaptic proteins (Figure S1B). Notably, the staining of PSD95, a postsynaptic protein, is present only in synaptosomes but essentially absent in free and docked synaptic vesicles, suggesting that the TrxR-Trx belongs to the presynaptic compartment (Figure S1B). Moreover, Figure 1C shows that SVs immunoisolated with an antibody specific for synaptophysin, a protein marker of SVs (Fykse et al., 1993), do contain TrxR. The TrxR-Trx redox system is bound extrinsically to the SV surface, as it is removed upon incubation with bicarbonate/carbonate buffer at pH 11 (Figure 1C). Such a location explains why these proteins were not detected before in thorough proteomics studies of SVs, because bicarbonate-washed SVs were employed (Boyken et al., 2013; Morciano et al., 2005; Takamori et al., 2006). At the same time, it indicates that the TrxR-Trx redox system may play an important role in neuroexocytosis.

Inhibitors of Thioredoxin Reductase Prevent the Intoxication of Neurons by Botulinum Neurotoxin Serotypes A, C, and E

Even if the role(s) of the TrxR-Trx system in SV function remains to be discovered, we used BoNT intoxication as readout of its functionality, following the demonstration that the cytosolic reduction of the single interchain disulfide bond is essential to enable their metalloprotease activity (Fischer and Montal, 2007; Schiavo et al., 1993). Here, and in the next sections, we show the effects of a large series of TrxR-Trx inhibitors on its capability to reduce the interchain disulfide bridge of BoNT/A, BoNT/C, and BoNT/E. These three botulinum neurotoxins were chosen because they have different structures (Kumaran et al., 2009; Lacy et al., 1998) and are implicated in human and animal botulism, and because BoNT/A is used in human therapy (Dressler, 2012; Hallett et al., 2013; Naumann et al., 2013).

Figure 2A shows that an antibody specific for the BoNT/A-truncated SNAP25 stains well a BoNT/A-treated primary culture of neurons consisting of more than 95% cerebellar granular neurons (CGNs), while no labeling was detectable when neurons were pretreated with the TrxR-specific inhibitors juglone

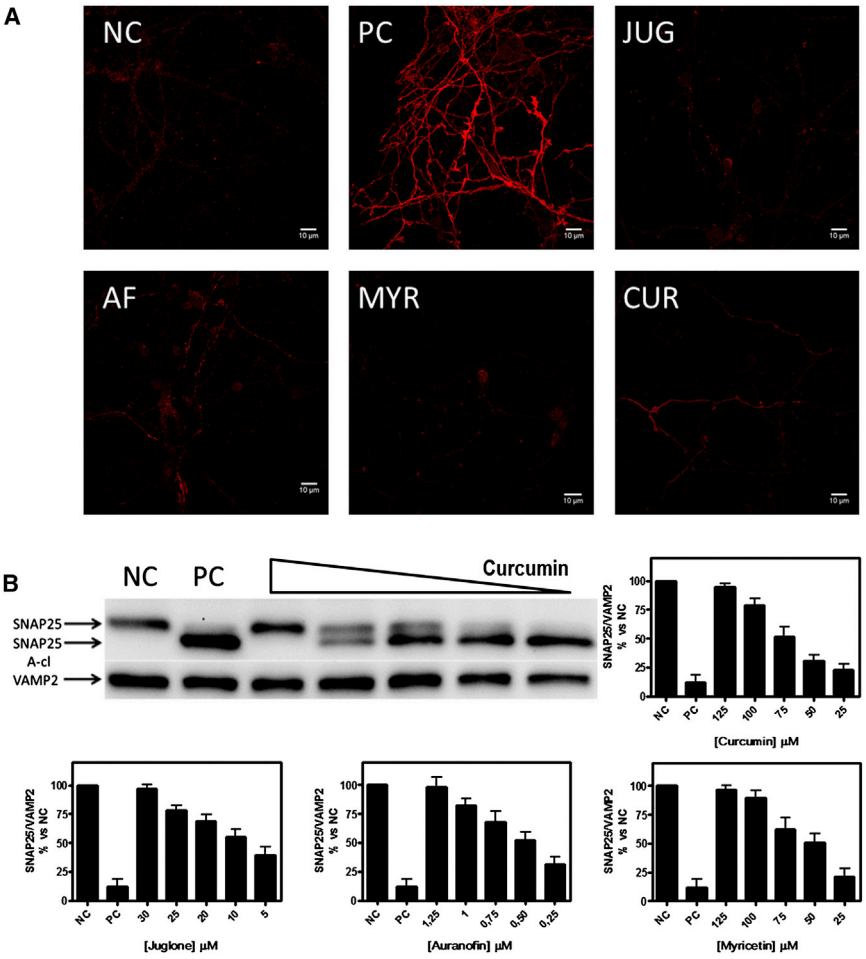

Figure 2. The BoNT/A-Induced Cleavage of SNAP25 Is Prevented in Cerebellar Granular Neurons by Thioredoxin Reductase Inhibitors

(A) CGNs were treated with TrxR inhibitors (JUG, 20 μM; AUR, 1 μM; MYR, 75 μM; CUR, 100 μM) or vehicle (NC, no toxin; PC, toxin treated) at 37°C. After 30 min, BoNT/A 1 nM was added for an additional 30 min to all samples except NC, and then neurons were washed and incubated in the presence of the same concentration of inhibitors for an additional 2 hr. Samples were fixed and stained with an antibody specific for the C terminus of the BoNT/A-cleaved SNAP25 ($SNAP25_{1–197}$). Anti-BoNT/A-cleaved SNAP25 was detected with an Alexa Fluor 555-conjugated secondary antibody. Images shown are representative of three independent sets of experiments. Scale bar, 10 μm. See also Figure S2A.

(B) Quantification of SNAP25 by immunoblotting. CGNs were preincubated for 30 min with the indicated concentration of inhibitor at 37°C, BoNT/A 1 nM was added for 15 min, cells were washed, and culture medium with the same concentration of inhibitor was restored and incubation prolonged for 12 hr at 37°C. Cells were lysed and the SNAP25 content was estimated with an antibody that recognizes both the cleaved and the intact form of SNAP25 and another one specific for VAMP2, as an internal control. The top left panel reports a typical immunoblot resulting from an experiment in which curcumin was present (NC, no toxin no inhibitor added; PC, no inhibitor plus BoNT/A 1 nM final concentration; the five right lanes refer to sample treated with the increasing curcumin concentrations indicated in the right panel). The other panels report the amount of SNAP25 determined as a ratio to VAMP2 staining, which serves as internal control, taking the value in nontreated cells (NC) as 100% in CGNs samples treated with the indicated amounts of the different inhibitors and with BoNT/A. SD values derive from three independent experiments performed in triplicate. See also Figures S2B and S2C. Similar results were obtained when 10 pM BoNT/A was left, together with inhibitors, for 12 hr at 37°C before cell lysis and evaluation of SNAP25 cleavage (not shown).

(JUG), auranofin (AUR), myricetin (MYR), and curcumin (CUR) (Cai et al., 2012; Fang et al., 2005; Lu and Holmgren, 2012; Lu et al., 2006; Omata et al., 2006; Rackham et al., 2011). Figure 2B shows that the inhibition of the BoNT/A-mediated cleavage of SNAP25 by these inhibitors is dose dependent. In control experiments, we found that these TrxR inhibitors do not significantly affect the viability of CGNs at the maximal doses used and that they do not affect the metalloproteolytic activity of BoNT/A tested in vitro with recombinant SNAP25 (not shown). Similar experiments were performed with CGNs treated with BoNT/E (Figure S2A and S2B) and BoNT/C (Figure S2C); in this latter case, the readout of inhibitor activity was also performed with an antibody specific for syntaxin, as BoNT/C cleaves both SNAP25 and syntaxin (Pantano and Montecucco, 2014). It should be noted that the TrxR inhibitors used here show similar dose-dependence patterns versus the three different neurotoxins, indicating that the step they inhibit has similar relevance for the display of the SNARE cleavage activity of the three different BoNTs.

Inhibitors of Thioredoxin Prevent the Intoxication of Neurons by Botulinum Neurotoxin Serotypes A, C, and E

The reduction of the protein disulfides in the cytosol by the TrxR-Trx system is the end result of the transfer of reducing equivalents from NADPH to TrxR and then to Trx (Arnér and Holmgren, 2000; Hanschmann et al., 2013; Lu and Holmgren, 2009). The majority of available inhibitors of this redox system are directed toward TrxR, but recently, specific inhibitors of Trx have been tested in humans: PX-12 is under clinical trial as and anticancer agent (Baker et al., 2013; Kirkpatrick et al., 1998; Ramanathan et al., 2011), and ebselen is under investigation as a postischemia and poststroke therapeutic (Aras et al., 2014; Yamaguchi et al., 1998; Zhao et al., 2002). PX-12 and ebselen (EBS) inhibit the BoNT/A-induced cleavage of SNAP25 in CGNs as detected by immunofluorescence (Figure 3A) and by quantitative immunoblotting (Figure 3B). At the same time, neither PX-12 nor ebselen affects the viability of CGNs at the maximal doses used here, nor do they show any effect on the metalloproteolytic activity of BoNT/A (not shown). A similar efficacy of these inhibitors was

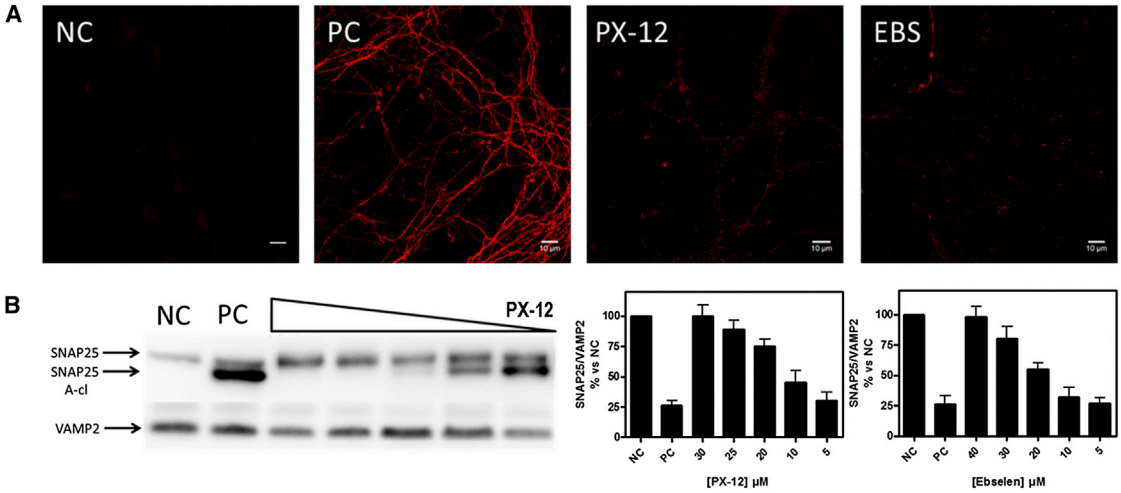

Figure 3. Inhibitors of Thioredoxin Prevent the SNAP25 Cleavage by BoNT/A in Cerebellar Granular Neurons

(A) CGNs were treated with Trx inhibitors (PX-12 25 μM or EBS 30 μM) or vehicle (NC, no toxin; PC, toxin treated) at 37°C. After 30 min, BoNT/A 1 nM was added for an additional 30 min, neurons were washed, and the incubation with the same concentration of inhibitors was prolonged for 2 hr. Treated neurons were fixed and stained with an antibody specific for the BoNT/A-cleaved form of SNAP25 ($SNAP25_{1-197}$). BoNT/A-cleaved SNAP25 was detected with an Alexa Fluor 555-conjugated secondary antibody. These images are representative of three independent sets of experiments. Scale bar, 10 μm. See also Figure S3A.

(B) Quantification of SNAP25 by immunoblotting. CGNs were preincubated for 30 min with the indicated concentration of inhibitor at 37°C, BoNT/A 1 nM (final concentration) was added for 15 min, cells were washed, and culture medium with the same concentration of inhibitor was restored and incubation prolonged for 12 hr at 37°C. Cells were lysed and the SNAP25 content was estimated with an antibody that recognizes both the cleaved and the intact form of SNAP25 and another one specific for VAMP2, as an internal control. The left panel reports the result of a typical experiment aimed at determining the effect of thioredoxin inhibitor PX-12. The right five lanes derive from samples exposed to increasing concentrations of PX-12 indicated in the middle panel. NC, no toxin added; PC, toxin added in absence of PX-12. The middle and right panels report the amount of SNAP25 determined as a ratio to VAMP2 staining, which serves as internal control in samples treated with the indicated amounts of the two inhibitors, taking the value of nontreated cells (NC) as 100%. SD values derive from three independent experiments performed in triplicates. See also Figures S3B and S3C. Similar results were obtained when 10 pM BoNT/A was left, together with inhibitors, for 12 hr at 37°C before cell lysis and evaluation of SNAP25 cleavage (not shown).

found in the prevention of the intoxication of CGNs by BoNT/E (Figures S3A and S3B) or by BoNT/C (Figure S3C).

Thioredoxin and Thioredoxin Reductase Inhibitors Inhibit the Peripheral Neuroparalysis Induced by Botulinum Neurotoxin Serotypes A and C

The panel of inhibitors of TrxR and of Trx used here have been extensively tested in animals and in humans as possible therapeutics in different diseases (Hanschmann et al., 2013; Holmgren and Lu, 2010; Mahmood et al., 2013) and are nontoxic at the doses used here. This encouraged us to test their activity in preventing BoNT toxicity in mice by using as a readout the digit abduction score (DAS) assay, which provides a reliable estimation of the paralysis induced by a local injection of toxin (Broide et al., 2013). Notably, such an experiment avoids the death of the animal and allows one to follow the rate of muscular activity recovery with time. In fact, this assay exploits a remarkable property of BoNTs; i.e., the complete reversibility of their peripheral neuroparalytic action (Rossetto et al., 2014). Figures 4 and S4 report these profiles for BoNT/A and BoNT/C, respectively, which are the two BoNT serotypes characterized by a long duration of action (Eleopra et al., 1997; Morbiato et al., 2007). It is clearly shown that the intramuscular injection of auranofin, myricetin, and curcumin (Figures 4A and S4A), and of PX-12 and ebselen (Figures 4B and S4B), are effective in lowering the neuroparalytic effect of these neurotoxins, permitting a more rapid recovery of the muscle activity. Notably, the latter two inhibitors, which act on Trx, are particularly effective. The DAS assay with BoNT/E does not provide significant results due to the very short duration of action of this BoNT serotype in mice (Rossetto et al., 2006). The inhibitory effect was also demonstrated by classical electrophysiological recordings (Molgo et al., 1990) of the treated hindlimbs. Figure 4C shows that the soleus muscle excided 1 week after BoNT/A local injection presents the complete blockade of the evoked end-plate potential (EEPP). On the other hand, muscles that were pretreated before the local injection of BoNT/A with myricetin, which acts upon TrxR, or with PX-12, which acts upon Trx, show an almost complete recovery of the EEPP, while the injection of the sole drugs does not alter the EEPP (Figure S4C). Finally, the possible inhibitory activity of these drugs in the prevention of death from botulism was evaluated. We used ebselen to perform this proof of principle, since this drug is representative of the other ones used here as it interacts with both TrxR and Trx (Zhao et al., 2002). More extensive trials of this kind were not allowed by the local animal ethical committee because of the large number of animals required. Figure 4D shows that a systemic pretreatment of mice with a well-tolerated dose of ebselen (7.5 mg/kg) (Meotti et al., 2003) significantly reduces the number of deaths induced by a 2-fold lethal dose of BoNT/A. Remarkably, this pretreatment also largely extends the life of the remaining animals, a figure of great significance for human botulism.

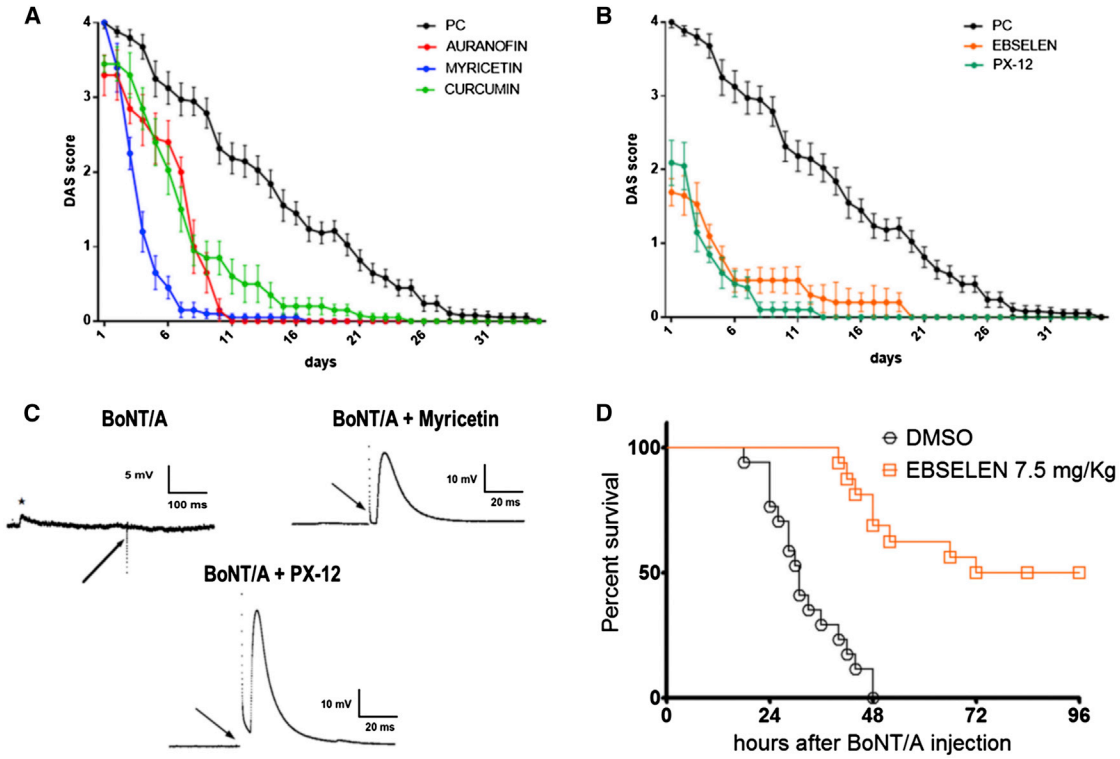

Figure 4. Thioredoxin and Thioredoxin Reductase Inhibitors Largely Prevent the Local Paralysis and Death Induced by BoNT/A

(A and B) Digit abduction score (DAS) changes with time after injection of 15 pg of BoNT/A in the mouse hindlimb after a previous injection with the indicated TrxR (A) or Trx (B) inhibitors or vehicle only (PC); DAS scores for animals treated with inhibitors only are not shown for clarity. All values are means ± SEM from four individual experiments using at least three animals per condition. See also Figures S4A and S4B.

(C) Representative traces of evoked postsynaptic potential by nerve stimulation in *soleus* muscles dissected at day 6 from mice treated as PC (left) or as in (A) (middle) or (B) (right). Traces represent intracellular recordings of evoked postsynaptic potentials following nerve stimulation (arrow), with resting membrane potential clamped at −70 mV. In BoNT/A-treated muscle fibers, no postsynaptic potentials could be evoked, indicating complete nerve block. Star indicates a spontaneous miniature end-plate potential. See also Figure S4C.

(D) Ebselen reduces BoNT/A lethality. Adult male CD1 mice preconditioned with ebselen 7.5 mg/kg (n = 15) or vehicle (n = 15) (see Experimental Procedures) were i.p. injected with 2 × MLD_{50} of BoNT/A. The animals were monitored every 4 hr for 96 hr. The survival curves were compared and found to be significantly different ($p < 0.0001$).

DISCUSSION

The control of the redox potentials of the cell cytosol and organelles is essential for cell life, and this is exerted via connected redox systems consisting of several redox couples. A cell redox event of particularly high relevance is the control of the formation and breakdown of protein disulfide bonds, which are implicated in controlling a variety of cell functions and are altered in a number of human diseases. In addition to glutathione and cysteine-dependent reducing systems, protein disulfide reduction is performed by the NADPH-thioredoxin reductase-thioredoxin system (Holmgren, 1989). The paramount importance of the TrxR-Trx system is indicated by its high conservation along biological evolution and by its localization in the nucleus and inside mitochondria, in addition to the cytosol (Hanschmann et al., 2013; Holmgren, 1985; Lu and Holmgren, 2012; Rigobello and Bindoli, 2010). This redox system is also expressed in neurons and Schwann cells, and it is axonally transported in both directions (Rozell et al., 1985; Stemme et al., 1985). All TrxR-Trx isoforms are essential for cell life as deduced by the fact that their suppression leads to cell death and are associated to various human diseases, including cancer (Arnér and Holmgren, 2000; Holmgren and Lu, 2010; Mukherjee and Martin, 2008). Accordingly, a large number of drugs were developed to be evaluated as candidates for clinical use. Using the TrxR-specific drug auranofin, which is currently used in the treatment of rheumatoid arthritis and seems to have a great potential for the treatment of other pathological conditions (Madeira et al., 2012), we have provided indirect evidence that the TrxR-Trx system reduces the disulfide bond linking the L and H chains of tetanus neurotoxin (Pirazzini et al., 2013). Building on this observation and on the fact that synaptic vesicles mediate the entry of tetanus neurotoxin inside neurons (Matteoli et al., 1996), we have investigated the association of the TrxR-Trx system with SVs and found that it is indeed present on the cytosolic surface of SVs as extrinsic proteins that can be removed with a high pH bicarbonate/carbonated buffer incubation. Intriguingly, TrxR-Trx is enriched in those synaptic vesicles that are docked to active zones and are ready to release their neurotransmitter content upon depolarization of the presynaptic membrane. This finding

suggests that the TrxR-Trx system may play a role in maintaining SV protein function by reducing protein disulfides. For example, the cysteine string protein forms disulfide-mediated dimers that may be noncompatible with its essential chaperone function (Braun and Scheller, 1995). Moreover, the cytosolic domains of several other SV proteins include cysteines (Morciano et al., 2009; Takamori et al., 2006). In addition, it should be recalled that Trx has the folding of primitive chaperones (Arnér and Holmgren, 2000; Berndt et al., 2008; Dekker et al., 2011; Ingles-Prieto et al., 2013), and a chaperone role on the cytosolic surface is a possibility to be considered. Previous careful studies of SV proteomics have not found TrxR and Trx (Boyken et al., 2013; Morciano et al., 2005, 2009), but this is explained by the present finding of the extrinsic nature of the binding of TrxR and Trx to SVs and by the fact that bicarbonate-stripped SVs were used in the mass spectrometry studies. Here, experimental evidence that TrxR and Trx have to be added to the complex composition and structure of the synaptic vesicles is provided. It also adds to the list of the several SV membrane components whose physiological role is still unknown. The identification of the SV protein substrates of Trx requires a study in itself. Nevertheless, here we have shown that this redox system is functional on the cytosolic surface of SVs by using botulinum neurotoxins, for which it was previously established that the reduction of the single interchain disulfide bridge is an essential prerequisite to free the metalloprotease activity of BoNT/A and BoNT/E (Fischer and Montal, 2007; Schiavo et al., 1993). Accordingly, a series of well-characterized inhibitors of TrxR and Trx prevent, in a concentration-dependent manner, the display of metalloproteolytic activity of the three different BoNTs tested in neuronal cultures. It is noteworthy that the scale of potency of the various inhibitors is closely similar for the three BoNT serotypes, indicating that the BoNTs are similarly dependent on disulfide reduction. Such data strongly support the rather general conclusion that the interchain disulfide reduction is a very essential molecular step of the intoxication process performed by all clostridial neurotoxins into neurons. Of even greater importance it is the finding that such inhibitors are very effective in lowering the paralysis induced by a local injection of BoNT/A and BoNT/C. Perhaps more importantly, ebselen elicits a remarkable protection of mice from a 2-fold lethal dose of BoNT/A, a serotype often associated with human botulism. As a consequence, the present experiments identify a class of inhibitors of BoNTs that should be active on all BoNTs independently of their different primary sequence and immunoreactivity, as the single interchain disulfide bond is strictly conserved. This class of inhibitors includes several compounds that have long been tested or are currently under validation for human therapy and that have a substantial record of safety. Our data therefore provide a proof of principle for using these BoNT inhibitors in the prevention and therapy of human botulism. Clearly, these inhibitors are not effective once the L chain is already in the cytosol, but it is known that in clinical botulism the neurotoxin remains in circulation for weeks after the initial symptoms (Fagan et al., 2009; Sheth et al., 2008), and these drugs may prevent further entry of BoNT L chains. This is more important in infant botulism, where there is a continuous supply of BoNT from the vegetative bacteria implanted into the intestine (Johnson and Montecucco, 2008). Remarkably, as these inhibitors act on a common step for all BoNTs, such a strategy may be used immediately after diagnosis, without the need for serotype identification. With the growing number of BoNTs (>40 types already reported) (Rossetto et al., 2014), this is a matter of concern with respect to the current use of BoNT antisera, and such a pharmacological approach could parallel and synergize with the antisera treatment. In addition, these drugs could be used as preventive therapy for individuals who have to enter environments where BoNTs have been released.

EXPERIMENTAL PROCEDURES

Purification of Synaptic Vesicles from Rat Cerebral Cortex

SV isolation was performed with established methods (Boyken et al., 2013; De Camilli et al., 1983; Morciano et al., 2005). Briefly, 15 rats were used. Cerebral cortices were pulled from the cerebellum, brainstem, and most of the midbrain and were mechanically homogenized in 320 mM sucrose, 4 mM HEPES (pH 7.3) supplemented with protease inhibitors (complete EDTA-free, Roche). After differential centrifugations, crude synaptic vesicles were separated through a continuous sucrose gradient (0.25–1.5 M sucrose, 4 mM HEPES [pH 7.3]) in a Beckmann XL-80 ultracentrifuge for 5 hr with a SW28 rotor. Vesicles sedimenting at about 300–400 mM sucrose (free SVs) and those sedimenting at 800–1,000 mM (docked SVs) were collected and pelleted by centrifugation in a 70Ti rotor (Morciano et al., 2005). These vesicle fractions were resuspended in SV buffer (4 mM HEPES, 300 mM glycine [pH 7.4] supplemented with protease inhibitors). In some experiments, free and docked SVs (1 mg of total protein) were incubated with 100 μl of G protein-coupled Dynabeads (Life Technology), previously coupled with anti-synaptophysin, and were immunoisolated by overnight incubation. The immunoisolated SVs were directly lysed in nonreducing loading sample buffer, subjected to SDS-PAGE, and transferred onto a nitrocellulose membrane. Proteins were then labeled with specific antibodies, as indicated in the legends of Figures 1B and S1B legends. In some cases, SV bound to the Dynabeads were removed, washed, and stripped of extrinsic proteins upon incubation with 100 mM Na-bicarbonate buffer adjusted to pH 11.0. After centrifugation, the supernatant and the pellets were subjected to SDS-PAGE and then western blotted with the antibodies indicated in the Figure 1C legend.

Botulinum Neurotoxin Inhibition Assay on CGNs

CGNs at 6–8 days in vitro (DIV) were preincubated for 30 min with increasing concentrations of the indicated inhibitors in basal medium Eagle (BME) 10% fetal bovine serum (FBS), 25 mM KCl at 37°C and 5% CO_2. The indicated toxin was then added and left for 15 min at 37°C (BoNT/A and BoNT/C, 1 nM; BoNT/E, 2 nM). Thereafter, the toxin was washed away and the normal culture medium was restored with the same indicated concentration of inhibitor for 12 hr at 37°C and 5% CO_2.

Alternatively, CGNs at 6–8 DIV were preincubated for 30 min with different concentrations of the indicated inhibitors in BME 10% FBS, 25 mM KCl at 37°C and 5% CO_2. The BoNT was then added (BoNT/A, 10 pM; BoNT/E and BoNT/C, 50 pM), in the same medium, and left for 12 hr at 37°C in the presence of inhibitors. In both cases, the translocation of the L chains of the various neurotoxins was evaluated following their specific proteolytic activity by immunoblotting with specific antibodies against their SNARE protein targets.

Immunocytochemistry and Immunohistochemistry

Neurons were seeded onto 24-well plates at a cell density of 2.5×10^5 cells per well. CGNs at 6–8 DIV were preincubated for 30 min with the indicated concentration of inhibitor in BME 10% FBS, 25 mM KCl at 37°C and 5% CO_2. BoNT/A 1 nM was added and the incubation carried out for 30 min, the neurons were washed, and the incubation with the proper inhibitor was prolonged for 2 hr. In the case of serotype E, the toxin was added (2 nM) and left for 2 hr. The translocation of the L chains of the two neurotoxins was evaluated following the generation of the cleaved form of SNAP25 with specific primary antibodies.

This was determined by fixing neurons for 15 min at room temperature with 4% paraformaldehyde in PBS, quenched (50 mM NH_4Cl in PBS) for 20 min, and permeabilized with 5% acetic acid in ethanol for 20 min at −20°C. Neurons were then incubated with primary antibodies specific for the BoNT/A- or BoNT/E-truncated forms of SNAP25 (Antonucci et al., 2008), using VAMP2 staining as internal control. For the identification of Trx and TrxR, CGNs were fixed at 6–8 DIV and immunostained with specific antibodies, which were detected with Alexa-conjugated secondary antibodies. Coverslips were mounted in 90% glycerol in PBS containing 3% N-propylgallate and examined by either confocal (Leica TCS SP5) or epifluorescence (Leica DMIRE2) microscopy.

Digit Abduction Score Assay

Swiss-Webster adult male CD1 mice weighing 26–28 g were housed under controlled light/dark conditions, and food and water were provided ad libitum. All experiments were performed in accordance with Italian guidelines (law n. 116/1992) and approved by the animal ethical committee of Padova University. Inhibitors were dissolved in an ethanol stock solution. Gastrocnemii muscles were injected (injection volume 50 μl) with 0.05 mg of auranofin, curcumin, PX-12, or ebselen (10% ethanol) or with 0.01 mg of myricetin (20% ethanol) or vehicle alone (20% ethanol in 0.9% NaCl). After 30 min, the muscles were further injected with 15 pg of BoNT/A or 20 pg of BoNT/C (injection volume 25 μl). Hindlimb paralysis was evaluated at least once per day and DAS score provided (Aoki, 2001; Broide et al., 2013).

Mouse Death Assay

Swiss-Webster adult male CD1 mice weighing 24–26 g were housed under controlled light/dark conditions, and food and water were provided ad libitum. All experiments were performed in accordance with Italian guidelines (law n. 116/1992) and were by the animal ethical committee of our university. Ebselen was dissolved in a stock solution (10 mg/ml) with DMSO. Mice were conditioned for 3 days with an intraperitoneal (i.p.) injection of ebselen 7.5 mg/kg or with vehicle every 12 hr. The fourth day, BoNT/A was prepared as a stock solution (1.75 pg/μl), and 30 min after the last inhibitor dose, mice were weighted and i.p. injected with 1 μl/g body weight, corresponding to 1.75 ng/kg BoNT/A (2 × MLD_{50}). Mice were monitored every 4 hr for 96 hr, after which the experiment was considered ended.

Electrophysiological Recordings

One week after inhibitor and toxin injections, the treated (left hind leg) and control nontreated (right hind leg) soleus muscles were quickly excised with particular care to leave a length of 1–1.5 mm of nerve stump. Excised muscles were immediately placed in a Tyrode physiological solution and bubbled with a 5% CO_2 95% O_2 gas mixture at room temperature (20–22°C). The composition of Tyrode solution was 139 mM NaCl, 12 mM $NaHCO_3$, 4 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM KH_2PO_4, and 11 mM glucose (pH 7.4). After 10 min incubation, muscles were transferred to a Sylgard-coated 35 mm Petri dish, placed with the region of nerve insertion up, and then pinned to the bottom using insect pins (Fine Science Tools). The dish was filled with Tyrode physiological solution bubbled with a 5% CO_2 95% O_2 gas mixture. A 3 μM final concentration of μ-Conotoxin GIIIB (Alomone Labs) was added from a stock solution to block muscle action potentials (Sons et al., 2006). Excitatory postsynaptic potentials were intracellularly recorded from single muscle fibers using borosilicate glass microelectrodes (inner diameter 0.86, outer diameter 1.5; 15 MOhm resistance) (Science Products). Intracellularly recorded signals were amplified using a SEVC amplifier (NPI electronic) in the current-clamp condition. Amplified signals were then sent to an A/D converter (National Instruments) and fed to a personal computer. Digitized recordings were analyzed offline using the WinEDR software for electrophysiology (Strathclyde and Pclamp6, Axon).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.08.017.

AUTHOR CONTRIBUTIONS

M.P., O.R., F.L., and C.M. conceived the project; D.A.T., G.Z., A.M., M.S., and M.P. performed and evaluated the experiments together with O.R., F.L., and C.M.; C.C.S., T.B., S.F., and O.R. produced, purified, and tested botulinum neurotoxins and other essential reagents and provided advice; and M.P. and C.M. wrote the paper with contributions of all other coauthors.

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REFERENCES

Antonucci, F., Rossi, C., Gianfranceschi, L., Rossetto, O., and Caleo, M. (2008). Long-distance retrograde effects of botulinum neurotoxin A. J. Neurosci. *28*, 3689–3696.

Aoki, K.R. (2001). A comparison of the safety margins of botulinum neurotoxin serotypes A, B, and F in mice. Toxicon *39*, 1815–1820.

Aras, M., Altaş, M., Meydan, S., Nacar, E., Karcıoğlu, M., Ulutaş, K.T., and Serarslan, Y. (2014). Effects of Ebselen on ischemia/reperfusion injury in rat brain. Int. J. Neurosci., Published online January 10, 2014 http://dx.doi.org/10.3109/00207454.2013.879581.

Arnér, E.S., and Holmgren, A. (2000). Physiological functions of thioredoxin and thioredoxin reductase. Eur. J. Biochem. *267*, 6102–6109.

Baker, A.F., Adab, K.N., Raghunand, N., Chow, H.H.S., Stratton, S.P., Squire, S.W., Boice, M., Pestano, L.A., Kirkpatrick, D.L., and Dragovich, T. (2013). A phase IB trial of 24-hour intravenous PX-12, a thioredoxin-1 inhibitor, in patients with advanced gastrointestinal cancers. Invest. New Drugs *31*, 631–641.

Berndt, C., Lillig, C.H., and Holmgren, A. (2008). Thioredoxins and glutaredoxins as facilitators of protein folding. Biochim. Biophys. Acta *1783*, 641–650.

Binz, T., and Rummel, A. (2009). Cell entry strategy of clostridial neurotoxins. J. Neurochem. *109*, 1584–1595.

Boyken, J., Grønborg, M., Riedel, D., Urlaub, H., Jahn, R., and Chua, J.J. (2013). Molecular profiling of synaptic vesicle docking sites reveals novel proteins but few differences between glutamatergic and GABAergic synapses. Neuron *78*, 285–297.

Braun, J.E., and Scheller, R.H. (1995). Cysteine string protein, a DnaJ family member, is present on diverse secretory vesicles. Neuropharmacology *34*, 1361–1369.

Broide, R.S., Rubino, J., Nicholson, G.S., Ardila, M.C., Brown, M.S., Aoki, K.R., and Francis, J. (2013). The rat Digit Abduction Score (DAS) assay: a physiological model for assessing botulinum neurotoxin-induced skeletal muscle paralysis. Toxicon *71*, 18–24.

Cai, W., Zhang, L., Song, Y., Wang, B., Zhang, B., Cui, X., Hu, G., Liu, Y., Wu, J., and Fang, J. (2012). Small molecule inhibitors of mammalian thioredoxin reductase. Free Radic. Biol. Med. *52*, 257–265.

CDC (Centers for Disease Control and Prevention), Department of Health and Human Services (HHS) (2012). Possession, use, and transfer of select agents and toxins; biennial review. Final rule. Fed. Regist. *77*, 61083–61115.

Colasante, C., Rossetto, O., Morbiato, L., Pirazzini, M., Molgó, J., and Montecucco, C. (2013). Botulinum neurotoxin type A is internalized and translocated from small synaptic vesicles at the neuromuscular junction. Mol. Neurobiol. *48*, 120–127.

Davletov, B., Bajohrs, M., and Binz, T. (2005). Beyond BOTOX: advantages and limitations of individual botulinum neurotoxins. Trends Neurosci. *28*, 446–452.

De Camilli, P., Harris, S.M., Jr., Huttner, W.B., and Greengard, P. (1983). Synapsin I (Protein I), a nerve terminal-specific phosphoprotein. II. Its specific association with synaptic vesicles demonstrated by immunocytochemistry in agarose-embedded synaptosomes. J. Cell Biol. *96*, 1355–1373.

Dekker, C., Willison, K.R., and Taylor, W.R. (2011). On the evolutionary origin of the chaperonins. Proteins *79*, 1172–1192.

Dolly, J.O., Black, J., Williams, R.S., and Melling, J. (1984). Acceptors for botulinum neurotoxin reside on motor nerve terminals and mediate its internalization. Nature *307*, 457–460.

Dressler, D. (2012). Clinical applications of botulinum toxin. Curr. Opin. Microbiol. *15*, 325–336.

Eleopra, R., Tugnoli, V., Rossetto, O., Montecucco, C., and De Grandis, D. (1997). Botulinum neurotoxin serotype C: a novel effective botulinum toxin therapy in human. Neurosci. Lett. *224*, 91–94.

Fagan, R.P., McLaughlin, J.B., and Middaugh, J.P. (2009). Persistence of botulinum toxin in patients' serum: Alaska, 1959-2007. J. Infect. Dis. *199*, 1029–1031.

Fang, J., Lu, J., and Holmgren, A. (2005). Thioredoxin reductase is irreversibly modified by curcumin: a novel molecular mechanism for its anticancer activity. J. Biol. Chem. *280*, 25284–25290.

Fischer, A., and Montal, M. (2007). Crucial role of the disulfide bridge between botulinum neurotoxin light and heavy chains in protease translocation across membranes. J. Biol. Chem. *282*, 29604–29611.

Fischer, A., and Montal, M. (2013). Molecular dissection of botulinum neurotoxin reveals interdomain chaperone function. Toxicon *75*, 101–107.

Fykse, E.M., Takei, K., Walch-Solimena, C., Geppert, M., Jahn, R., De Camilli, P., and Südhof, T.C. (1993). Relative properties and localizations of synaptic vesicle protein isoforms: the case of the synaptophysins. J. Neurosci. *13*, 4997–5007.

Hallett, M., Albanese, A., Dressler, D., Segal, K.R., Simpson, D.M., Truong, D., and Jankovic, J. (2013). Evidence-based review and assessment of botulinum neurotoxin for the treatment of movement disorders. Toxicon *67*, 94–114.

Hanschmann, E.M., Godoy, J.R., Berndt, C., Hudemann, C., and Lillig, C.H. (2013). Thioredoxins, glutaredoxins, and peroxiredoxins—molecular mechanisms and health significance: from cofactors to antioxidants to redox signaling. Antioxid. Redox Signal. *19*, 1539–1605.

Harper, C.B., Martin, S., Nguyen, T.H., Daniels, S.J., Lavidis, N.A., Popoff, M.R., Hadzic, G., Mariana, A., Chau, N., McCluskey, A., et al. (2011). Dynamin inhibition blocks botulinum neurotoxin type A endocytosis in neurons and delays botulism. J. Biol. Chem. *286*, 35966–35976.

Hill, K.K., and Smith, T.J. (2013). Genetic diversity within Clostridium botulinum serotypes, botulinum neurotoxin gene clusters and toxin subtypes. Curr. Top. Microbiol. Immunol. *364*, 1–20.

Holmgren, A. (1985). Thioredoxin. Annu. Rev. Biochem. *54*, 237–271.

Holmgren, A. (1989). Thioredoxin and glutaredoxin systems. J. Biol. Chem. *264*, 13963–13966.

Holmgren, A., and Lu, J. (2010). Thioredoxin and thioredoxin reductase: current research with special reference to human disease. Biochem. Biophys. Res. Commun. *396*, 120–124.

Ingles-Prieto, A., Ibarra-Molero, B., Delgado-Delgado, A., Perez-Jimenez, R., Fernandez, J.M., Gaucher, E.A., Sanchez-Ruiz, J.M., and Gavira, J.A. (2013). Conservation of protein structure over four billion years. Structure *21*, 1690–1697.

Johnson, E.A., and Montecucco, C. (2008). Botulism. Handb. Clin. Neurol. *91*, 333–368.

Kirkpatrick, D.L., Kuperus, M., Dowdeswell, M., Potier, N., Donald, L.J., Kunkel, M., Berggren, M., Angulo, M., and Powis, G. (1998). Mechanisms of inhibition of the thioredoxin growth factor system by antitumor 2-imidazolyl disulfides. Biochem. Pharmacol. *55*, 987–994.

Kumaran, D., Eswaramoorthy, S., Furey, W., Navaza, J., Sax, M., and Swaminathan, S. (2009). Domain organization in Clostridium botulinum neurotoxin type E is unique: its implication in faster translocation. J. Mol. Biol. *386*, 233–245.

Lacy, D.B., Tepp, W., Cohen, A.C., DasGupta, B.R., and Stevens, R.C. (1998). Crystal structure of botulinum neurotoxin type A and implications for toxicity. Nat. Struct. Biol. *5*, 898–902.

Lu, J., and Holmgren, A. (2009). Selenoproteins. J. Biol. Chem. *284*, 723–727.

Lu, J., and Holmgren, A. (2012). Thioredoxin system in cell death progression. Antioxid. Redox Signal. *17*, 1738–1747.

Lu, J., Papp, L.V., Fang, J., Rodriguez-Nieto, S., Zhivotovsky, B., and Holmgren, A. (2006). Inhibition of Mammalian thioredoxin reductase by some flavonoids: implications for myricetin and quercetin anticancer activity. Cancer Res. *66*, 4410–4418.

Madeira, J.M., Gibson, D.L., Kean, W.F., and Klegeris, A. (2012). The biological activity of auranofin: implications for novel treatment of diseases. Inflammopharmacology *20*, 297–306.

Mahmood, D.F., Abderrazak, A., El Hadri, K., Simmet, T., and Rouis, M. (2013). The thioredoxin system as a therapeutic target in human health and disease. Antioxid. Redox Signal. *19*, 1266–1303.

Masuyer, G., Chaddock, J.A., Foster, K.A., and Acharya, K.R. (2014). Engineered botulinum neurotoxins as new therapeutics. Annu. Rev. Pharmacol. Toxicol. *54*, 27–51.

Matteoli, M., Verderio, C., Rossetto, O., Iezzi, N., Coco, S., Schiavo, G., and Montecucco, C. (1996). Synaptic vesicle endocytosis mediates the entry of tetanus neurotoxin into hippocampal neurons. Proc. Natl. Acad. Sci. USA *93*, 13310–13315.

Meotti, F.C., Borges, V.C., Zeni, G., Rocha, J.B.T., and Nogueira, C.W. (2003). Potential renal and hepatic toxicity of diphenyl diselenide, diphenyl ditelluride and Ebselen for rats and mice. Toxicol. Lett. *143*, 9–16.

Molgo, J., Comella, J.X., Angaut-Petit, D., Pecot-Dechavassine, M., Tabti, N., Faille, L., Mallart, A., and Thesleff, S. (1990). Presynaptic actions of botulinal neurotoxins at vertebrate neuromuscular junctions. J. Physiol. (Paris) *84*, 152–166.

Montal, M. (2010). Botulinum neurotoxin: a marvel of protein design. Annu. Rev. Biochem. *79*, 591–617.

Montecucco, C., and Molgó, J. (2005). Botulinal neurotoxins: revival of an old killer. Curr. Opin. Pharmacol. *5*, 274–279.

Morbiato, L., Carli, L., Johnson, E.A., Montecucco, C., Molgó, J., and Rossetto, O. (2007). Neuromuscular paralysis and recovery in mice injected with botulinum neurotoxins A and C. Eur. J. Neurosci. *25*, 2697–2704.

Morciano, M., Burré, J., Corvey, C., Karas, M., Zimmermann, H., and Volknandt, W. (2005). Immunoisolation of two synaptic vesicle pools from synaptosomes: a proteomics analysis. J. Neurochem. *95*, 1732–1745.

Morciano, M., Beckhaus, T., Karas, M., Zimmermann, H., and Volknandt, W. (2009). The proteome of the presynaptic active zone: from docked synaptic vesicles to adhesion molecules and maxi-channels. J. Neurochem. *108*, 662–675.

Mukherjee, A., and Martin, S.G. (2008). The thioredoxin system: a key target in tumour and endothelial cells. Br. J. Radiol. *81*, S57–S68.

Naumann, M., Dressler, D., Hallett, M., Jankovic, J., Schiavo, G., Segal, K.R., and Truong, D. (2013). Evidence-based review and assessment of botulinum neurotoxin for the treatment of secretory disorders. Toxicon *67*, 141–152.

Omata, Y., Folan, M., Shaw, M., Messer, R.L., Lockwood, P.E., Hobbs, D., Bouillaguet, S., Sano, H., Lewis, J.B., and Wataha, J.C. (2006). Sublethal concentrations of diverse gold compounds inhibit mammalian cytosolic thioredoxin reductase (TrxR1). Toxicol. In Vitro *20*, 882–890.

Pantano, S., and Montecucco, C. (2014). The blockade of the neurotransmitter release apparatus by botulinum neurotoxins. Cell. Mol. Life Sci. *71*, 793–811.

Pirazzini, M., Bordin, F., Rossetto, O., Shone, C.C., Binz, T., and Montecucco, C. (2013). The thioredoxin reductase-thioredoxin system is involved in the

entry of tetanus and botulinum neurotoxins in the cytosol of nerve terminals. FEBS Lett. *587*, 150–155.

Rackham, O., Shearwood, A.-M.J., Thyer, R., McNamara, E., Davies, S.M.K., Callus, B.A., Miranda-Vizuete, A., Berners-Price, S.J., Cheng, Q., Arnér, E.S.J., and Filipovska, A. (2011). Substrate and inhibitor specificities differ between human cytosolic and mitochondrial thioredoxin reductases: Implications for development of specific inhibitors. Free Radic. Biol. Med. *50*, 689–699.

Ramanathan, R.K., Abbruzzese, J., Dragovich, T., Kirkpatrick, L., Guillen, J.M., Baker, A.F., Pestano, L.A., Green, S., and Von Hoff, D.D. (2011). A randomized phase II study of PX-12, an inhibitor of thioredoxin in patients with advanced cancer of the pancreas following progression after a gemcitabine-containing combination. Cancer Chemother. Pharmacol. *67*, 503–509.

Rigobello, M.P., and Bindoli, A. (2010). Mitochondrial thioredoxin reductase purification, inhibitor studies, and role in cell signaling. Methods Enzymol. *474*, 109–122.

Rossetto, O., Morbiato, L., Caccin, P., Rigoni, M., and Montecucco, C. (2006). Presynaptic enzymatic neurotoxins. J. Neurochem. *97*, 1534–1545.

Rossetto, O., Pirazzini, M., and Montecucco, C. (2014). Botulinum neurotoxins: genetic, structural and mechanistic insights. Nat. Rev. Microbiol. *12*, 535–549. http://dx.doi.org/10.1038/nrmicro3295.

Rozell, B., Hansson, H.A., Luthman, M., and Holmgren, A. (1985). Immunohistochemical localization of thioredoxin and thioredoxin reductase in adult rats. Eur. J. Cell Biol. *38*, 79–86.

Schiavo, G., Santucci, A., Dasgupta, B.R., Mehta, P.P., Jontes, J., Benfenati, F., Wilson, M.C., and Montecucco, C. (1993). Botulinum neurotoxins serotypes A and E cleave SNAP-25 at distinct COOH-terminal peptide bonds. FEBS Lett. *335*, 99–103.

Sheth, A.N., Wiersma, P., Atrubin, D., Dubey, V., Zink, D., Skinner, G., Doerr, F., Juliao, P., Gonzalez, G., Burnett, C., et al. (2008). International outbreak of severe botulism with prolonged toxemia caused by commercial carrot juice. Clin. Infect. Dis. *47*, 1245–1251.

Sons, M.S., Busche, N., Strenzke, N., Moser, T., Ernsberger, U., Mooren, F.C., Zhang, W., Ahmad, M., Steffens, H., Schomburg, E.D., et al. (2006). alpha-Neurexins are required for efficient transmitter release and synaptic homeostasis at the mouse neuromuscular junction. Neuroscience *138*, 433–446.

Stemme, S., Hansson, H.A., Holmgren, A., and Rozell, B. (1985). Axoplasmic transport of thioredoxin and thioredoxin reductase in rat sciatic nerve. Brain Res. *359*, 140–146.

Takamori, S., Holt, M., Stenius, K., Lemke, E.A., Grønborg, M., Riedel, D., Urlaub, H., Schenck, S., Brügger, B., Ringler, P., et al. (2006). Molecular anatomy of a trafficking organelle. Cell *127*, 831–846.

Yamaguchi, T., Sano, K., Takakura, K., Saito, I., Shinohara, Y., Asano, T., and Yasuhara, H.; Ebselen Study Group (1998). Ebselen in acute ischemic stroke: a placebo-controlled, double-blind clinical trial. Stroke *29*, 12–17.

Zhao, R., Masayasu, H., and Holmgren, A. (2002). Ebselen: a substrate for human thioredoxin reductase strongly stimulating its hydroperoxide reductase activity and a superfast thioredoxin oxidant. Proc. Natl. Acad. Sci. USA *99*, 8579–8584.

Cell Reports, Volume *8*

Supplemental Information

Thioredoxin and Its Reductase Are Present on Synaptic Vesicles, and Their Inhibition Prevents the Paralysis Induced by Botulinum Neurotoxins

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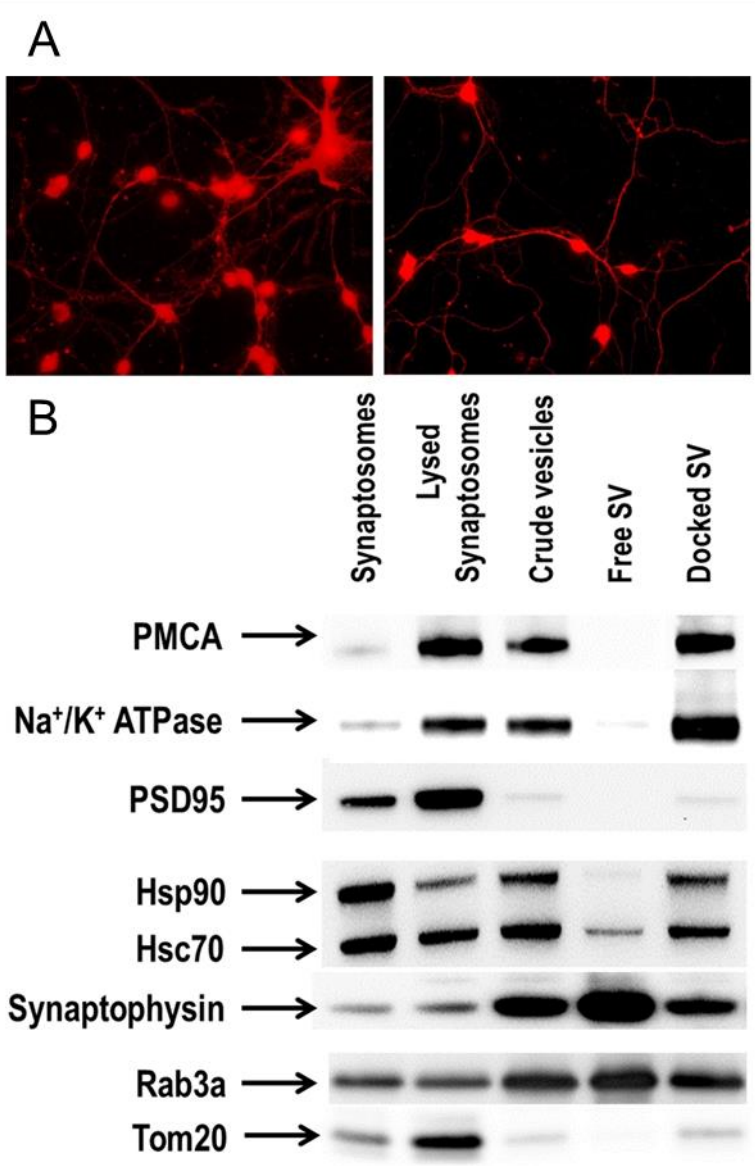

Figure S1. Thioredoxin Reductase and Thioredoxin are present in nerve terminals. (A) Confocal images showing thioredoxin reductase (left) and thioredoxin (right) highly expressed in primary cultures of Cerebellar Granular Neurons. The staining is present in cell bodies as well as inside nerve projections and terminals. (B) Post synaptic (PSD95), mitochondrial (Tom20) or presynaptic markers (all the others) protein content into rat brain synaptosomes and their sub-fractions (indicated on the top of the lanes, 10µg of total lysate proteins per lane). PMCA, Plasma Membrane Calcium ATPase; NA^+/K^+ ATPase, Sodium-Potassium pump; PSD95, Post Synaptic Density protein 95; Hsp90, Heat shock protein 90; Hsc70, Heat shock cognate 70; Rab3a, Ras-related protein 3a; Tom20, translocase of outer mitochondrial membranes 20 kDa. Related to Figure 1.

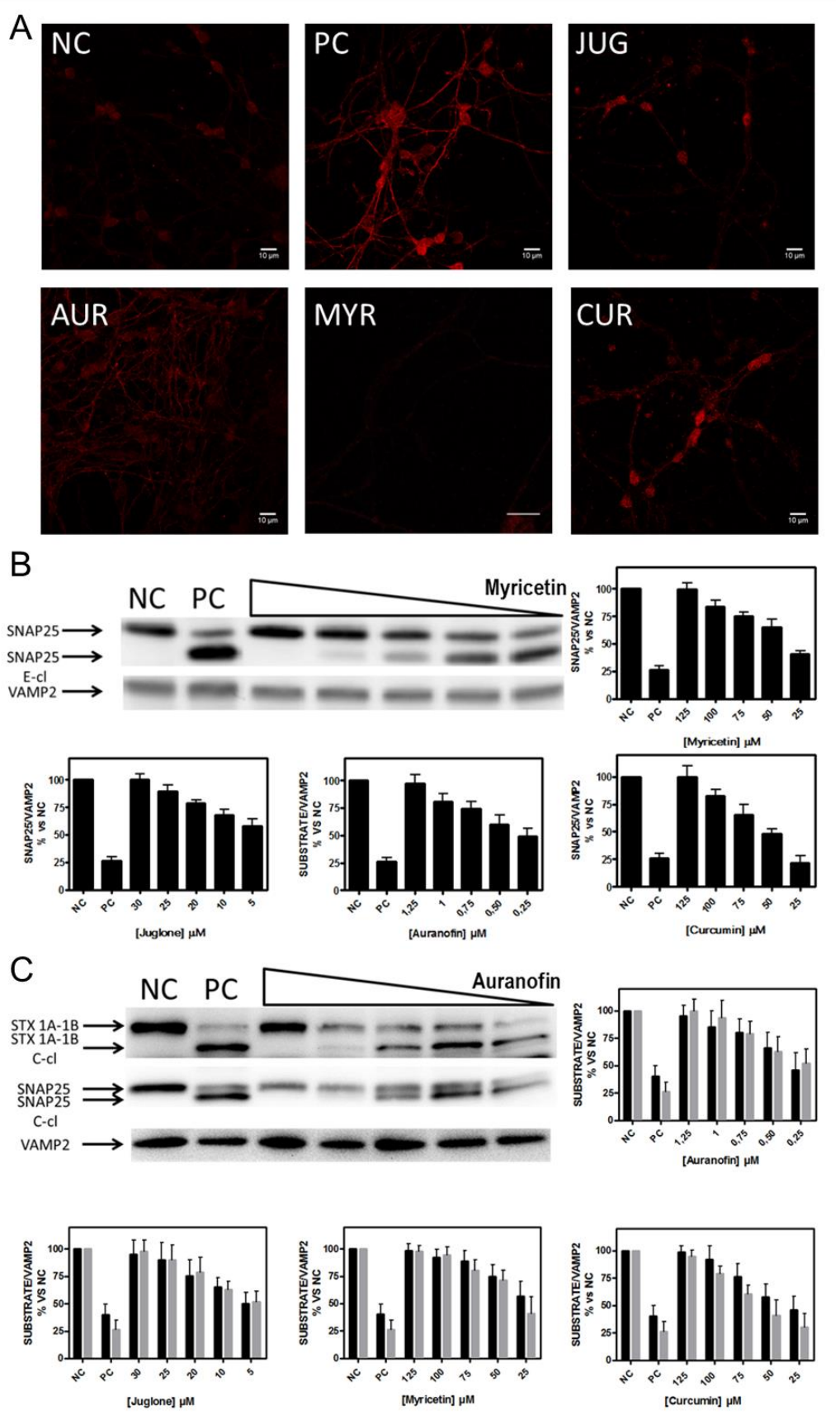

Figure S2. The BoNT/E and BoNT/C induced cleavage of SNARE proteins is prevented in cerebellar granular neurons by Thioredoxin Reductase inhibitors. (A)

CGNs were treated with TrxR inhibitors (JUG 20 µM; AUR 1 µM; MYR 75 µM; CUR 100 µM) or vehicle (NC, no toxin and PC, toxin treated) at 37 °C. After 30 minutes, BoNT/E 2 nM was added for further 120 min to all samples except NC. Samples were washed, fixed and stained with an antibody specific for the C terminus of the BoNT/E-cleaved SNAP25 ($SNAP25_{1-180}$). Anti BoNT/E-cleaved SNAP25 was detected with an Alexa555-conjugated secondary antibody. Images shown are representative of three independent sets of experiments. Bar =10 µm. (B) Quantification of SNAP25 cleavage induced by BoNT/E: CGNs were pre-incubated for 30 min with the indicated concentration of inhibitor at 37 °C, BoNT/E 2 nM was added for 15 min, cells were washed and culture medium with the same concentration of inhibitor was restored and incubation prolonged for 12 h at 37 °C. Cells were lysed and the SNAP25 content was estimated with an antibody which recognizes both the cleaved and the intact form of SNAP25 and another one specific for VAMP2, as internal control. The top left panel reports a typical immunoblot resulting from an experiments in which myricetin was present (NC, no toxin no inhibitor added, PC no inhibitor plus BoNT/E 2 nM final concentration, the five right lanes refer to sample treated with increasing myricetin concentrations, indicated in the side panel). The other panels report the amount of SNAP25 determined as a ratio to VAMP2 staining which serves as internal control, taking the value in non-treated cells (NC) as 100%, in CGNs samples treated with the indicated amounts of the different inhibitors and with BoNT/E. S.D. values derive from at least three independent experiments performed in triplicates. Closely similar results were obtained when 50 picoMolar BoNT/E was left, together with inhibitors, for 12 h at 37 °C before cell lysis and evaluation of SNAP25 cleavage (not shown). (C) Quantification of SNAP25 and syntaxin 1A-1B cleavage induced by BoNT/C (1nM): samples were treated as in B, with the only exception that immunoblotting was performed also against syntaxin 1A-1B. S.D. values derive from at least three independent experiments performed in triplicates. Closely similar results were obtained when 50 picoMolar BoNT/C was left, together with inhibitors, for 12 h at 37 °C before cell lysis and evaluation of SNAREs cleavage (not shown). Related to Figure 2.

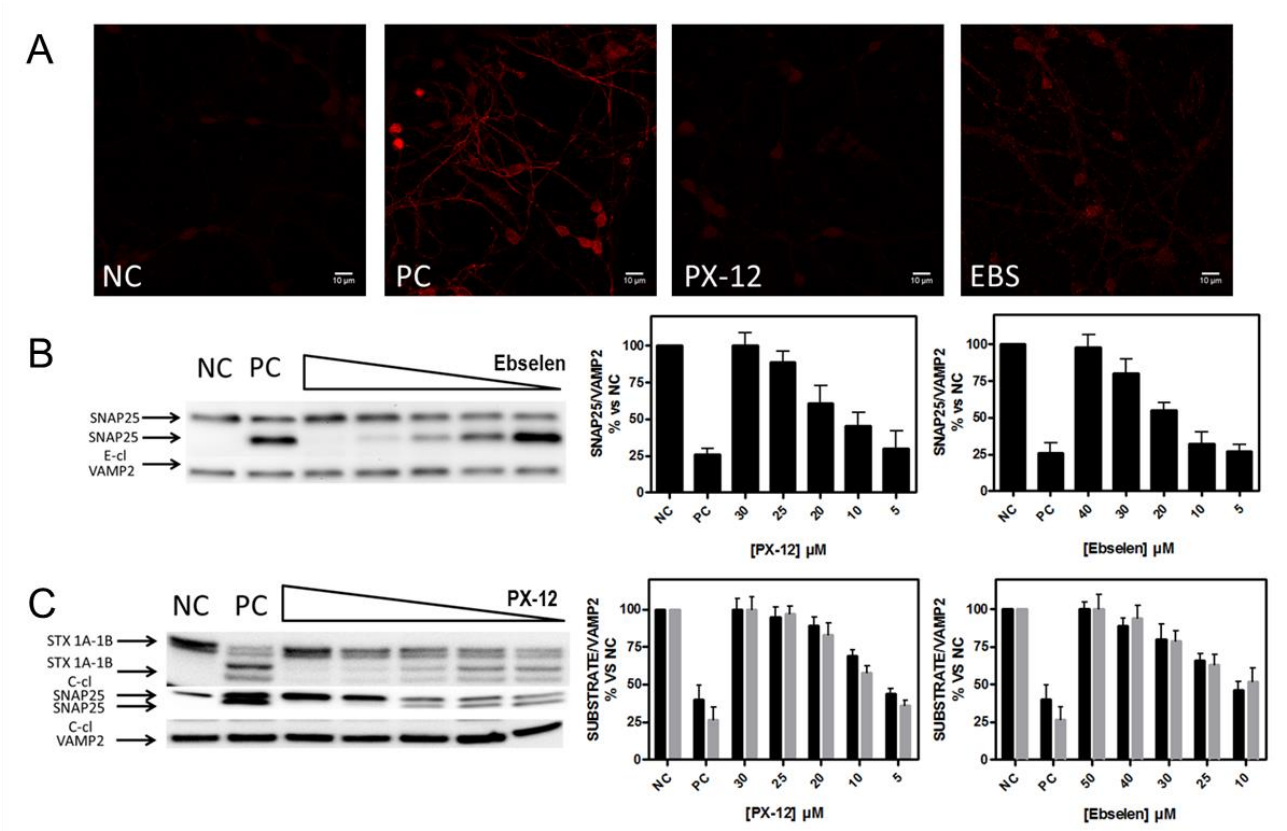

Figure S3. The BoNT/E and BoNT/C induced cleavage of SNARE proteins is prevented by Thioredoxin inhibitors in cerebellar granular neurons. (A) CGNs were treated with the thioredoxin inhibitors (PX-12 25 µM or EBS 30 µM) or the vehicle (NC, no toxin and PC, toxin treated) at 37 °C. After 30 minutes, BoNT/E 2 nM was added and the incubation prolonged for 2 h. Treated neurons were fixed and stained with an antibody against $SNAP25_{1-180}$ and VAMP2. BoNT/E cleaved SNAP25 was detected with an Alexa555 conjugated secondary antibody while VAMP2 with an Alexa488 secondary antibody (not shown for clarity). Images shown are representative from three independent experiments. Bar =10 µm. (B) Quantification of SNAP25 cleavage induced by BoNT/E: CGNs were pre-incubated for 30 min with the indicated concentration of inhibitor at 37 °C, BoNT/E 2 nM was added for 15 min, cells were washed and culture medium with the same concentration of inhibitor was restored and incubation prolonged for 12 h at 37 °C. Cells were lysed and the SNAP25 content was estimated with an antibody which recognizes both the cleaved and the intact form of SNAP25 and another one specific for VAMP2, as loading control. The left panel shows a typical immunoblot obtained with increasing concentrations of ebselen (reported on the side panel). The other panels report the quantification of residual SNAP25 with the indicated concentrations of inhibitors, plotted as a ratio with respect to VAMP2 staining, used as an internal standard, taking the value in non-treated cells (NC) as 100%. S.D. values derive from three independent experiments

performed in triplicates. Closely similar results were obtained when 50 picoMolar BoNT/E was left, together with inhibitors, for 12 h at 37 °C (not shown). (C) Quantification of SNAP25 and syntaxin 1A-1B cleavage induced by BoNT/C (1nM): samples were treated as in B, with the only exception that immunoblotting was performed also against syntaxin 1A-1B. S.D. values derive from at least three independent experiments performed in triplicates. Closely similar results were obtained when 50 picoMolar BoNT/C was left, together with inhibitors, for 12 h at 37 °C before cell lysis and evaluation of SNAREs cleavage (not shown). Related to Figure 3.

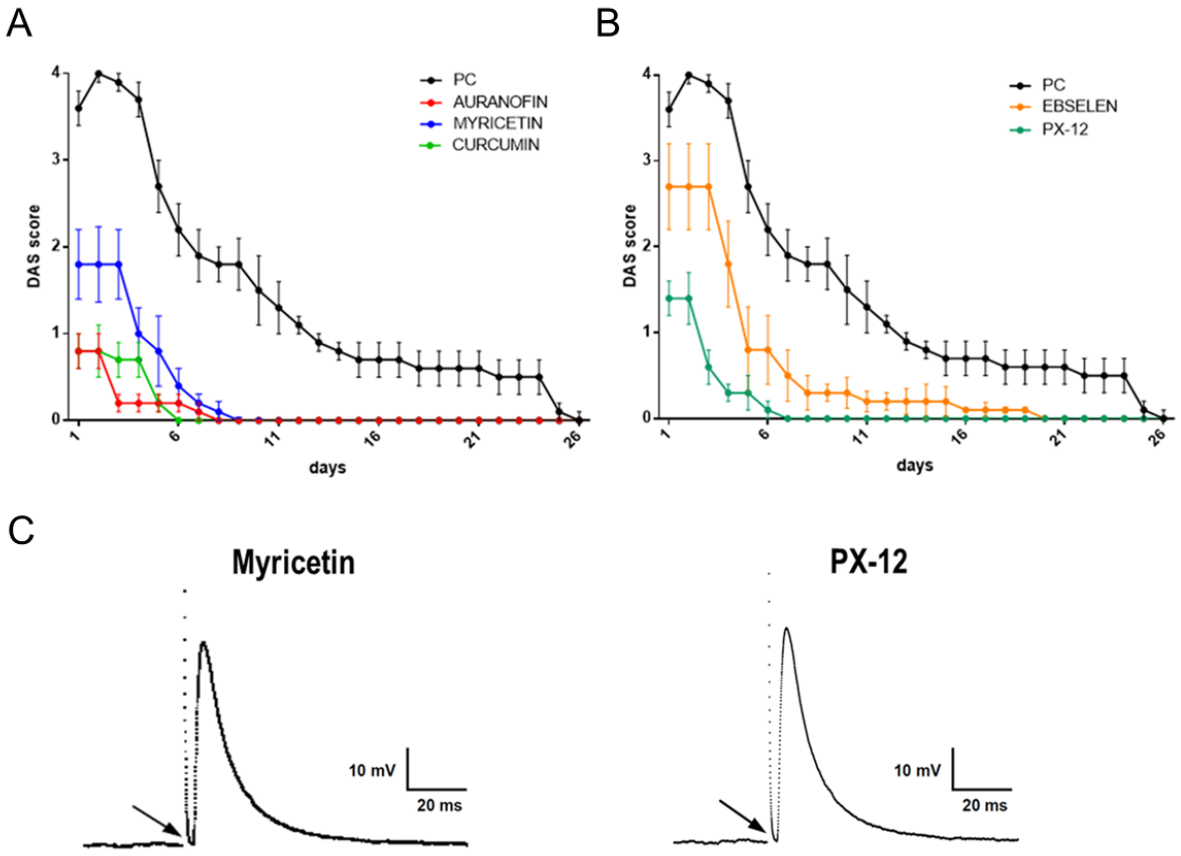

Figure S4. Thioredoxin or Thioredoxin Reductase inhibitors largely prevent and shorten the muscle paralysis induced by BoNT/C in mice. DAS changes with time induced by 20pg of BoNT/C injected in the mouse hind limb after pre-injection in the leg of different TrxR (A) or Trx (B) inhibitors or vehicle (PC). Values are the means ±SEM derived from four experiments using at least three animals per condition. (C) Representative traces of post-synaptic potential evoked by nerve stimulation in mouse soleus muscles dissected 6 days after injection with the indicated inhibitors. Traces represent intracellular recordings of EPPP following nerve stimulation (arrow). Resting membrane potential was clamped at -70 mV. Related to Figure 4.

Supplementary Experimental procedures

Reagents

Auranofin [1-Thio-β-D-glucopyranosatotriethylphosphine gold-2,3,4,6-tetraacetate], Myricetin [3,3′,4′,5,5′,7-Hexahydroxyflavone], Curcumin [(E,E)-1,7-bis(4-Hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione], Juglone [5-Hydroxy-1,4-naphtoquinone], cytosine β-D-arabinoside, DNAse I, poly-L-lysine were purchased from Sigma Aldrich. PX-12 [2-[(1-Methylpropyl)dithio]-1H-imidazole] and Ebselen [2-Phenyl-1,2-benzisoselenazol-3(2H)-one] were purchased from Santa Cruz Biotechnology. Anti-VAMP2 (104 211) and anti SNAP25 (SMI81, ab24737) monoclonal antibodies were from Synaptic System and Abcam, respectively. Anti-BoNT/A cleaved SNAP25, anti-BoNT/E cleaved SNAP25 and anti-syntaxin 1A-1B polyclonal antibodies have been previously characterized (Antonucci et al., 2008; Schiavo et al., 1995), anti PMCA (plasma membrane calcium pump ATPase) was from Thermo Scientific, $Na^{+/}K^{+}$ ATPase was from Abcam, anti-synaptophysin mAb (clone SY38) was from Dako, anti-thioredoxin reductase 1 (07-613) was from Merck Millipore, anti-thioredoxin 1 Mab (clone EPR6111) was from GeneTex, anti-PSD95 Mab was from Sigma Aldrich, anti-Hsc70 Mab was from Synaptic Sytem, anti-Hsp90 Mab was from BD transduction Laboratories^{TM}, anti-tom20 (FL-145) was from SantaCruz Biotechnology. BoNT/A and /C were prepared and purified as described (Schiavo and Montecucco, 1995; Shone and Tranter, 1995) whilst BoNT/E was produced in *Escherichia coli* via recombinant methods (Binz et al., 1994).

Cell cultures

Primary cultures of rat cerebellar granule neurons (CGNs) were prepared from 6- to 8-days-old rats as previously described (Rigoni et al., 2004). Cerebella of 6 days post-natal rats were mechanically disrupted and then trypsinized in the presence of DNase I. Cells were collected and plated into 24 well plates, coated with poly-L-lysine (50 µg/mL), at a cell density of 4×10^5 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 gentamicin. To arrest growth of non-neuronal cells, cytosine arabinoside (10 µM) was added to the medium 18–24 h after plating.

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% or 10-20% NuPage gel (Invitrogen) and separated by electrophoresis in 1X MES buffer (Invitrogen) or 1X Tris-Glycine buffer. Proteins were then transferred onto Protran nitrocellulose membranes (Whatman) and saturated for 1 h in PBST (PBS containing 0.1% Tween20) supplemented with 5% non-fatty milk. Incubation with primary antibodies was performed overnight at 4°C. The membranes were washed three times with PBST and incubated with secondary HRP-conjugated antibodies. Finally, membranes were washed several times with PBST and visualization was carried out using Luminata Crescendo (Merck Millipore).

In vitro proteolytic activity

BoNT/A or BoNT/E (2 µg) was reduced with reducing buffer (150 mM NaCl, 10 mM NaH_2PO_4, 15 mM DTT pH 7.4) in the presence of indicated Trx-TrxR inhibitor for 30 minutes at 37° C. 5 µg of recombinant GST-SNAP25 was added to the reduced toxins and the reaction was carried out for 12 hours at 37° C. SNAP25 cleavage was assessed by SDS-PAGE and immunoblotting with an anti SNAP25 antibody.

Viability test

CGNs were seeded in a 96 wells plates at a cell density 10^5 cells per well. After 6 div, different concentration of Trx or TrxR inhibitors, ranging from 0 to the maximum concentration used in the experiments, were added and left for 12 hours. Neurons were the washed and MTS assay (Promega) performed according to manufacturer indication.

Supplemental references

Antonucci, F., Rossi, C., Gianfranceschi, L., Rossetto, O., and Caleo, M. (2008). Long-distance retrograde effects of botulinum neurotoxin A. J Neurosci. *28*, 3689-3696.

Binz, T., Blasi, J., Yamasaki, S., Baumeister, A., Link, E., Südhof, T.C., Jahn, R., and Niemann, H. (1994). Proteolysis of SNAP-25 by types E and A botulinal neurotoxins. J Biol Chem *269*, 1617-1620.

Rigoni, M., Schiavo, G., Weston, A.E., Caccin, P., Allegrini, F., Pennuto, M., Valtorta, F., Montecucco, C., and Rossetto, O. (2004). Snake presynaptic neurotoxins with

phospholipase A2 activity induce punctate swellings of neurites and exocytosis of synaptic vesicles. J Cell Sci. *117*, 3561-3570.

Schiavo, G., and Montecucco, C. (1995). Tetanus and botulism neurotoxins: isolation and assay. Methods Enzymol *248*, 643-652.

Schiavo, G., Shone, C.C., Bennett, M.K., Scheller, R.H., and Montecucco, C. (1995). Botulinum neurotoxin type C cleaves a single Lys-Ala bond within the carboxyl-terminal region of syntaxins. J Biol Chem. *270*, 10566-10570.

Shone, C.C., and Tranter, H.S. (1995). Growth of clostridia and preparation of their neurotoxins. Curr Top Microbiol Immunol *195*, 143-160.

Inhibition of Botulinum Neurotoxins Interchain Disulfide Bond Reduction Prevents the Peripheral Neuroparalysis of Botulism

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Inhibition of botulinum neurotoxins interchain disulfide bond reduction prevents the peripheral neuroparalysis of botulism

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ABSTRACT

Botulinum neurotoxins (BoNTs) form a growing family of metalloproteases with a unique specificity either for VAMP, SNAP25 or syntaxin. The BoNTs are grouped in seven different serotypes indicated by letters from A to G. These neurotoxins enter the cytosol of nerve terminals *via* a 100 kDa chain which binds to the presynaptic membrane and assists the translocation of a 50 kDa metalloprotease chain. These two chains are linked by a single disulfide bridge which plays an essential role during the entry of the metalloprotease chain in the cytosol, but thereafter it has to be reduced to free the proteolytic activity. Its reduction is mediated by thioredoxin which is continuously regenerated by its reductase. Here we show that inhibitors of thioredoxin reductase or of thioredoxin prevent the specific proteolysis of VAMP by the four VAMP-specific BoNTs: type B, D, F and G. These compounds are effective not only in primary cultures of neurons, but also in preventing the *in vivo* mouse limb neuroparalysis. In addition, one of these inhibitors, Ebselen, largely protects mice from the death caused by a systemic injection. Together with recent results obtained with BoNTs specific for SNAP25 and syntaxin, the present data demonstrate the essential role of the thioredoxin–thioredoxin reductase system in reducing the interchain disulfide during the nerve intoxication mechanism of all serotypes. Therefore its inhibitors should be considered for a possible use to prevent botulism and for treating infant botulism.

1. Introduction

Several species of anaerobic bacteria of the genus *Clostridium* produce botulinum neurotoxins which belong to seven different serotypes (BoNT/A-/G) [1,2]. Their number is rapidly growing and many different sub-serotypes are presently known. The biological and toxicological properties of these novel BoNTs are poorly understood, but the limited amount of experimental data indicate that they act predominantly at peripheral cholinergic nerve terminals, causing a long lasting blockade of acetylcholine release with ensuing paralysis of skeletal and autonomic nerve terminals, characteristic of botulism [3]. Apart from BoNT/D [4–6], BoNTs are the most toxic poisons for humans and are classified as potential bioterrorist weapons [7,8]. This extremely high toxicity results from their neurospecificity and from their catalytic activity, which leads to knock-out of proteins essential to the neurotransmitter release apparatus [2,9]. All BoNTs consist of a metalloprotease light chain (L, 50 kDa) and a heavy chain (H, 100 kDa) linked by a strictly conserved interchain disulfide bond. This molecular structure has been shaped during evolution in order to exploit essential physiological features of the vertebrate nervous system. Indeed BoNTs bind specifically to peripheral nerve terminals presynaptic membrane [10] *via* the C-terminus of the H chain which interacts with polysialogangliosides leading to toxin accumulation. The subsequent binding to a protein receptor, transiently exposed on the membrane, is harnessed for their endocytosis [11,12]. In the case of BoNT/A the endocytic organelles were identified as synaptic vesicles [13,14]. Similar data are not available for the other BoNTs, but several experiments performed with vacuolar ATPase proton pump inhibitors clearly indicate that all these neurotoxins enter the lumen of an acidic compartment [15,16]. Indeed it is established that all serotypes have to undergo a low-pH driven

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membrane translocation of the L chain, mediated by the N-terminal part of H chain [9,17,18]. Once on the cytosolic side, the L metalloprotease remains attached to the H chain *via* the interchain disulfide bridge. This bond is strictly conserved among serotypes, sub-serotypes and also tetanus neurotoxin, which is structurally and functionally related to BoNTs. Remarkably, the premature reduction of this disulfide completely abrogates the toxicity of all clostridial neurotoxins, underscoring its fundamental role in the intoxication process [19–23]. The reduction of this bond is essential to release the catalytic activity of the L metalloprotease within the cytosol versus the three SNARE proteins [20]. Indeed, also in the test-tube BoNTs cannot cleave their recombinant substrates unless this linkage is reduced [24,25]. Once enabled through reduction, the L chain of BoNT/B, /D, /F and /G cleave VAMP at different peptide bonds, BoNT/A and /E cleave SNAP25, while BoNT/C is particular because it is the only one capable to cleave two substrates, SNAP25 and syntaxin [26,27].

We recently reported that the thioredoxin reductase (TrxR)–thioredoxin (Trx) redox system is present on the cytosolic surface of synaptic vesicles and that its inhibition with specific drugs very effectively prevented the neuroparalysis induced by the three SNAP25 specific BoNTs (A, C and E) [28,29]. Here, we extended the study to the four VAMP-specific BoNTs (B, D, F and G) [16,30] using the four chemicals whose structures are shown in Fig. 1 and which are well characterized inhibitors of TrxR–Trx system. Myricetin is a flavonoid which reacts with the selenium atom present in the active site of the reduced TrxR, providing its irreversible inhibition [31]. Curcumin is a polyphenol of vegetal origin that irreversibly inhibits TrxR forming a 1:2 adduct [32]. In both cases, the direct consequence of inhibition is the loss of Trx reducing potential. PX12 acts mainly on thioredoxin by alkylating a non-catalytic cysteine residue, generating a steric hindrance that prevents the interaction with its reductase. As a result, Trx remains permanently in the oxidized, inactive, form [33,34]. Ebselen acts on both members of the redox couple, as it is an excellent substrate for the mammalian TrxR and a highly efficient oxidant of reduced Trx. Thus, Ebselen prevents the normal function of both enzymes [35].

Together with our previous reports [28,36], the present results provide a strong indication that the reduction of the single interchain disulfide bond is a newly identified key event in nerve intoxication of all BoNTs. We therefore propose that TrxR–Trx inhibitors can be considered as a novel and general class of anti-BoNTs drugs and discuss their possible use in humans.

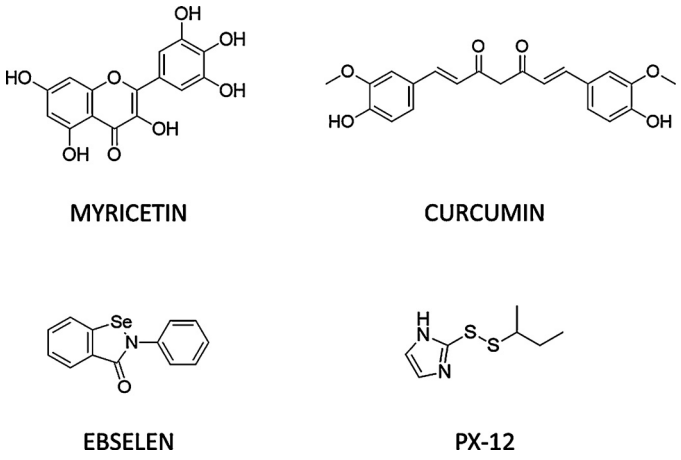

Fig. 1. Thioredoxin–thioredoxin reductase inhibitors used in this study. 3,3′,4′,5,5′,7-Hexahydroxyflavone (Myricetin) and (E,E)-1,7-bis(4-hydroxy-3 methoxyphenyl)-1,6 heptadiene-3,5-dione (Curcumin) preferentially inhibit thioredoxin reductase, 2-phenyl-1,2-benzisoselenazol-3(2H)-one (Ebselen) both thioredoxin and thioredoxin reductase and 2-[(1-methylpropyl) dithio]-1H-imidazole (PX12) inhibits thioredoxin.

2. Materials and methods

2.1. Reagents

3,3′,4′,5,5′,7-Hexahydroxyflavone (Myricetin), (E,E)-1,7-bis(4-hydroxy-3 methoxyphenyl)-1,6 heptadiene-3,5-dione (Curcumin), cytosine β-D-arabinoside, DNAse I and poly-L-lysine were purchased from Sigma– Aldrich. 2-[(1-Methylpropyl) dithio]-1H-imidazole (PX12) was purchased from Santa Cruz Biotechnology and 2-phenyl-1,2-benzisoselenazol-3(2H)-one (Ebselen) was purchased from Cayman Chemical. Antibodies: VAMP2 (104 211) and Syntaxin-1A (110 111) were from Synaptic System, SNAP25 (SMI81, ab24737) was from Abcam. Botulinum neurotoxins B, D and G were produced in *Escherichia coli via* recombinant methods [37–39] whilst BoNT/F was purified as previously described [40].

2.2. Neuronal cultures

Primary cultures of rat cerebellar granule neurons (CGNs) were prepared from 6- to 8-days-old rats as previously described [41]. Briefly, cerebella were isolated, mechanically disrupted and then trypsinized in the presence of DNase I. Cells were then collected and plated into 24 well plates, pre-coated with poly-L-lysine (50 μg/ml), at a cell density of 4×10^5 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 gentamicin (hereafter indicated as complete culture medium). To arrest growth of non-neuronal cells, cytosine arabinoside (10 μM) was added to the medium 18–24 h after plating.

2.3. Botulinum neurotoxins inhibition assay on CGNs

CGNs at 6–8 days *in vitro* (DIV) were incubated with increasing concentrations of the indicated inhibitor in complete culture medium for 30 min at 37 °C. Thereafter, the indicated toxin was diluted in complete culture medium and added to CGNs in order to obtain the following final concentrations: BoNT/B (2 nM) or BoNT/F (4 nM) or BoNT/G (4 nM). Incubation was prolonged for 12 h at 37 °C. In the case of BoNT/D, owing to its potency, the toxin was added at a final concentration of 0.025 nM and incubated for 15 min at 37 °C. The neuronal culture was then washed and the culture medium with the same concentration of inhibitor was restored for 12 h. Toxicity was evaluated following the specific proteolytic activity of BoNTs *via* immunoblotting with antibodies specific for VAMP2, SNAP25 and syntaxin. All inhibitors were dissolved in DMSO and stored at −80 °C.

2.4. Immunoblotting

Cells were directly lysed with Laemmli sample buffer containing protease inhibitors (complete Mini EDTA-free, Roche). Cell lysates were loaded onto a 4–12% NuPage gel (Life technologies) and separated by electrophoresis in 1X MES buffer (Life technologies). Proteins were transferred onto Protran nitrocellulose membranes (Whatman) and saturated for 1 h in PBST (PBS, 0.1% Tween 20) supplemented with 5% non-fatty milk. Incubation with primary antibodies was performed overnight at 4 °C. The membranes were then washed three times with PBST and incubated with secondary HRP-conjugated antibodies for 1 h. Finally, membranes were washed twice with PBST and once with PBS; visualization was carried out using Luminata Crescendo (Merck Millipore).

2.5. Immunocytochemistry

Neurons were seeded onto 13 mm round glasses in 24-well plates at a cell density of 4×10^5 cells per well. CGNs at 6–8 DIV were pre-incubated for 30 min with the indicated concentration of inhibitor in complete culture medium at 37 °C and 5% CO_2. BoNT/D was added to reach a final concentration of 0.2 nM and the incubation carried out for 10 min at 4 °C. Neurons were washed and the incubation with the indicated inhibitor in the same medium was prolonged for 90 min at 37 °C and 5% CO_2. After treatments, isolated CGNs were fixed for 10 min with 4% paraformaldehyde in PBS and processed for immunocytochemistry. Coverslips were mounted using Fluorescent Mounting Medium (Dako) and examined by epifluorescence (Leica DMIRE2) microscopy. BoNT/D activity was evaluated following the decrease of VAMP2 staining, detected with an antibody specific for the intact form of the protein.

2.6. Digit abduction score assay

Swiss-Webster adult male CD1 mice weighing 26–28 g were housed under controlled light/dark conditions, and food and water were provided *ad libitum*. All experiments were performed in accordance with the European Communities Council Directive n° 2010/63/UE and approved by the Italian Ministry of Health. Curcumin and PX12 were dissolved in ethanol to make stock solutions (10 mg/ml and 5 mg/ml, respectively and stored at −20 °C), whilst Ebselen was dissolved in DMSO (10 mg/ml and stored at −20 °C). A freshly opened aliquot of Ebselen must be used. Hind limb skeletal muscles were injected (total injection volume 25 μl) with 0.02 mg of Curcumin or PX12 or Ebselen or vehicle alone (8% ethanol or DMSO in 0.9% NaCl with 0.2% gelatin). After 30 min, muscles were further injected with BoNT/B (0.5 pg/g) or BoNT/D (0.02 pg/g) or BoNT/F (2 pg/g) or BoNT/G (5 pg/g) or vehicle alone (0.9% NaCl with 0.2% gelatin). Hind limbs paralysis was evaluated at least once per day using the Digit Abduction Score (DAS) assay, performed as previously reported [42,43].

2.7. Lethality assay

Swiss-Webster adult male CD1 mice weighing 24–26 g were housed under controlled light/dark conditions, and food and water were provided *ad libitum*. All experiments were performed in accordance with the European Communities Council Directive n° 2010/63/UE and approved by the Italian Ministry of Health. A stock solution of Ebselen in DMSO was prepared (7.5 mg/ml). Mice were conditioned for 3 days with intraperitoneal (i.p.) injections of Ebselen at a dose of 7.5 mg/kg or with vehicle (DMSO) every 12 h. Each experiment was conducted with a freshly opened aliquot of Ebselen. The third day, BoNT/B or BoNT/D or BoNT/F was prepared as a stock solution (BoNT/B 0.9 pg/μl, BoNT/D 0.04 pg/μl and BoNT/F 2.5 pg/μl in 0.9% NaCl with 0.2% gelatin), and 30 min after the injection of the last inhibitor dose, mice were weighted and i.p. injected with 1 μl/g body weight, roughly corresponding to a 2 fold MLD_{50} for each toxin. The respective MLD_{50} have been determined through preliminary experiments: BoNT/B 0.45 ng/kg, BoNT/D 0.02 ng/kg and BoNT/F 1.25 ng/kg). Mice were monitored every 4 h for 96 h, at which the experiment was considered ended.

3. Results

3.1. Inhibitors of thioredoxin reductase and thioredoxin prevent cleavage of VAMP by botulinum neurotoxins type B, D, F and G in cultured neurons

The most convenient and rapid way to screen the ability of TrxR–Trx inhibitors in blocking the VAMP-specific BoNTs toxicity is the use of sensitive neuronal cultures. Fig. 2 shows that, upon overnight incubation of primary cultures of cerebellar granular neurons, 2 nM BoNT/B cleaves its substrate, as evaluated by

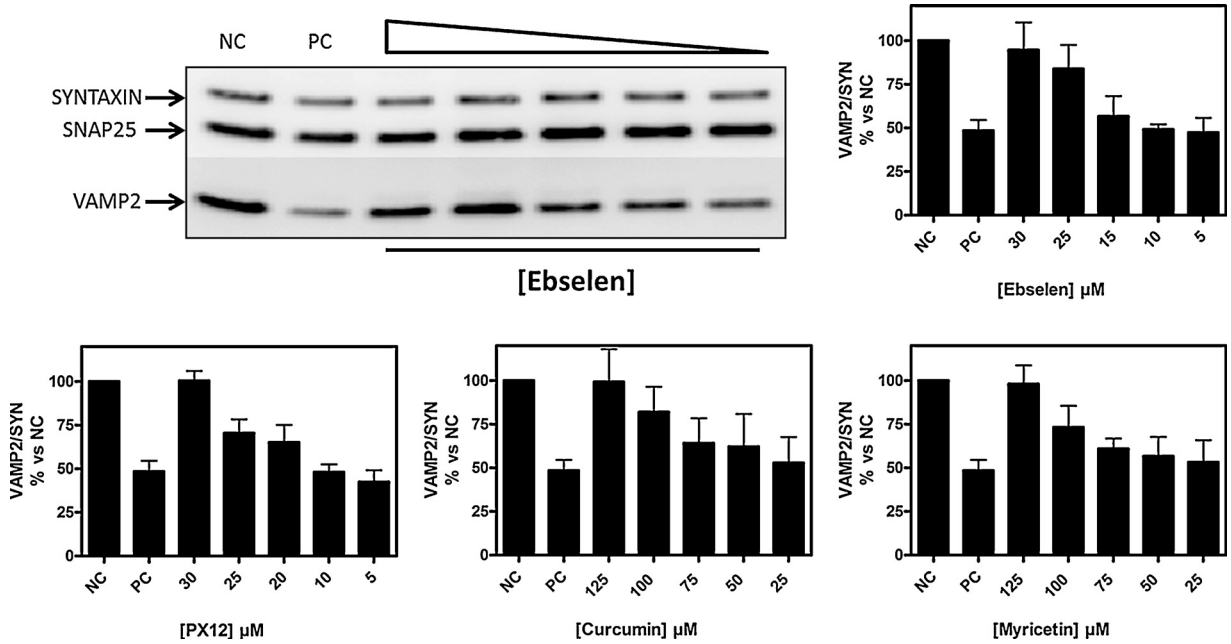

Fig. 2. Thioredoxin–thioredoxin reductase inhibitors prevent the BoNT/B-induced cleavage of VAMP2 in neuronal culture. CGNs were incubated with the indicated concentrations of inhibitors at 37 °C for 30 min. 2 nM BoNT/B was then added and incubation prolonged for 12 h at 37 °C; cells were then lysed and the VAMP2 content was estimated with an antibody recognizing the intact form of VAMP2. Syntaxin and SNAP25 staining was used as loading control. Upper left panel shows a representative immunoblot, obtained in experiments with Ebselen (NC, no toxin added; PC, only toxin added). Graphs show the quantification, of the experiments performed with the reported inhibitors, for VAMP2 determined as a ratio to Syntaxin staining, taking the value of non-treated cells as 100%. SD values derive from at least three independent experiments.

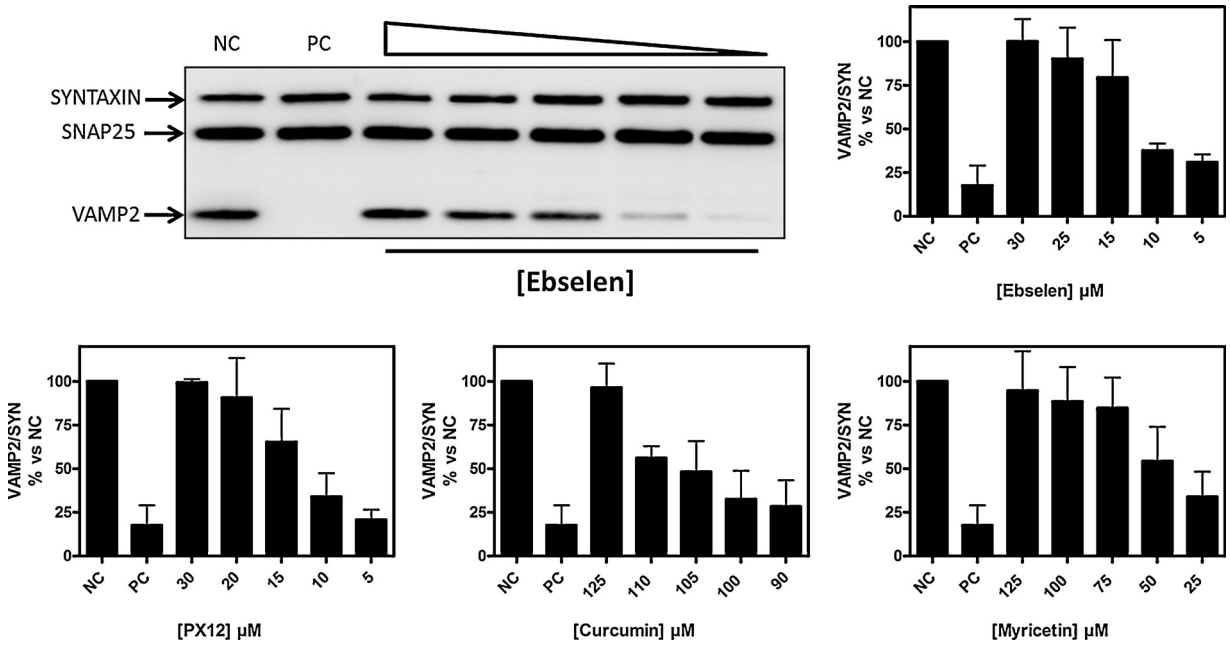

Fig. 3. Thioredoxin–thioredoxin reductase inhibitors prevent the BoNT/D-induced cleavage of VAMP2 in neuronal culture. CGNs were incubated with the indicated concentrations of inhibitor at 37 °C for 30 min. BoNT/D 0.025 nM was added for 15 min, cells were washed, and culture medium with the same concentration of inhibitor was restored and incubation prolonged for 12 h at 37 °C. Cells were then lysed and the VAMP2 content was estimated with an antibody recognizing the intact form of VAMP2. Syntaxin and SNAP25 staining was used as loading control. Upper left panel shows a representative immunoblot, obtained in experiments with Ebselen (NC, no toxin added; PC, only toxin added). Graphs show the quantification, of the experiments performed with the reported inhibitors, for VAMP2 determined as a ratio to Syntaxin staining, taking the value of non-treated cells as 100%. SD values derive from at least three independent experiments.

western blotting with an antibody specific for the intact form of the protein; a residual portion of VAMP2 not accessible to the metalloprotease L chain of BoNT/B is a common finding [19,44,45]. At the same time, Ebselen, PX12, Curcumin and Myricetin prevent the cleavage of VAMP2 in a dose dependent mode. Importantly, at the doses used here, these drugs do not affect cell viability, nor do they inhibit the cleavage of a recombinant VAMP2 (not shown).

Fig. 3 shows that BoNT/D is particularly active in CGNs and a concentration as low as 0.025 nM cleaves nearly all VAMP2. Nevertheless, the four compounds are effective in blocking such an activity with a profile of concentration dependence similar to that found with BoNT/B (Fig. 2), as determined by immunoblotting. This is even more evident in Fig. 4, which documents their effect using an immunofluorescence analysis. Importantly, the two assays provide fully consistent results even though different toxin concentrations, binding and incubation times were used. This reinforces the conclusion about the preventing activity of the four drugs used here.

We have no a direct explanation for the much higher potency of BoNT/D in VAMP2 cleavage with respect to BoNT/B, but it could be ascribed to a faster entry into the cytosol of BoNT/D than BoNT/B, which was reported to be particularly slow in cell cultures [44]. This may also explain why, when TrxR is not completely inhibited as in the case of lower concentrations of Curcumin, BoNT/D activity appears to be less blocked as compared to BoNT/B. Nevertheless, the comparable inhibition profile of these two BoNTs strongly indicates that the reduction of the interchain bond, catalyzed by the TrxR–Trx system, is of similar and essential importance to enable their intraneuronal catalytic activity. This conclusion is reinforced by the similar data obtained using BoNT/F and BoNT/G, whose inhibition profiles are reported in Fig. 5 and Fig. 6, respectively. Importantly, we achieved this result by using four different compounds, which belong to different chemical classes, have different molecular structures as well as different mechanisms to inhibit TrxR–Trx system.

3.2. Inhibitors of thioredoxin reductase and thioredoxin effectively lower the reversible flaccid paralysis induced by botulinum neurotoxins type B, D, F and G in mice

One remarkable aspect of BoNTs action *in vivo* is that they induce a reversible peripheral neuroparalysis. This property is clearly documented by the black traces in the panels of Fig. 7 which shows the recovery time course of mouse limb muscles function after the paralysis induced by a single local injection of BoNT/B, /D, /F and /G in sub-lethal doses. The neuromuscular paralytic effect was evaluated over time with the well-established DAS assay [42,43], which assigns a score to the severity of muscles paralysis according to the capability of the mouse to move the hind limb fingers, upon injection of BoNTs close to the EDL (*Extensor Digitoris Longus*) muscle. This score ranges from 0 (no paralysis) up to 4 (all fingers are paralyzed). Even though the paralytic effect exerted by the four BoNTs has different durations, it is worth noting that the local injection of the TrxR–Trx inhibitors effectively reduced both the severity and the duration of the paralysis. Owing to the very large number of sampling required by this type of analysis, we used three different inhibitors to dissect the entire TrxR–Trx system: Curcumin (inhibitor of TrxR), PX12 (inhibitor of Trx), and Ebselen (inhibitor of TrxR and Trx). All of them very effectively prevented the peripheral neuroparalysis induced by the four different BoNTs, with Ebselen being slightly more potent (Fig. 7).

3.3. Ebselen effectively prevents mice death caused by botulinum neurotoxins type B, D and F

On the basis of the results reported above, it became very relevant to test the capacity of the TrxR–Trx inhibitors in preventing the development of botulism upon systemic delivery of the four neurotoxins. Since such tests would have required a very large number of animals, we confined the experiments to one of the drugs. Ebselen was chosen because it best protected against paralysis in the DAS assay and because it has been used in clinical

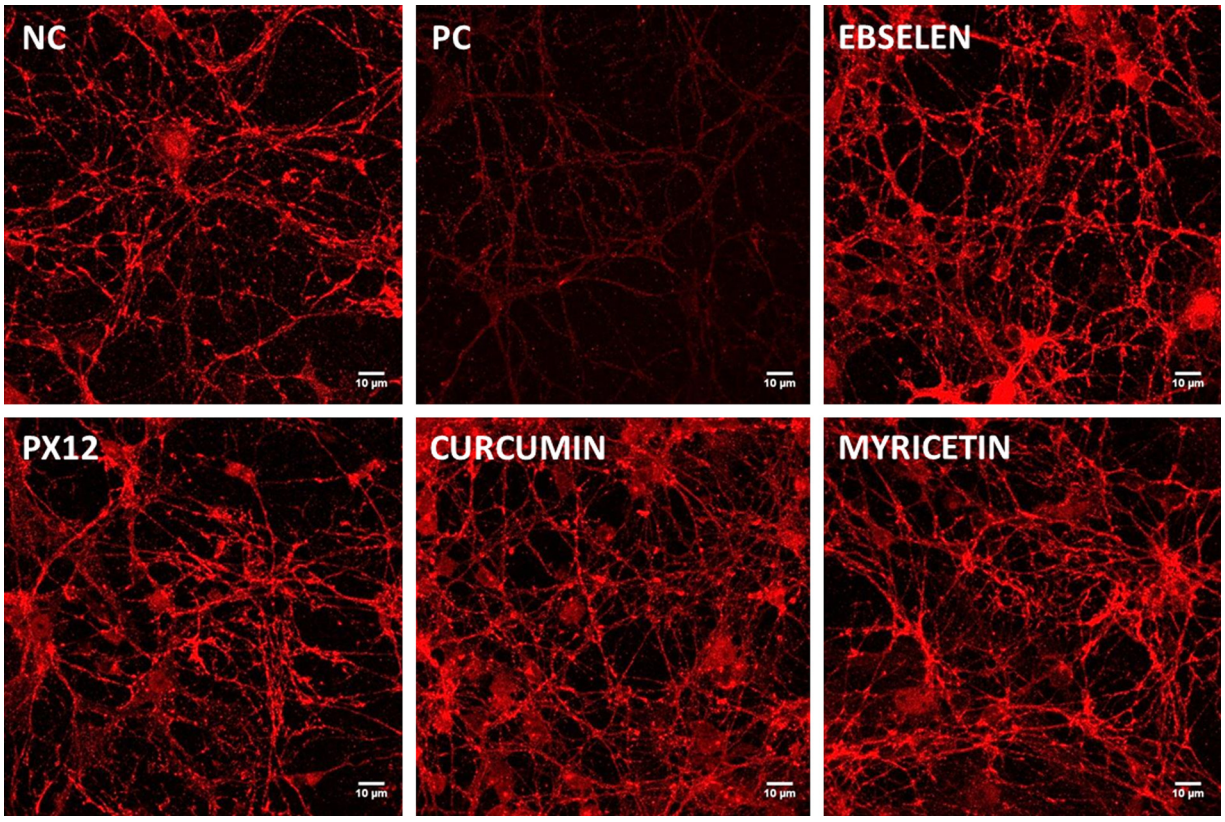

Fig. 4. Immunocytochemical evaluation of BoNT/D activity blockage by Inhibitors of thioredoxin–thioredoxin reductase system. CGNs were treated with Trx–TrxR inhibitors (Curcumin 100 μM, Ebselen 30 μM, Myricetin 100 μM or PX12 25 μM) or vehicle (NC, no toxin added; PC, only toxin added) at 37 °C. After 30 min, BoNT/D 0.2 nM was added for 10 min at 4 °C after which neurons were washed and incubated with the same concentration of inhibitors for further 90 min at 37 °C. Thereafter neurons were fixed and VAMP2 cleavage was assessed using a specific antibody. The images are representative of three independent sets of experiments (scale bar 10 μm).

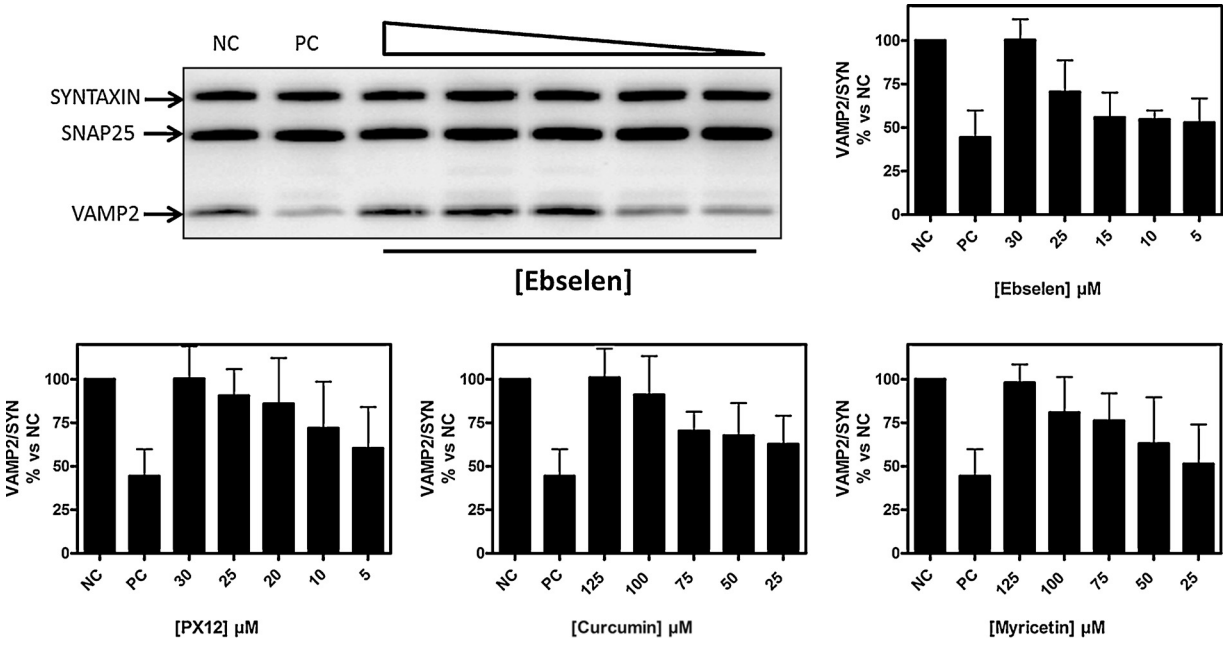

Fig. 5. Thioredoxin–thioredoxin reductase inhibitors prevent the BoNT/F-induced cleavage of VAMP2 in in neuronal culture. CGNs were incubated with the indicated concentrations of inhibitor at 37 °C for 30 min. 4 nM BoNT/F was then added and incubation prolonged for 12 h at 37 °C, cells were then lysed and the VAMP2 content was estimated with an antibody recognizing the intact form of VAMP2. Syntaxin and SNAP25 staining was used as loading control. Upper left panel shows a representative immunoblot, obtained in experiments with Ebselen (NC, no toxin added; PC, only toxin added). Graphs show the quantification, of the experiments performed with the reported inhibitors, for VAMP2 determined as a ratio to Syntaxin staining, taking the value of non-treated cells as 100%. SD values derive from at least three independent experiments.

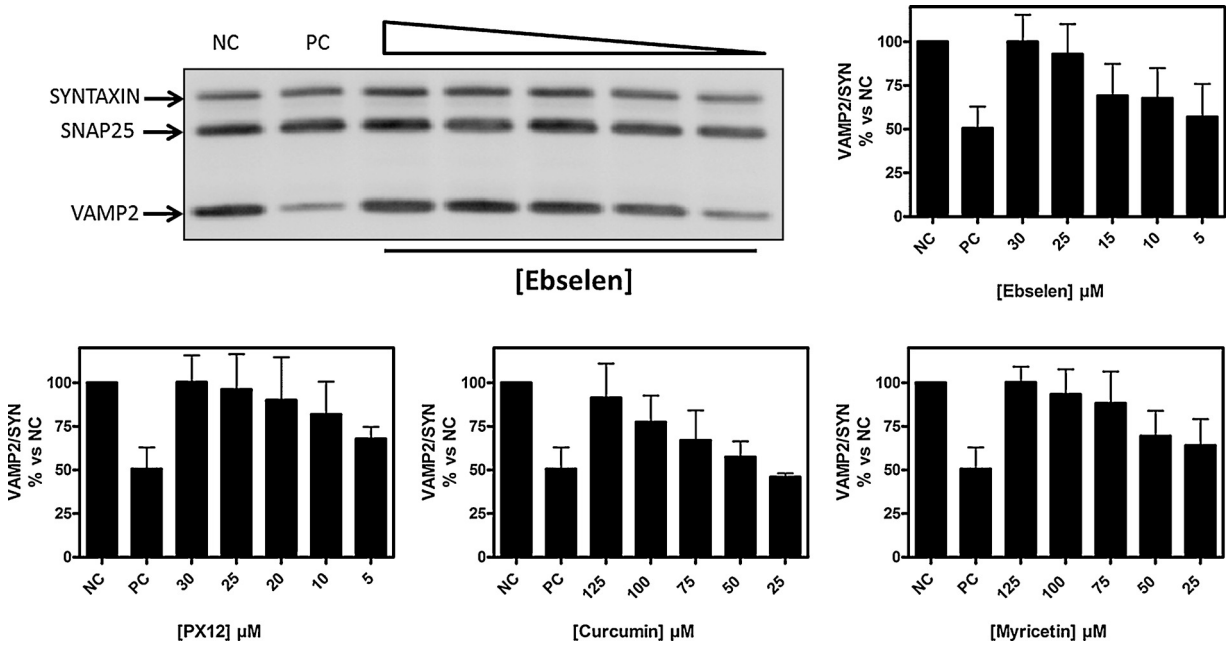

Fig. 6. Thioredoxin–thioredoxin reductase inhibitors prevent the BoNT/G-induced cleavage of VAMP2 in neuronal culture. CGNs were incubated with the indicated concentrations of inhibitor at 37 °C for 30 min. 4 nM BoNT/G was then added and incubation prolonged for 12 h at 37 °C, cells were then lysed and the VAMP2 content was estimated with an antibody recognizing the intact form of VAMP2. Syntaxin and SNAP25 staining was used as loading control. Upper left panel shows a representative immunoblot, obtained in experiments with Ebselen (NC, no toxin added; PC, only toxin added). Graphs show the quantification, of the experiments performed with the reported inhibitors, for VAMP2 determined as a ratio to Syntaxin staining, taking the value of non-treated cells as 100%. SD values derive from at least three independent experiments.

trials in humans for other diseases [46,47]. In addition, we focused our attention on BoNT/B and BoNT/F, because these serotypes are involved in human botulism and BoNT/D because it is often associated to animal botulism [2,3]. Type G was not tested because

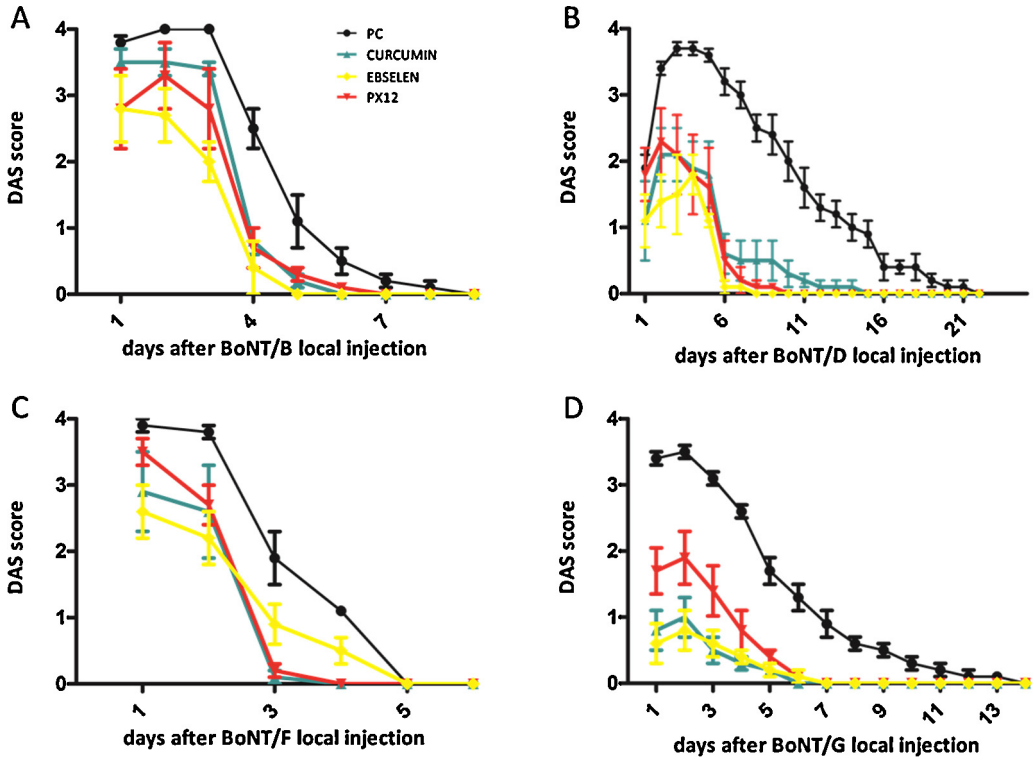

Fig. 7. Thioredoxin–thioredoxin reductase inhibitors decrease the local paralysis induced by BoNT/B, /D, /F and G. 0.02 mg of Curcumin (green traces) or PX12 (red traces) or Ebselen (yellow traces) or vehicle (PC, black traces) were injected in the hind limb of adult male CD1 mice. After 30 min the same hind limbs were injected with 0.5 pg/g of BoNT/B (A) or 0.2 pg/g of BoNT/D (B) or 2 pg/g of BoNT/F (C) or 5 pg/g of BoNT/G (D) and the severity of local paralysis was evaluated and reported as DAS score (see Section 2). DAS score of animals treated with only inhibitors are not shown for clarity. DAS values are means ± SEM from three individual experiments of at least eight animals per condition.

it has not been reported to cause botulism in humans or animals. Fig. 8 shows that Ebselen substantially protects mice from intraperitoneally injected BoNT/B, or BoNT/D or BoNT/F. Noteworthy, all mice treated displayed the symptoms typical of botulism, such as a generalized flaccid paralysis of muscles, but Ebselen-treated animals displayed milder symptoms and in several cases they were not severe enough to cause the respiratory failure or to prevent drinking and feeding that generally contribute to death of injected mice in the animal cage. Importantly, survivors fully recovered from developed symptoms in few days, suggesting that large amounts of the injected toxin did not reached VAMP.

4. Discussion

The results presented here are very relevant in several respects. The first one is that they provide experimental evidence that thioredoxin reductase-thioredoxin disulfide reducing system cleaves the single interchain disulfide bond of the four BoNT serotypes whose L chain proteolyses VAMP: BoNT/B, /D, /F and /G. Together with our previous reports on the SNAP25 and syntaxin cleaving BoNTs [28,36], it can now be concluded that this molecular event, which takes place on the cytosolic face of an intracellular acidic compartment [20], is an essential step of the cellular mechanism of intoxication of all BoNT serotypes. So essential, that it is sufficient to inhibit the TrxR–Trx redox system to completely prevent the toxicity of these very powerful neurotoxins in cultured neurons. This conclusion is even more important if one considers the large number of novel BoNTs that are being discovered [48,49]. They can be classified as subtypes of the main seven serotypes, which have now all been analyzed with respect to disulfide reduction (the present paper and [28,36]). Therefore, it can be concluded that the release in the cytosol of the L chain metalloprotease activity of all clostridial neurotoxins requires reduction of the interchain disulfide bridge by the TrxR–Trx system.

An important feature of BoNTs is their reversibility of action. This remarkable property has been exploited to evaluate the respective potency and duration of the different serotypes *in vivo* [50,51] through the DAS assay. This test is based on the intramuscular injection of a limited amount of BoNTs which can induce the local paralysis of mice hind legs without causing their death [42,43]. This is facilitated by a very limited diffusion from the site of injection, a feature which becomes very relevant in the therapeutic use of BoNTs, particularly when small muscles are injected [52,53]. Given that the intracellular degradation of the L chain seems to be the main reason of BoNTs reversibility of action, the duration of paralysis primarily depends on the amount L chains which have entered the nerve terminals, beside the intrinsic properties of the different L chains may also play a significant role [27,54,55]. Therefore, the second valuable result described here is that inhibitors of the TrxR–Trx system are general inhibitors of all BoNTs and are capable of preventing to a large extent their local neuroparalytic action in mice by reducing the number of L chains which have entered the nerve terminals. Accordingly, this result indicates the possible employment of these inhibitors in accidental events of over dosage of a BoNT during its therapeutic use.

We also assayed the efficacy of TrxR–Trx inhibitors in protecting mice from a systemic injection of BoNTs. This assay better recapitulates botulism, i.e., a generalized peripheral neuroparalysis which generally develops following the toxin absorption from a large organ such as the intestine (alimentary botulism) [2]. Botulism is reversible, provided that the intoxicated patient is mechanically ventilated to prevent death by respiratory muscles paralysis. Because of local regulations on experimentation involving animals, we could not assay the efficacy of all the inhibitors tested in neuronal cultures and capable of preventing local neuroparalysis (DAS assay), in mice lethality tests. However, using Ebselen we have provided a proof of principle that inhibitors of the TrxR–Trx system can effectively prevent the development of the flaccid paralysis caused by BoNT and protect a sizeable fraction of animals from deadly effects. Notably, the survivors recovered completely. This result is very relevant as it suggests that Ebselen, and probably the other TrxR–Trx inhibitors, can be used to prevent botulism in humans and in animals. This is also valid in the case of BoNT/D which, in our hands, is the most toxic of all BoNTs in mice with a MLD_{50} of 0.02 ng/kg to be compared with the literature data of 0.4 ng/kg [56].

On the basis of the present knowledge about the BoNTs mechanism of neuron intoxication, it is clear that once the reduction of the interchain disulfide bond has released the L chain metalloprotease activity, the inhibitors tested here are no longer effective. In other words, these inhibitors cannot be considered for the use after the symptoms of botulism have developed. Therefore the drugs tested here are to be considered as prophylactic and the limitation of a prophylaxis has been discussed before [57]. Notwithstanding, if given soon after diagnosis, these inhibitors may lessen symptoms severity by preventing the entry of circulating BoNT, and therefore shorten the period of hospitalization which is associated with the high costs of intensive care. It is indeed known that in adult botulism caused by the ingestion of BoNT poisoned food, there is a long persistence of the toxin in the general circulation [58,59]. Moreover, having a good record of safety in humans, as deduced from previous trials [60–65], these drugs may have a great potential in the treatment of human botulism where a continuous release of freshly produced BoNT takes place, which is the case of infant botulism [66]. In this form of the disease, the BoNT producing Clostridia colonize the intestine,

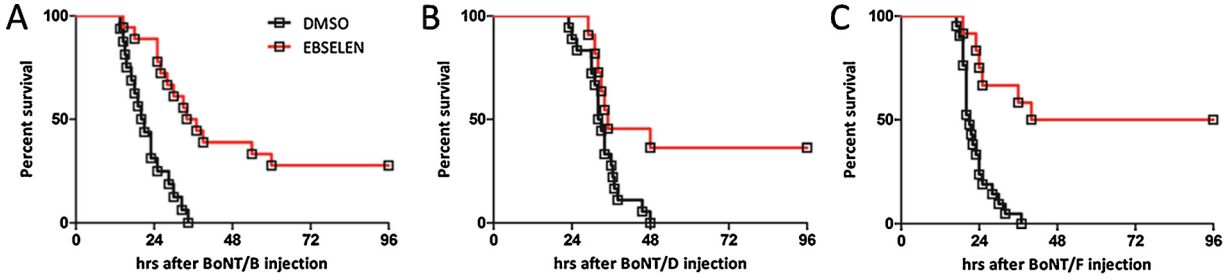

Fig. 8. Ebselen prevents the lethality of BoNT/B, /D and /F. Adult male CD1 mice were preconditioned with Ebselen 7.5 mg/kg ($n = 15$) or vehicle ($n = 15$) as described in Section 2. Thereafter, 2xMLD_{50} of BoNT/B (A) or BoNT/D (B) or BoNT/F (C) were injected i.p. Animals were monitored every 4 h for 96 h, after which the experiment was considered concluded. Graphs report the survival curves analyzed with the Log-rank (Mantel–Cox) test, and found to be significantly different (BoNT/B: $p < 0.0001$; BoNT/D: $p < 0.0058$; BoNT/F: $p < 0.0022$) from inhibitor free controls.

owing to the lack of competitive bacterial flora, and release the toxin in the general circulation for long periods of time [2,67].

There is growing interest in finding new mechanism-based antidotes against BoNTs, and some molecules were found to have beneficial potential (Fischer, Nakai et al., 2009). The main advantage of such molecules is that they act regardless of the serotypes causing envenomation. This is the more important in light of the large number of different BoNTs that are being discovered. These drugs can be administered without knowing the BoNT serotype and sub-type, thus saving the time required for toxin characterization. This is also relevant to those cases of botulism caused by Clostridia producing more than one BoNT (Barash and Arnon 2004, Barash and Arnon 2013, Dover, Barash et al., 2013, Maslanka, Lúquez et al., 2015).

Authors contributions

(1) Study conception and design: M.P. and C.M.
(2) Acquisition, analysis and/or interpretation of data: G.Z., D.A.T., M.P. and C.M.
(3) Drafting/revision of the work for intellectual content and context: M.P., G.Z., D.A.T., O.R., T.B. and C.M.
(4) Final approval and overall responsibility for the published work: G.Z., D.A.T., M.P., T.B., C.C.S., S.F., F.L., O.R. and C.M.

Conflict of interests

The authors declare that they have no conflict of interests.

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References

[1] K.K. Hill, T.J. Smith, Genetic diversity within *Clostridium botulinum* serotypes, botulinum neurotoxin gene clusters and toxin subtypes, Curr. Top. Microbiol. Immunol. 364 (2013) 1–20.
[2] O. Rossetto, M. Pirazzini, C. Montecucco, Botulinum neurotoxins: genetic, structural and mechanistic insights, Nat. Rev. Microbiol. 12 (2014) 535–549.
[3] E.A. Johnson, C. Montecucco, Botulism, Handb. Clin. Neurol. 91 (2008) 333–368.
[4] J.A. Coffield, N. Bakry, R.D. Zhang, J. Carlson, L.G. Gomella, L.L. Simpson, In vitro characterization of botulinum toxin types A, C and D action on human tissues: combined electrophysiologic, pharmacologic and molecular biologic approaches, J. Pharmacol. Exp. Ther. 280 (1997) 1489–1498.
[5] R. Eleopra, C. Montecucco, G. Devigili, C. Lettieri, S. Rinaldo, L. Verriello, et al., Botulinum neurotoxin serotype D is poorly effective in humans: an in vivo electrophysiological study, Clin. Neurophysiol. 124 (2013) 999–1004.
[6] S. Pellett, W.H. Tepp, J.M. Scherf, C.L. Pier, E.A. Johnson, Activity of botulinum neurotoxin type D (strain 1873) in human neurons, Toxicon 101 (2015) 63–69.
[7] S.S. Arnon, R. Schechter, T.V. Inglesby, D.A. Henderson, J.G. Bartlett, M.S. Ascher, et al., Botulinum toxin as a biological weapon: medical and public health management, JAMA 285 (2001) 1059–1070.
[8] Centers for Disease Control and Prevention DoHaHS, Possession, use, and transfer of select agents and toxins; biennial review. Final rule, Fed. Regist. 77 (2012) 61083–61115.
[9] M. Montal, Botulinum neurotoxin: a marvel of protein design, Annu. Rev. Biochem. 79 (2010) 591–617.
[10] J.O. Dolly, J. Black, R.S. Williams, J. Melling, Acceptors for botulinum neurotoxin reside on motor nerve terminals and mediate its internalization, Nature 307 (1984) 457–460.
[11] A. Rummel, Double receptor anchorage of botulinum neurotoxins accounts for their exquisite neurospecificity, Curr. Top. Microbiol. Immunol. 364 (2013) 61–90.
[12] R.A. Kammerer, R.M. Benoit, Botulinum neurotoxins: new questions arising from structural biology, Trends Biochem. Sci. 39 (2014) 517–526.
[13] C. Colasante, O. Rossetto, L. Morbiato, M. Pirazzini, J. Molgo, C. Montecucco, Botulinum neurotoxin type A is internalized and translocated from small synaptic vesicles at the neuromuscular junction, Mol. Neurobiol. 48 (2013) 120–127.
[14] C.B. Harper, S. Martin, T.H. Nguyen, S.J. Daniels, N.A. Lavidis, M.R. Popoff, et al., Dynamin inhibition blocks botulinum neurotoxin type A endocytosis in neurons and delays botulism, J. Biol. Chem. 286 (2011) 35966–35976.
[15] L.L. Simpson, Ammonium chloride and methylamine hydrochloride antagonize clostridial neurotoxins, J. Pharmacol. Exp. Ther. 225 (1983) 546–552.
[16] G. Schiavo, M. Matteoli, C. Montecucco, Neurotoxins affecting neuroexocytosis, Physiol. Rev. 80 (2000) 717–766.
[17] A. Fischer, M. Montal, Molecular dissection of botulinum neurotoxin reveals interdomain chaperone function, Toxicon 75 (2013) 101–107.
[18] M. Pirazzini, D.A. Tehran, O. Leka, G. Zanetti, O. Rossetto, C. Montecucco, On the translocation of botulinum and tetanus neurotoxins across the membrane of acidic intracellular compartments, Biochim. Biophys. Acta (BBA) (2015) , doi: http://dx.doi.org/10.1016/j.bbamem.2015.08.014.
[19] M. Pirazzini, O. Rossetto, P. Bolognese, C.C. Shone, C. Montecucco, 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. 13 (2011) 1731–1743.
[20] A. Fischer, M. Montal, Crucial role of the disulfide bridge between botulinum neurotoxin light and heavy chains in protease translocation across membranes, J. Biol. Chem. 282 (2007) 29604–29611 Epub 2007 July 31.
[21] A. de Paiva, B. Poulain, G.W. Lawrence, C.C. Shone, L. Tauc, J.O. Dolly, A role for the interchain disulfide or its participating thiols in the internalization of botulinum neurotoxin A revealed by a toxin derivative that binds to ecto-acceptors and inhibits transmitter release intracellularly, J. Biol. Chem. 268 (1993) 20838–20844.
[22] G. Schiavo, E. Papini, G. Genna, C. Montecucco, An intact interchain disulfide bond is required for the neurotoxicity of tetanus toxin, Infect. Immun. 58 (1990) 4136–4141.
[23] A. Kistner, D. Sanders, E. Habermann, Disulfide formation in reduced tetanus toxin by thioredoxin: the pharmacological role of interchain covalent and noncovalent bonds, Toxicon 31 (1993) 1423–1434.
[24] G. Schiavo, F. Benfenati, B. Poulain, O. Rossetto, P. Polverino de Laureto, B.R. DasGupta, et al., Tetanus and botulinum-B neurotoxins block neurotransmitter release by proteolytic cleavage of synaptobrevin, Nature 359 (1992) 832–835.
[25] G. Schiavo, O. Rossetto, S. Catsicas, P. Polverino de Laureto, B.R. DasGupta, F. Benfenati, et al., Identification of the nerve terminal targets of botulinum neurotoxin serotypes A, D, and E, J. Biol. Chem. 268 (1993) 23784–23787.
[26] T. Binz, Clostridial neurotoxin light chains: devices for SNARE cleavage mediated blockade of neurotransmission, Curr. Top. Microbiol. Immunol. 364 (2013) 139–157.
[27] S. Pantano, C. Montecucco, The blockade of the neurotransmitter release apparatus by botulinum neurotoxins, Cell. Mol. Life Sci. 71 (2014) 793–811.
[28] M. Pirazzini, T. Azarnia, D. ehran, G. Zanetti, A. Megighian, M. Scorzeto, S. Fillo, et al., Thioredoxin and its reductase are present on synaptic vesicles, and their inhibition prevents the paralysis induced by botulinum neurotoxins, Cell Rep. 8 (2014) 1870–1878.
[29] M. Montal, Redox regulation of botulinum neurotoxin toxicity: therapeutic implications, Trends Mol. Med. 20 (2014) 602–603.
[30] T. Binz, S. Sikorra, S. Mahrhold, Clostridial neurotoxins: mechanism of SNARE cleavage and outlook on potential substrate specificity reengineering, Toxins 2 (2010) 665–682.
[31] J. Lu, L.V. Papp, J. Fang, S. Rodriguez-Nieto, B. Zhivotovsky, A. Holmgren, Inhibition of mammalian thioredoxin reductase by some flavonoids: implications for myricetin and quercetin anticancer activity, Cancer Res. 66 (2006) 4410–4418.
[32] J. Fang, J. Lu, A. Holmgren, Thioredoxin reductase is irreversibly modified by curcumin: a novel molecular mechanism for its anticancer activity, J. Biol. Chem. 280 (2005) 25284–25290.
[33] D.L. Kirkpatrick, M. Kuperus, M. Dowdeswell, N. Potier, L.J. Donald, M. Kunkel, et al., Mechanisms of inhibition of the thioredoxin growth factor system by antitumor 2-imidazolyl disulfides, Biochem. Pharmacol. 55 (1998) 987–994.
[34] A. Mukherjee, S.G. Martin, The thioredoxin system: a key target in tumour and endothelial cells, Br. J. Radiol. 81 (2008) Spec No 1:S57–68.
[35] R. Zhao, H. Masayasu, A. Holmgren, Ebselen a substrate for human thioredoxin reductase strongly stimulating its hydroperoxide reductase activity and a superfast thioredoxin oxidant, Proc. Natl. Acad. Sci. U. S. A. 99 (2002) 8579–8584.
[36] M. Pirazzini, F. Bordin, O. Rossetto, C.C. Shone, T. Binz, C. Montecucco, The thioredoxin reductase-thioredoxin system is involved in the entry of tetanus and botulinum neurotoxins in the cytosol of nerve terminals, FEBS Lett. 587 (2013) 150–155.
[37] A. Rummel, S. Mahrhold, H. Bigalke, T. Binz, The HCC-domain of botulinum neurotoxins A and B exhibits a singular ganglioside binding site displaying serotype specific carbohydrate interaction, Mol. Microbiol. 51 (2004) 631–643.
[38] A. Rummel, T. Karnath, T. Henke, H. Bigalke, T. Binz, Synaptotagmins I and II act as nerve cell receptors for botulinum neurotoxin G, J. Biol. Chem. 279 (2004) 30865–30870 Epub 2004 April 30.
[39] S. Bade, A. Rummel, C. Reisinger, T. Karnath, G. Ahnert-Hilger, H. Bigalke, et al., Botulinum neurotoxin type D enables cytosolic delivery of enzymatically active cargo proteins to neurones via unfolded translocation intermediates, J. Neurochem. 91 (2004) 1461–1472.

[40] C.C. Shone, H.S. Tranter, Growth of clostridia and preparation of their neurotoxins, Curr. Top. Microbiol. Immunol. 195 (1995) 143–160.
[41] M. Rigoni, G. Schiavo, A.E. Weston, P. Caccin, F. Allegrini, M. Pennuto, et al., Snake presynaptic neurotoxins with phospholipase A2 activity induce punctate swellings of neurites and exocytosis of synaptic vesicles, J. Cell Sci. 117 (2004) 3561–3570.
[42] K.R. Aoki, A comparison of the safety margins of botulinum neurotoxin serotypes A, B, and F in mice, Toxicon 39 (2001) 1815–1820.
[43] R.S. Broide, J. Rubino, G.S. Nicholson, M.C. Ardila, M.S. Brown, K.R. Aoki, et al., The rat digit abduction score (DAS) assay: a physiological model for assessing botulinum neurotoxin-induced skeletal muscle paralysis, Toxicon 71 (2013) 18–24.
[44] S. Sun, W.H. Tepp, E.A. Johnson, E.R. Chapman, Botulinum neurotoxins B and E translocate at different rates and exhibit divergent responses to GT1b and low pH, Biochemistry 51 (2012) 5655–5662.
[45] S. Sun, S. Suresh, H. Liu, W.H. Tepp, E.A. Johnson, J.M. Edwardson, et al., Receptor binding enables botulinum neurotoxin B to sense low pH for translocation channel assembly, Cell Host Microbe 10 (2011) 237–247.
[46] T. Yamaguchi, K. Sano, K. Takakura, I. Saito, Y. Shinohara, T. Asano, et al., Ebselen in acute ischemic stroke: a placebo-controlled, double-blind clinical trial: Ebselen study group, Stroke 29 (1998) 12–17.
[47] I. Saito, T. Asano, K. Sano, K. Takakura, H. Abe, T. Yoshimoto, et al., Neuroprotective effect of an antioxidant, ebselen, in patients with delayed neurological deficits after aneurysmal subarachnoid hemorrhage, Neurosurgery 42 (1998) 269–277.
[48] C. Montecucco, M.B. Rasotto, On botulinum neurotoxin variability, MBio (2015) 2015.
[49] T.J. Smith, K.K. Hill, B.H. Raphael, Historical and current perspectives on *Clostridium botulinum* diversity, Res. Microbiol. 166 (2015) 290–302.
[50] Y. Torii, Y. Goto, M. Takahashi, S. Ishida, T. Harakawa, T. Sakamoto, et al., Quantitative determination of biological activity of botulinum toxins utilizing compound muscle action potentials (CMAP), and comparison of neuromuscular transmission blockage and muscle flaccidity among toxins, Toxicon 55 (2010) 407–414.
[51] F.r.A. Meunier, G. Lisk, D. Sesardic, J.O. Dolly, Dynamics of motor nerve terminal remodeling unveiled using SNARE-cleaving botulinum toxins: the extent and duration are dictated by the sites of SNAP-25 truncation, Mol. Cell. Neurosci. 22 (2003) 454–466.
[52] L. Carli, C. Montecucco, O. Rossetto, Assay of diffusion of different botulinum neurotoxin type a formulations injected in the mouse leg, Muscle Nerve 40 (2009) 374–380.
[53] R. Eleopra, V. Tugnoli, R. Quatrale, O. Rossetto, C. Montecucco, Different types of botulinum toxin in humans, Mov. Disord. 19 (Suppl. 8) (2004) S53–S59.
[54] S. Pellett, W.H. Tepp, R.C.M. Whitemarsh, M. Bradshaw, E.A. Johnson, In vivo onset and duration of action varies for botulinum neurotoxin A subtypes 1–5, Toxicon (2015) , doi:http://dx.doi.org/10.1016/j.toxicon.2015.06.021.
[55] C.B. Shoemaker, G.A. Oyler, Persistence of Botulinum neurotoxin inactivation of nerve function, Curr. Top. Microbiol. Immunol. 364 (2013) 179–196.
[56] D.M. Gill, Bacterial toxins: a table of lethal amounts, Microbiol. Rev. 46 (1982) 86–94.
[57] B. Thyagarajan, N. Krivitskaya, J.G. Potian, K. Hognason, C.C. Garcia, J.J. McArdle, Capsaicin protects mouse neuromuscular junctions from the neuroparalytic effects of botulinum neurotoxin a, J. Pharmacol. Exp. Ther. 331 (2009) 361–371.
[58] R.P. Fagan, J.B. McLaughlin, J.P. Middaugh, Persistence of botulinum toxin in patients' serum: Alaska, 1959–2007, J. Infect. Dis. 199 (2009) 1029–1031.
[59] A.N. Sheth, P. Wiersma, D. Atrubin, V. Dubey, D. Zink, G. Skinner, et al., International outbreak of severe botulism with prolonged toxemia caused by commercial carrot juice, Clin. Infect. Dis. 47 (2008) 1245–1251.
[60] A.F. Baker, K.N. Adab, N. Raghunand, H.H.S. Chow, S.P. Stratton, S.W. Squire, et al., A phase IB trial of 24-hour intravenous PX-12, a thioredoxin-1 inhibitor, in patients with advanced gastrointestinal cancers, Invest. New Drugs 31 (2013) 631–641.
[61] R.K. Ramanathan, J. Abbruzzese, T. Dragovich, L. Kirkpatrick, J.M. Guillen, A.F. Baker, et al., A randomized phase II study of PX-12, an inhibitor of thioredoxin in patients with advanced cancer of the pancreas following progression after a gemcitabine-containing combination, Cancer Chemother. Pharmacol. 67 (2011) 503–509.
[62] M.J. Parnham, H. Sies, The early research and development of ebselen, Biochem. Pharmacol. 86 (2013) 1248–1253.
[63] M. Parnham, H. Sies, Ebselen prospective therapy for cerebral ischaemia, Expert Opin. Investig. Drugs 9 (2000) 607–619.
[64] A. Goel, A.B. Kunnumakkara, B.B. Aggarwal, Curcumin as Curecumin: from kitchen to clinic, Biochem. Pharmacol. 75 (2008) 787–809.
[65] E. Middleton, C. Kandaswami, T.C. Theoharides, The effects of plant flavonoids on mammalian cells:implications for inflammation, heart disease, and cancer, Pharmacol. Rev. 52 (2000) 673–751.
[66] R. Koepke, J. Sobel, S.S. Arnon, Global occurrence of infant botulism, 1976–2006, Pediatrics 122 (2008) e73–e82.
[67] S.S. Arnon, Infant botulism, Annu. Rev. Med. 31 (1980) 541–560.

A Novel Inhibitor Prevents the Peripheral Neuroparalysis of Botulinum Neurotoxin

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A Novel Inhibitor Prevents the Peripheral Neuroparalysis of Botulinum Neurotoxins

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Botulinum neurotoxins (BoNTs) form a large class of potent and deadly neurotoxins. Given their growing number, it is of paramount importance to discover novel inhibitors targeting common steps of their intoxication process. Recently, EGA was shown to inhibit the action of bacterial toxins and viruses exhibiting a pH-dependent translocation step in mammalian cells, by interfering with their entry route. As BoNTs act in the cytosol of nerve terminals, the entry into an appropriate compartment wherefrom they translocate the catalytic moiety is essential for toxicity. Herein we propose an optimized procedure to synthesize EGA and we show that, *in vitro*, it prevents the neurotoxicity of different BoNT serotypes by interfering with their trafficking. Furthermore, in mice, EGA mitigates botulism symptoms induced by BoNT/A and significantly decreases the lethality of BoNT/B and BoNT/D. This opens the possibility of using EGA as a lead compound to develop novel inhibitors of botulinum neurotoxins.

The most potent human poisons are the botulinum neurotoxins (BoNTs), which are neurospecific metalloproteases acting inside peripheral nerve terminals. They are synthesized by different species of Clostridia and have been grouped in seven serotypes (BoNT/A to/G) based on their immunological properties. All known BoNTs act by interrupting the release of neurotransmitter acetylcholine at peripheral cholinergic terminals causing a long lasting paralysis that may lead to death by respiratory failure[1]. Nonetheless, mechanically ventilated patients can fully recover in a time period which strongly depends on the toxin serotypes and on the amount of toxin molecules entered in the nerve terminals[2]. According to their extreme potency, and with the fact that they can be easily produced in large amounts, BoNTs are considered potential bioweapons[3,4]. On the other hand, due to their neurospecificity, reversibility and lack of diffusion from the site of injection, BoNT/A has worldwide become one of the safest therapeutics used for the treatment of a growing list of human syndromes, characterized by the hyperactivity of peripheral nerve terminals[5,6]. BoNTs consist of two polypeptide chains (L and H), kept together by a single disulphide bond. The overall structure can be subdivided in three 50 kDa domains which accomplish different tasks along the mechanism of neuron intoxication[2]. The L chain is the N-terminal domain endowed with metalloprotease activity. The C-terminal domain (HC) is responsible for the neurospecific binding to the presynaptic membrane of nerve endings, whilst the intermediate domain (HN) is involved in membrane translocation of L. The current view of BoNT mechanism of

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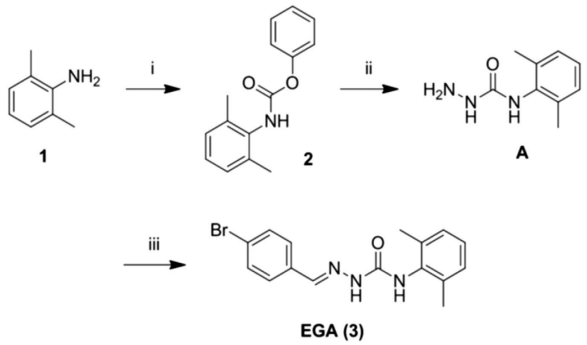

Figure 1. Synthesis of EGA. Synthesis of 2-(4-bromobenzylidene)-N-(2,6-dimethylphenyl) hydrazinecarboxamide (EGA); Reagents and conditions (i) $ClCO_2Ph$, Py, DCM (96% isolated yield); (ii) $NH_2NH_2{\cdot}H_2O$, DME (iii) 4-bromobenzaldehyde, $CHCl_3$ (88% isolated yield, passages ii and iii).

action envisages a first interaction with polysialogangliosides, which mediate the toxin binding to the plasma membrane. This is followed by lateral movements that make possible the encounter with a protein receptor which is the lumenal part of a synaptic vesicle (SV) protein[2,7,8]. The protein receptor has been identified as synaptotagmin I and II for BoNT/B,/DC and/G[8,9], and SV2 for BoNT/A,/E and F[8,10]; the protein receptor for the remaining serotypes remains to be established. This latter binding is preliminary to the internalization of the toxin-receptors complex inside an acidic intracellular compartment whose nature has been identified as SV only for tetanus neurotoxin and for BoNT/A[11–13]. Little is known on the nature of the endocytic vesicles/compartment used by the other serotypes, but considerable evidence indicate that the acidification of its lumen generally triggers a structural change of L and HN together with membrane lipids which ultimately leads to the translocation of the L chain into the cytosol[14–16]. This process is completed by the reduction of the interchain disulphide bond, on the cytosolic side of the acidic compartment performed by the thioredoxin reductase–thioredoxin system[17–20] . The released L metalloprotease specifically cleaves one of the three SNARE proteins thereby preventing the Ca^{2+} induced release of the neurotransmitter contained inside SVs[21,22]. Many novel BoNTs have been recently discovered and their sequences are present in databases, but many more have not yet been deposited. All known novel BoNTs are classified as subtypes, and indicated with an Arabic number added to the parental serotype (e.g. A2, A3 etc., when their amino acid sequences differ by more than 2.4% from the parental serotype A1)[2], or as mosaic BoNTs, and indicated with a double capital letter, e.g. BoNT/DC,/CD,/FA, when they are chimeras of the different serotypes. Due to their different origin, BoNT variants exhibit different antigenicity and are neutralized to a different degree by existing serotype specific antisera[23,24]. Accordingly, it is possible that the therapy with humanized monoclonal antibodies raised versus a BoNT subtype may not neutralize variants of the same serotype[25,26]. This situation calls for increased efforts in the identification of inhibitors effective in preventing the neuroparalytic action of BoNTs irrespectively of their serotype and subtype which could be used without knowing the particular type of BoNT involved. Recently, Gillespie *et al.* (2013), performing a high-throughput screening, identified 4-bromobenzaldehyde N-(2,6-dimethylphenyl)semicarbazone (abbreviated as EGA) as an inhibitor of pathogens that enter cells via intracellular acid compartments[27]. Since BoNTs toxicity is also strictly dependent on the passage through an acidic environment[2], we decided to test the activity of EGA on BoNT action in the light of the urgency and importance to find inhibitors capable of interfering with the large and still growing number of BoNTs with undefined immunological properties. Here, we focused our attention on BoNT/A and BoNT/B because most frequently associated with human botulism and used in human therapy[1,2]. We also considered BoNT/D, which scarcely affects humans[28], but it is very frequently involved in animal botulism. Here we show that EGA drastically hinders BoNTs activity on neuronal cultures, without interfering with specific steps of their cellular mechanism of intoxication. More importantly, this compound is also very effective in reducing neurotoxicity *in vivo*. Together, our results suggest that EGA represents a new tool for studying BoNTs trafficking and a good candidate for the development of new inhibitors. Notably, we also report an optimized procedure for the synthesis of EGA, which involves milder reaction conditions and provides much higher overall yield than previously reported[29].

Results

High yield synthesis of 4-bromobenzaldehyde N-(2,6-dimethylphenyl) semicarbazone (EGA). The reported approach by Jung in 2014 for the preparation of EGA has been adapted and improved to obtain higher yields. The synthesis involves the three steps reported in Fig. 1. In the first one (i), 2,6-dimethylaniline (1) is allowed to react with phenyl chloroformate to give the corresponding phenylcarbamate (2), which is next subjected to hydrazinolysis to give semicarbazide (indicated as A) (ii). The final step (iii) is the reaction of A with 4-bromobenzaldehyde to form the desired semicarbazone (3,

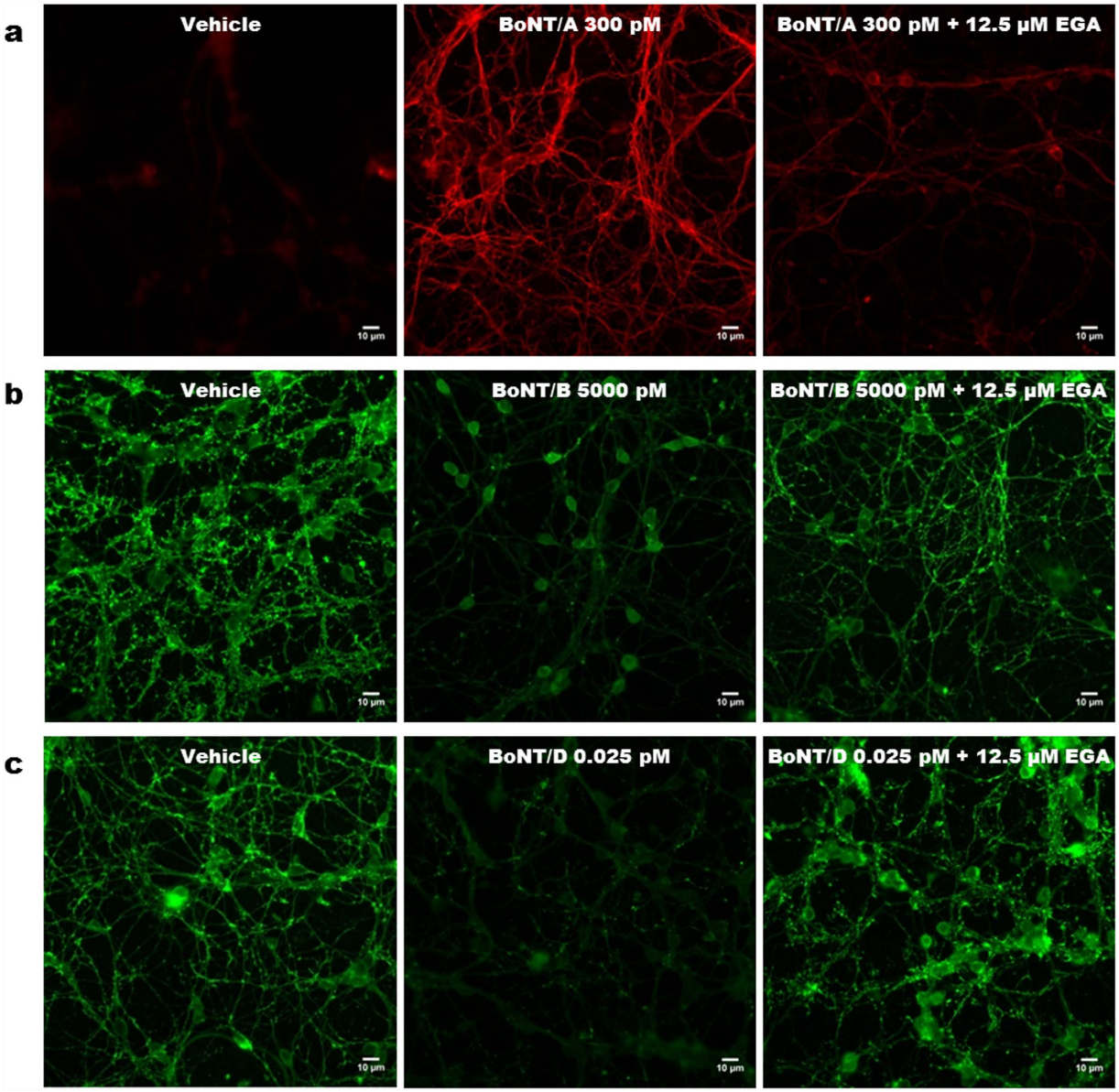

Figure 2. Immunocytochemical evaluation of EGA inhibition against different serotypes of BoNT in CGNs. (**a–c**) CGNs were treated with EGA 12.5 μM or vehicle (DMSO) at 37 °C for 30 min. Thereafter, the indicated amount of BoNT was added for 12 hrs. Samples were fixed and stained with an antibody specific for BoNT/A-cleaved SNAP25 (**a**) or intact VAMP2, (**b,c**). BoNT/A-cleaved SNAP25 was detected with an Alexa Fluor 555 goat anti-rabbit, while VAMP2 with an Alexa Fluor 488 goat anti-mouse. Images shown are representative of three independent experiments. Scale bar, 10 μm.

EGA). The procedure described by Jung *et al.* (2014) involves the isolation of A and rather drastic conditions (acidic solution and high temperature) in the last step, leading to an overall yield of 27%. A much higher overall yield (84%) is obtained by the new procedure that we have devised: 2 is isolated, whereas steps (ii) and (iii) are performed in one-pot without the isolation of A. Furthermore, much milder conditions are used in the last synthetic step. Details are found in the Supplementary information section.

EGA prevents the botulinum neurotoxins cleavage of SNARE proteins in cultured neurons. The use of cultured cerebellar granular neurons (CGNs) offers a simple and rapid way to screen the efficacy of candidate molecules in inhibiting BoNTs activity. The overnight incubation with 0.3 nM BoNT/A induces the cleavage of SNAP25, as assessed by the appearance in immunofluorescence (Fig. 2a, middle panel) and western blot (Fig. 3a, bottom panel) of its truncated form, revealed with a specific antibody. This toxin concentration is sufficient to induce a complete cleavage of its substrate, as evaluated using

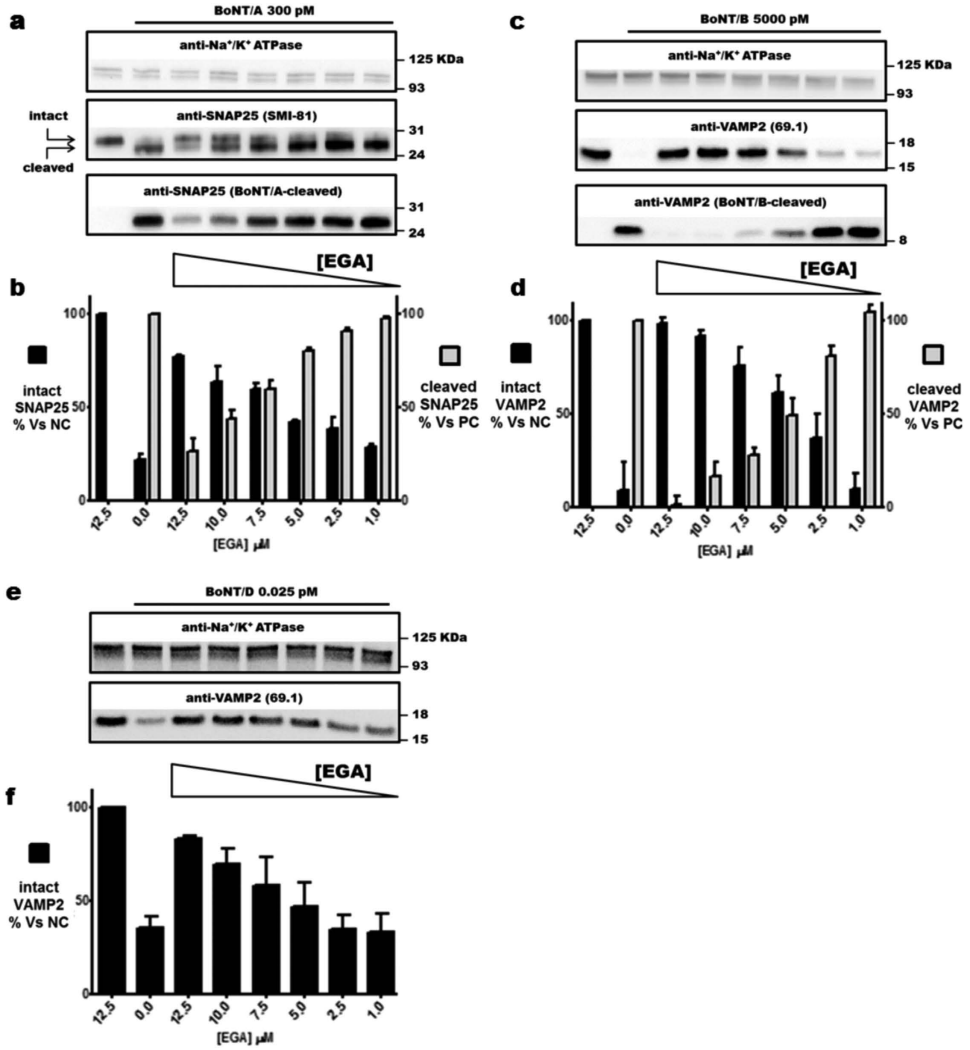

Figure 3. EGA interferes with the BoNT/A,/B and/D toxicity in CGNs. (**a,c,e**) CGNs were preincubated for 30 min with the indicated concentrations of EGA at 37 °C. Where indicated, BoNTs were added at the reported concentrations for 12 hrs. Then cells were lysed and the SNARE content was estimated with the indicated antibodies: (**a**) SMI81 recognizes both the full length and the cleaved form of SNAP25; BoNT/A-cleaved recognizes only BoNT/A-truncated SNAP25; (**c,e**) VAMP2 (69.1) recognizes the intact form of VAMP2 and, BoNT/B-cleaved recognizes only BoNT/B-cleaved VAMP2. In all experiments an antibody against the Na^+/K^+ ATPase antibody was used as loading control. Blots are representative of a typical experiment. (**b,d,f**) Densitometry analysis of western blots obtained in (**a,c**) and (**e**) respectively. All data are presented as mean values and error bars indicated the standard deviation obtained from at least three independent experiments.

an antibody recognizing both forms (intact and truncated) of SNAP25 (Fig. 3a, middle panel, SMI-81). Figure 3b shows that such activity is however inhibited by EGA in a concentration dependent manner, with a maximal effect at 12.5 μM. The inhibitory effect, though substantial, is not complete as a small amount of cleaved SNAP25 is still generated (Fig. 2a, right panel and Fig. 3b).

Figures 2b and 3c,d show that similar results are obtained with BoNT/B. Notably, to achieve the substantial cleavage of VAMP2, which is normally concentrated at synaptic contacts (Fig. 2b, left panel), BoNT/B had to be used at a concentration of 5 nM (Fig. 2b, middle panel). Nevertheless, the pre-treatment with 12.5 μM EGA is sufficient to abrogate its cleavage (Fig. 2b, right panel). EGA prevents this toxicity in a concentration dependent manner, as shown by the inhibition of VAMP2 cleavage, determined with two different antibodies (Fig. 3c middle and bottom panels). Interestingly, despite the high amount of toxin used, in this case the effect of EGA, at higher concentration, is complete (Fig. 3d).

The same set of experiments was replicated using BoNT/D. This serotype is the most potent in rodents[28] and, in CGNs, a minimal concentration (0.025 pM) induces the almost complete cleavage of VAMP2 (Fig. 2c, middle panel). Similar to what found for BoNT/A, we found that EGA substantially prevents the action of this potent neurotoxin (Fig. 2c, right panel), and this inhibition is dependent on

the amount of the chemical, as estimated in western blot with an antibody specific for the intact form of VAMP2 (Fig. 3e, bottom panel and Fig. 3f).

Importantly, Figure S1 shows that neurons viability is not significantly affected, even at the highest concentration of EGA used.

EGA does not interfere with the four basic steps of BoNTs' mechanism of action. The cellular target of EGA is not known and we investigated whether any of the four main steps of the BoNTs' cellular mechanism of action is directly impacted by the action of the drug.

The first step of intoxication is the specific binding of BoNTs to peripheral nerve endings followed by their internalization via endocytosis[2]. Given its chemical nature, EGA could in principle intercalate among lipids and alter the properties of the presynaptic membrane, making it less receptive for BoNTs binding. To investigate this possibility, we took advantage of two constructs consisting of the HC domain of BoNT/A and of BoNT/B fused to a fluorescent protein (cpV-HC/A) or tagged by a c-Myc epitope (c-Myc-HC/B), respectively. These chimeras fully maintain the capability of parental BoNTs to bind to the presynaptic membrane of neurons[8,30] and to become endocytosed[12,13]. We found that EGA, used at the concentration which displayed the maximum efficacy in protecting CGNs, does not interfere with the binding and the endocytosis of both BoNT/A and BoNT/B, as assessed by the internalization of their respective derivatives which show the same pattern regardless of drug presence (Fig. 4a and Figure S2a). Intriguingly, the two HCs displayed clearly different patterns of staining, suggesting that they may be internalized inside different compartments. Figure 4b and Figure S2b show via western blot analyses the quantitation of the results. We performed this experiment with higher concentrations of HCs to meet the sensitivity requirements of the antibodies used in Western Blot. Consistently, Fig. 4b show that the previous treatment of CGNs with BoNT/D, which cleaves VAMP1/2 thus impairing SVs recycling, significantly decreases the uptake of HC/A, as reported elsewhere[31,32]. At variance, the uptake of HC/B was only partially affected by VAMP1/2 cleavage (Figure S2b), leaving open the possibility of a different trafficking of BoNT/B with respect to BoNT/A.

Nevertheless, the fact that BoNT/A and BoNT/B use synaptic vesicle proteins as receptors (SV2A/B/C and Synaptotagmin I-II, respectively) strongly suggests that they exploit SVs for their initial step of endocytosis. Accordingly, we decided to test the possible interference of EGA with SVs dynamics using a well-established assay[33]. As shown in Figure S2c, EGA does not affect SVs endocytosis as an antibody specific for the lumenal domain of the synaptic vesicle marker Synaptotagmin I, is internalized at the same extent as controls. On the contrary, if neurons are previously treated with BoNT/D, the uptake of the same antibody is prevented. The quantitation of the result is shown in Figure S2d. Taken together, these results demonstrate that BoNTs binding and internalization through SV cycling are not perturbed by EGA.

The BoNT exposure to an intracellular acidic compartment is the next essential step for the neuron intoxication by all BoNTs[2]. Using Lysotracker Red DND-99, a highly sensitive probe of acidic organelles in live cells, we found that EGA does not significantly interfere with the maturation of acidic compartments, both within CGNs cell body and along neurites, where BoNTs act (Fig. 4c). At the same time, bafilomycin A1, which prevents BoNTs toxicity by inhibiting the vacuolar-type H^+-ATPase proton pump[12,34,35], completely blocks the acidification of intracellular organelles. This suggests that the essential conditions needed for BoNTs translocation, i.e. an acidic environment, are maintained in the presence of EGA.

The final step of the nerve intoxication, the one responsible for neuroparalysis, is the cleavage of SNARE proteins by the L chain. BoNT/A chops off the last 9 amino acids of SNAP25, whereas BoNT/B and BoNT/D cleave at two different sites VAMP1/2. This proteolytic activity can be easily assayed *in vitro* by using recombinant substrates. As shown in Fig. 4d (left panel), upon reduction of the interchain disulphide bond, BoNT/A cleaves SNAP25, as shown by the shift of its molecular weight in SDS-PAGE (compare lane 1 and 2, upper panels). This activity is however not affected by 12.5 μM EGA (Fig. 4d, compare lane 2 and 3). The same result was obtained using an antibody specific for the cleaved form of SNAP25 and western blotting as a read out (Fig. 4d). Figure S3a and Figure S3b show that similar results were obtained with BoNT/B and BoNT/D, respectively, suggesting that the enzymatic activity of BoNT L chains is not affected by the drug.

EGA interferes with BoNTs trafficking within neurons. Gillespie *et al.* (2013) reported that EGA prevents the toxicity of bacterial toxins and viruses by blocking their trafficking from early to late endosomes. Here, we have shown that EGA inhibits BoNTs without interfering with the main events along their mechanism of action. As a consequence, we reasoned that EGA could alter the trafficking of BoNTs after their internalization, possibly preventing them to reach their translocation-competent compartment. If it is the case, EGA should not be capable of inhibiting BoNTs toxicity when their trafficking is bypassed by inducing the entry of the L chain across the plasma membrane of neurons[36]. As this experimental approach strongly depends on the binding to both receptors at the plasma membrane[36], we could perform this experiment only with BoNT/B, using an established PC12 cell line expressing on the plasma membrane the lumenal domain of Synaptotagmin I, the BoNT/B protein receptor[9], and with BoNT/D, whose binding domain harbors two ganglioside binding sites[37], using CGNs[36]. Figure 5a,b show that a low pH jump in the extracellular medium induces the translocation of BoNT/B and BoNT/D L chains

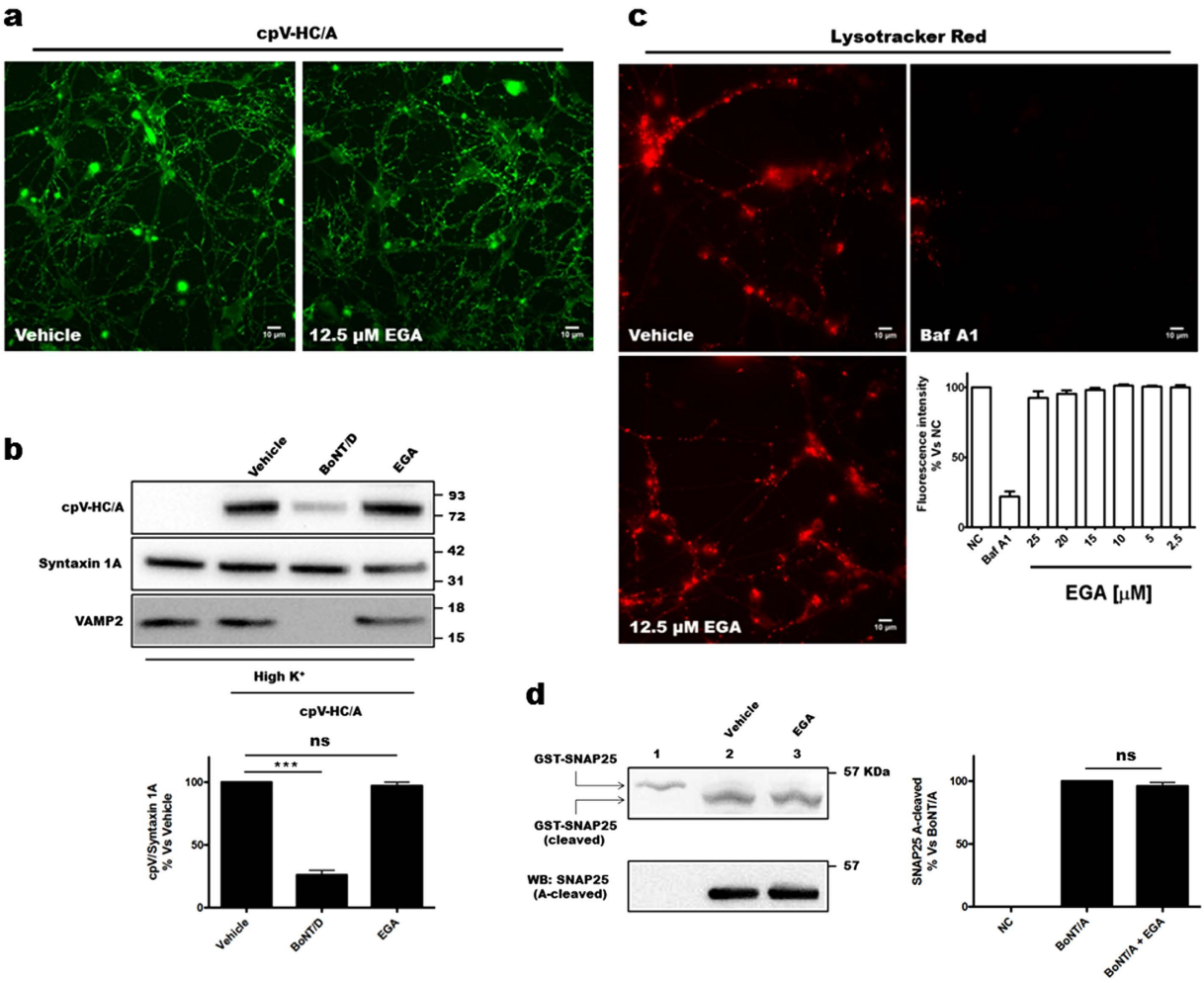

Figure 4. EGA does not inhibit the known steps of BoNT intoxication. (**a**) CGNs were treated with EGA (12.5 μM) or vehicle in culture medium at 37 °C. After 30 min, 100 nM cpV-HC/A was added in high K^+ buffer for 1 h. Neurons were then washed, fixed, and directly imaged. These images are representative of at least three independent sets of experiments. Scale bar, 10 μm. (**b**) CGNs were treated as in (**a**) with 250 nM cpV-HC/A and then lysed. The cpV-HC/A content was estimated with a GFP specific antibody. Syntaxin 1A serving as internal control was detected with a specific antibody and an anti-VAMP2 was used to assess BoNT/D cleavage. The amount of cpV-HC/A was determined as a ratio to Syntaxin 1A staining taking the value in non-treated cells (vehicle) as 100%. All data are presented as mean values and error bars indicated the standard deviation obtained from three independent experiments (***$p < 0.0001$; ns – non significant). (**c**) CGNs were treated with vehicle or 12.5 μM EGA or 10 nM Bafilomycin A1 for 30 min at 37 °C. Lysotracker Red was then added and the incubation prolonged for further 90 min. Cells were imaged by fluorescence microscopy. The graph shows the quantification of fluorescence intensity of acid compartments (% versus non-treated neurons) arising from CGNs treated with the indicated amount of EGA. Mean and standard deviation values refer to four different experiments. Scale bar, 10 μm. (**d**) 0.25 μg BoNT/A was reduced in the presence of 12.5 μM EGA for 30 min at 37 °C. 1 μg of GST-SNAP25 was added, the concentration of inhibitor was restored, and the reaction was carried out for 12 hrs at 37 °C. SNAP25 cleavage was assessed by SDS-PAGE and Coomassie staining (top-left panel) or immunoblotting (bottom-left panel) with an antibody specific for the BoNT/A-cleaved form of SNAP25. Lane 1 shows untreated GST-SNAP25. Right panel shows the densitometry analysis of western blots, tacking the value of BoNT/A without EGA (vehicle) as 100%. All data are presented as mean values and error bars indicated the standard deviation obtained from three independent experiments.

across the plasma membrane as evaluated by the cleavage of VAMP2. In agreement with our hypothesis, the same experiment performed in the presence of EGA showed the identical activity of both BoNTs on VAMP2. This suggests that, when these neurotoxins bypass their canonical entry routes, EGA cannot

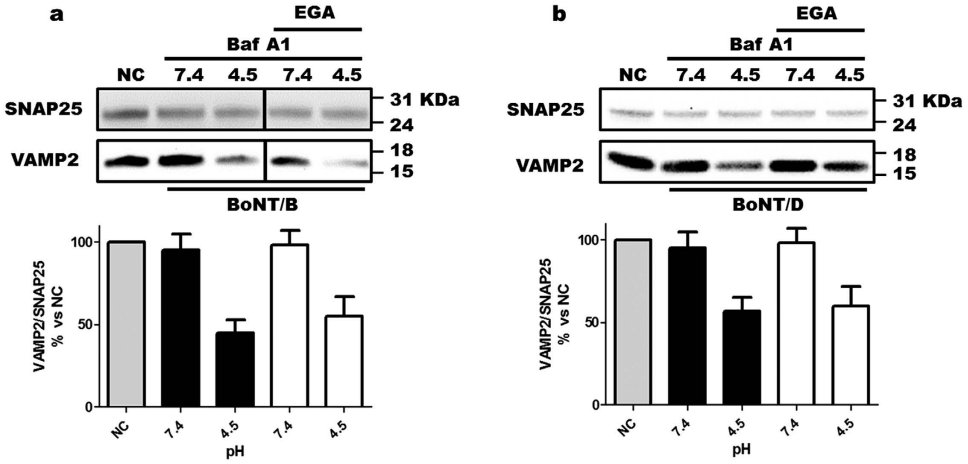

Figure 5. EGA does not inhibit the translocation and the reduction step. (**a**) PC12 cells expressing the lumenal domain of synaptotagmin I on their surface were pre-incubated with a mixture of gangliosides for 24 hrs. Cells were washed and, where indicated, treated with 12.5 μM EGA or vehicle for 30 min at 37 °C. Thereafter, BoNT/B (10 nM) was added in the cold for 15 min. Cells were then washed and incubated with medium A buffered at indicated pH at 37 °C for 10 min, in the presence or absence of EGA. Then, cells were washed and the incubation in culture medium containing 50 nM Bafilomycin A1 and the same concentration of EGA prolonged for 24 hrs at 37° C. The translocation of BoNT/B was assessed by monitoring the cleavage of VAMP2, determined via western blotting (top panel), and quantified (bottom panel) through the densitometry of VAMP2 as a ratio to SNAP25 staining which served as internal control, taking the value in non-treated cells (NC) as 100%. All data are presented as mean values and error bars indicated the standard deviation obtained from three independent experiments (ns – non significant).
(**b**) CGNs were treated with 12.5 μM EGA or vehicle for 30 min at 37 °C. Then, neurons were incubated with BoNT/D (2.5 pM) at 4 °C for 15 min, washed and incubated at 37 °C with buffers at different pH value (7.4 or 4.5) for 10 min; after washing, the neurons were incubated for 24 h with standard medium in the presence of 50 nM bafilomycin A1 and where indicated EGA. Then the SNARE protein content was estimated by immunoblotting with specific antibodies. Values are reported as the ratio between the staining with the antibody specific for VAMP2 and the staining with an antibody specific for SNAP25, and normalized vs. non-treated neurons (NC). All data are presented as mean values and error bars indicated the standard deviation obtained from three independent experiments (ns – non significant).

impact on their activity anymore. Taken together the results presented here indicate that EGA prevents the activity of BoNTs by inhibiting their intraneuronal trafficking.

EGA interferes with the neuroparalytic activity of BoNT/A, BoNT/B and BoNT/D at the mouse hemidiaphragm assay and *in vivo*. The main aim of the present work was to test the inhibitory capacity of EGA against BoNTs toxicity *in vivo*. Therefore, after the *in vitro* approach, we used the mouse hemidiaphragm muscle paralysis model, an *ex vivo* preparation which represents the standard method to assay the neuroparalytic activity of BoNTs at the neuromuscular junction. In this experimental set up, BoNTs induce a decrease in the twitch capability of the diaphragmatic muscle by exerting its metalloprotease activity within the attached phrenic nerve. This decay is followed over time, and is used to evaluate BoNT potency, but can also be adapted to determine the inhibitory capacity of antitoxins[38]. As shown in Fig. 6(a–c, black traces), BoNT/A, BoNT/B and BoNT/D induce a rapid drop in the twitch capability of the diaphragm muscle. On the other hand, the pre-treatment with 12.5 μM EGA, strongly delays the neuroparalytic activity of the three BoNTs (red traces). This inhibitory effect can be appreciated also by comparing the different parameters reported in Table S1, and the $t_{50\%}$ values in particular (Fig. 6d–f), namely the time needed to halve the muscle twitch capacity, which results greatly increased by the treatment with the drug, and found to be significantly different (Table S1).

We then tested the inhibitory effect of EGA *in vivo*. A wide range of doses from 7.5 mg/kg to 40 mg/kg per day was administered via b.i.d. intraperitoneal injections in mice: even after one week of treatment with this regimen, the drug was well tolerated by mice which did not show any sign of decreased vitality in terms of breathing, eating and drinking nor in terms of motility as compared with vehicle injected controls. The lethality of our preparations of BoNT/A,/B and/D was evaluated in preliminary experiments, and a dose of 0.5 ng/kg (BoNT/A), 0.9 ng/kg (BoNT/B) and 0.045 ng/kg (BoNT/D) was sufficient to progressively induce the classical symptoms of botulism (fur ruffling, sides musculature collapse, generalized weakness, labored breathing) and cause the deadly respiratory failure within 48 hours post injection (black traces of Fig. 6 panels g–i). The red traces of the same figure (panels h and i) show

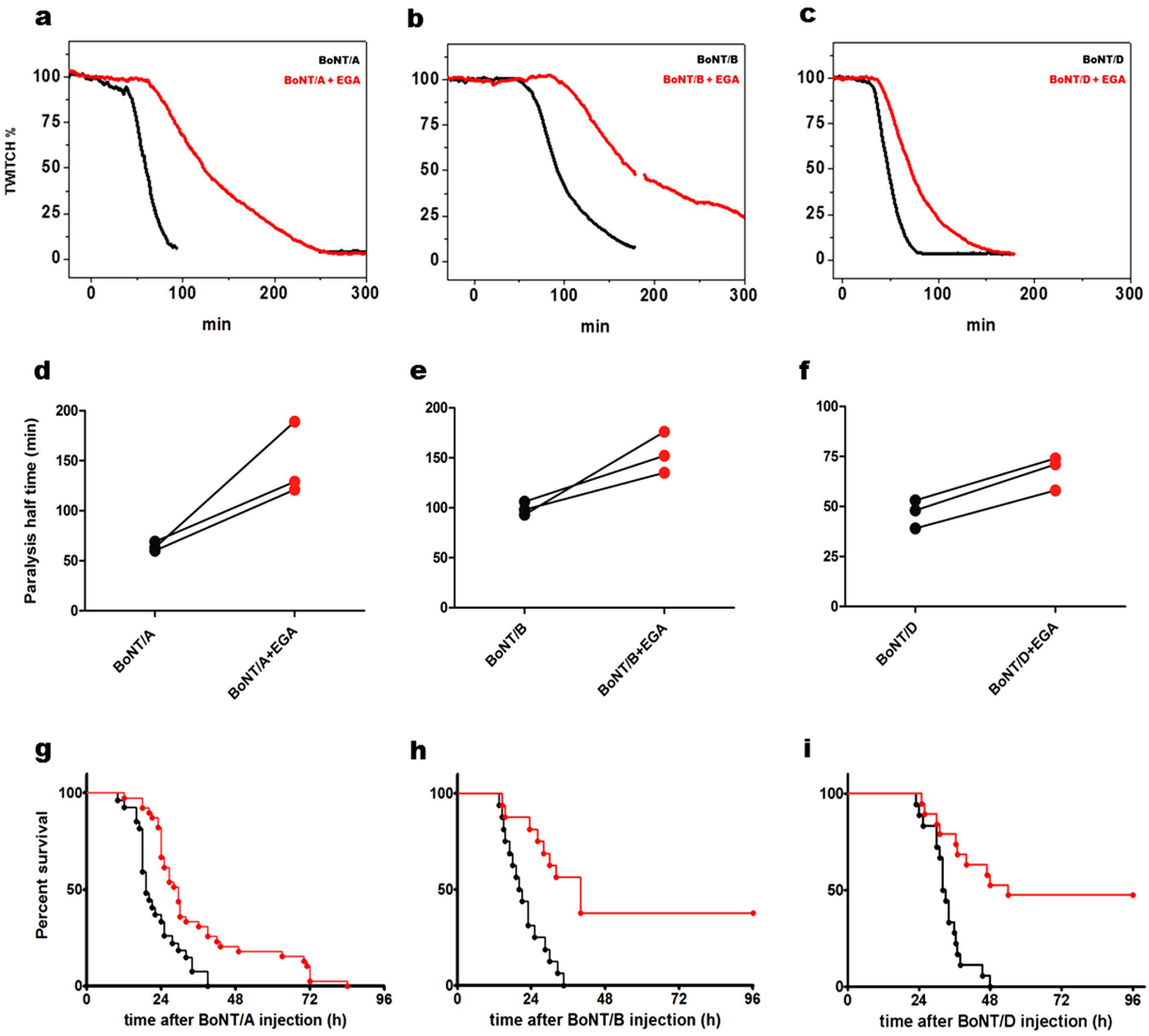

Figure 6. EGA provides protection against different serotypes of BoNTs in the phrenic nerve-hemidiaphragm twitch model, delays death induced by BoNT/A and strongly protect against death induced by BoNT/B and BoNT/D. (**a–c**) For each experiment, 12.5 μM EGA or vehicle (DMSO) was added to the nerve-muscle preparations in the bath at 37 °C; after 30 min, 10 pM BoNT/A (**a**) or 10 pM BoNT/B (**b**) or 100 pM BoNT/D (**c**) was added (time = 0). Muscle twitch was induced by nerve stimulation and monitored until paralysis. A representative experiment is reported for each toxin, showing the progressive twitch decrease in the presence of vehicle (black trace) or EGA (red trace). (**d–f**) Each experiment performed for (**a–c**) was expressed as the time required to decrease the twitch to 50% of the initial value (paralysis half time) in vehicle (black points) or EGA treated muscles (red points). (**g–i**) Adult CD1 mice preconditioned with EGA 12.5 mg/Kg (n = 20) or vehicle alone (n = 20) were i.p. injected with $2 \times MLD_{50}$ of BoNT/A (**g**) or BoNT/B (**h**) or BoNT/D (**i**). The animals were monitored every 4 hrs for 96 hrs. The survival curves were compared and found to be significantly different ($p < 0.0001$).

that EGA is particularly efficacious in preventing death from botulism induced by BoNT/B and BoNT/D. Importantly, in those mice that eventually died, the symptoms occurred with delay and were less pronounced. This was the case also for BoNT/A injected mice, where symptoms developed later and were milder, but without a reduced toxin lethality (Fig. 6g, red trace).

Discussion

The main result reported here is simple and very relevant at the same time. EGA is a potent inhibitor of the neuroparalytic activity of botulinum neurotoxins *in vitro* and *in vivo*, at doses that cause no apparent toxicity. This result indicates that EGA is the lead of a novel class of inhibitors potentially capable of preventing the activity of BoNTs in humans. This is the more relevant considering that the recent years have

witnessed the discovery of a large number of novel BoNTs, with different immunoreactivity[2,39,40], suggesting the possibility of the identification of BoNT variants that may be poorly neutralized by currently available antisera. This situation calls for the discovery of inhibitors capable of preventing the activity of all BoNTs. Necessarily, these novel inhibitors must be non-toxic to humans and must be effective *in vivo*. Notwithstanding long efforts of many laboratories, this goal has only partially been achieved[41]. We recently reported on inhibitors of the Thioredoxin reductase–Thioredoxin redox couple that effectively prevent the neuroparalytic activity of all BoNT serotypes without causing toxic effects in mice[19,20,42]

Here we add another lead compound with a different mechanism of inhibition. Despite our efforts using primary cultures of neurons and neuromuscular junction preparation, we have not identified the target of EGA, but we did not note toxic effects in mice treated with a dose that largely prevents the action of the three BoNTs used here. We have found that the main steps of BoNTs mechanism of action, i.e. binding, internalization, acidification of intracellular compartment, L chain translocation, disulphide reduction and substrate proteolysis, are not affected by this compound (Figs 4 and 5, Figure S2 and Figure S3). Notably, the range of concentration that block BoNTs in cultured neurons is the same previously found to inhibit the toxicity of different toxins and viruses in primary and immortalized macrophages. This suggests that, rather than having a direct effect on BoNTs (or on the other pathogens), EGA interferes with an intracellular host target responsible for their trafficking. This conclusion is reinforced by the result showing that EGA had no effect on the translocation of the L chain from the plasma membrane, when the canonical internalization route was bypassed (Fig. 5).

All known protein receptors of BoNTs are the lumenal domains of integral proteins of synaptic vesicles which suggests the general conclusion that all BoNTs are endocytosed inside these organelles at nerve terminals. However, the following trafficking of synaptic vesicles is not fully understood, though there is evidence that they may fuse with synaptic endosomes where they are quality controlled and then released to re-enter the synaptic vesicle cycle[43–48]. As a consequence, the fact that the three different serotypes considered here are differently protected by EGA, which inhibits the maturation of early endosomes[27], is an interesting aspect of the current study, because it revives the possibility that different BoNT may be trafficked through different routes inside the nerve terminals. Indeed, the diverse protein receptors of BoNTs may account for distinct fates of each toxin-receptor complexes, which have not yet been determined case by case. An alternative explanation is suggested by the finding that part of BoNT/A may enter terminals independently from SVs endocytosis[31,32], which is supported by studies showing that BoNTs display toxicity independently of the stimulation of SVs recycling[34,49–53]. The fact that EGA completely inhibits the activity of BoNT/B, although used at a concentration much higher than that of BoNT/A, opens the possibility that the activity of this toxin is dependent on a trafficking through endosomes and does not translocate its catalytic part into the cytosol across the SV membrane. This is a surprising finding which was unexpected on the basis of the knowledge that the SV protein synaptotagmin mediates the entry of BoNT/B[8,9]. However, considering that synaptotagmin can be trafficked through early endosome[54], the possibility that also BoNT/B may need the passage through this organelle to reach a membrane translocation-competent compartment becomes plausible. It is also in keeping with its slow time course of entry into cultured neurons as compared with other serotypes[34,55]. Moreover, a considerable amount of synaptotagmin molecules remains exposed on the plasma membrane surface, in a steady-state with those recycled through sorting endosomes[56], which makes possible that BoNT/B forms a toxin-receptor complex on the plasma membrane, rather than within SVs. This fits well with the present findings that: i) the internalization of c-Myc-HC/B was much less affected compared to that of cpV-HC/A, by the pre-treatment with BoNT/D (Fig. 4b and Figure S2b) and ii) the different staining pattern of the BoNT/A and BoNT/B binding domains (Fig. 4a and Figure S2a). This possibility is also supported by the *in vivo* finding that EGA has a remarkable effect against the lethality of BoNT/B and a lower one on BoNT/A (Fig. 6g,h).

The behavior of BoNT/D in response to inhibition of the endosomal pathway by EGA, in cultured neurons is more similar to that of BoNT/A rather than BoNT/B, as VAMP2 cleavage was not completely prevented (Figs 2c and 3e,f). On the other hand, BoNT/D was efficaciously inhibited by EGA *in vivo,* with an inhibitory profile similar to that of BoNT/B (Fig. 6i). The mechanism of BoNT/D binding to neurons is poorly understood and therefore its internalization and trafficking properties are not entirely clear[37,57], and as a consequence it is even more difficult to envisage how this toxin could be internalized and trafficked. The obtained results clearly show that the observations of cell culture experiments cannot be transferred *tout court* to *in vivo* conditions.

The present lack of knowledge on the biochemical target of EGA does not prevent research aimed at finding more potent inhibitors of the BoNT neuroparalytic action. Clearly, EGA action is a preventive one, as it cannot affect those L chains that have already translocated in the cytosol. Nevertheless, it can alleviate the symptoms of botulism after diagnosis because a considerable amount of BoNT remains in the general circulation of botulism patient for weeks after the first diagnosis[58–60]. Perhaps, more importantly, the present findings are relevant for infant botulism where a continuous entry of BoNT into the general circulation occurs via adsorption of the toxin produced by Clostridia that have colonized the gastrointestinal tract of infants owing to the reduced intestinal flora competing with Clostridia[2,61].

We would like to conclude by pointing out that the search for novel EGA-derived analogues is made simpler by the design of the novel method of synthesis of this compound described here, which provides a much higher yield with respect to the recently described method[29]. This procedure allowed us to rapidly

and efficiently synthesize large quantities of EGA, an essential pre-requisite to produce the considerable amount necessary for a possible employment of this or related compounds in humans.

Methods

Chemical Synthesis. Detailed protocol for EGA chemical synthesis is available in Supplementary Information.

Botulinum neurotoxin inhibition assay. EGA was dissolved in DMSO to prepare a stock solution (12.5 mM). CGNs at 6–8 days *in vitro* (DIV) were treated for 30 min with the indicated concentrations of EGA in complete culture medium at 37 °C and 5% CO_2. 0.3 nM BoNT/A, 5 nM BoNT/B or 0.025 pM BoNT/D was added, in the presence of the same concentration of inhibitor, and left for 12 hr at 37 °C and 5% CO_2. Further details can be found in the Supplementary Information.

cpV-HC/A and c-Myc-HC/B binding assay. CGNs were treated with EGA 12.5 μM or vehicle (DMSO) in culture medium at 37 °C. After 30 min, for immunocytochemistry experiments, 100 nM cpV-HC/A or c-Myc-HC/B was added in stimulating culture medium (complete culture medium, 57 mM KCl), for 1 hr. The same protocol was used with 250 nM of cpV-HC/A or c-Myc-HC/B but neurons were then lysed and immunoblotted to obtain a quantitative result. Details are in the Supplementary Information.

Low pH induced translocation of BoNT/B and BoNT/D across the plasma membrane. Experiment was conducted as previously described[36]. Detailed protocol is available in Supplementary Information.

Mouse diaphragm and lethality assay. All experiments were performed in accordance with the European Communities Council Directive n° 2010/63/UE and approved by the Italian Ministry of Health. Mouse diaphragms were isolated from CD-1 mice weighing about 20–25 g and halved into two contralateral hemi-diaphragms still innervated with the own phrenic nerve, and were treated as described in the Supplemental Experimental Procedures. Lethality assays were performed using Swiss-Webster adult male CD1 mice weighing 26–28 g as described in Supplementary Information.

Statistical analysis. For all the experiments, data are presented as mean values. Bars indicated the standard deviation. Significance was calculated by Student's t test (unpaired, two-side). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.0001$. Only values below 0.05 were considered significant (ns – non significant).

References

1. Johnson, E. A. & Montecucco, C. Botulism. *Handb Clin Neurol* **91,** 333–368 (2008).
2. Rossetto, O., Pirazzini, M. & Montecucco, C. Botulinum neurotoxins: genetic, structural and mechanistic insights. *Nat Rev Microbiol* **12,** 535–549 (2014).
3. Arnon, S. S. *et al.* Botulinum toxin as a biological weapon: medical and public health management. *JAMA*. **285,** 1059–1070 (2001).
4. Centers for Disease Control and Prevention. D. o. H. a. H. S. Possession, use, and transfer of select agents and toxins; biennial review. Final rule. *Fed Regist* **77,** 61083–61115 (2012).
5. Davletov, B., Bajohrs, M. & Binz, T. Beyond BOTOX: advantages and limitations of individual botulinum neurotoxins. *Trends Neurosci.* **28,** 446–452 (2005).
6. Dressler, D. Clinical applications of botulinum toxin. *Curr Opin Microbiol.* **15,** 325–336 (2012).
7. Rummel, A. Double receptor anchorage of botulinum neurotoxins accounts for their exquisite neurospecificity. *Curr Top Microbiol Immunol* **364,** 61–90 (2013).
8. Binz, T. & Rummel, A. Cell entry strategy of clostridial neurotoxins. *J Neurochem.* **109,** 1584–1595 (2009).
9. Nishiki, T. *et al.* Identification of protein receptor for Clostridium botulinum type B neurotoxin in rat brain synaptosomes. *J Biol Chem.* **269,** 10498–10503 (1994).
10. Dong, M. *et al.* SV2 is the protein receptor for botulinum neurotoxin A. *Science.* **312,** 592–596 (2006).
11. Matteoli, M. *et al.* Synaptic vesicle endocytosis mediates the entry of tetanus neurotoxin into hippocampal neurons. *Proc Natl Acad Sci USA* **93,** 13310–13315 (1996).
12. Colasante, C. *et al.* Botulinum neurotoxin type A is internalized and translocated from small synaptic vesicles at the neuromuscular junction. *Mol Neurobiol* **48,** 120–127 (2013).
13. Harper, C. B. *et al.* Dynamin inhibition blocks botulinum neurotoxin type A endocytosis in neurons and delays botulism. *J Biol Chem.* **286,** 35966–35976 (2011).
14. Montal, M. Botulinum neurotoxin: a marvel of protein design. *Annu Rev Biochem* **79,** 591–617 (2010).
15. Pirazzini, M. *et al.* On the translocation of botulinum and tetanus neurotoxins across the membrane of acidic intracellular compartments. *Biochimica et Biophysica Acta (BBA) - Biomembranes*, doi: http://dx.doi.org/10.1016/j.bbamem.2015.08.014.
16. Montecucco, C., Schiavo, G. & Dasgupta, B. R. Effect of pH on the interaction of botulinum neurotoxins A, B and E with liposomes. *Biochem J.* **259,** 47–53 (1989).
17. Fischer, A. & Montal, M. Crucial role of the disulfide bridge between botulinum neurotoxin light and heavy chains in protease translocation across membranes. *J Biol Chem.* **282,** 29604–29611 (2007).
18. Pirazzini, M. *et al.* The thioredoxin reductase-thioredoxin system is involved in the entry of tetanus and botulinum neurotoxins in the cytosol of nerve terminals. *FEBS Lett* **587,** 150–155 (2013).
19. Pirazzini, M. *et al.* Thioredoxin and its reductase are present on synaptic vesicles, and their inhibition prevents the paralysis induced by botulinum neurotoxins. *Cell Rep* **8,** 1870–1878 (2014).
20. Zanetti, G. *et al.* Inhibition of botulinum neurotoxins interchain disulfide bond reduction prevents the peripheral neuroparalysis of botulism. *Biochem Pharmacol*, doi: 10.1016/j.bcp.2015.09.023 (2015).

21. Binz, T. Clostridial neurotoxin light chains: devices for SNARE cleavage mediated blockade of neurotransmission. *Curr Top Microbiol Immunol* **364,** 139–157 (2013).
22. Pantano, S. & Montecucco, C. The blockade of the neurotransmitter release apparatus by botulinum neurotoxins. *Cell Mol Life Sci*, **71,** 793–811 (2013).
23. Kozaki, S., Miyazaki, S. & Sakaguchi, G. Development of antitoxin with each of two complementary fragments of Clostridium botulinum type B derivative toxin. *Infect Immun* **18,** 761–766 (1977).
24. Barash, J. R. & Arnon, S. S. A Novel Strain of Clostridium botulinum That Produces Type B and Type H Botulinum Toxins. *J Infect Dis*, **209,** 183–191 (2013).
25. Smith, T. J. *et al.* Sequence Variation within Botulinum Neurotoxin Serotypes Impacts Antibody Binding and Neutralization. *Infection and Immunity* **73,** 5450–5457.(2005).
26. Webb, R. P., Smith, T. J., Wright, P., Brown, J. & Smith, L. A. Production of catalytically inactive BoNT/A1 holoprotein and comparison with BoNT/A1 subunit vaccines against toxin subtypes A1, A2, and A3. *Vaccine* **27,** 4490–4497 (2009).
27. Gillespie, E. J. *et al.* Selective inhibitor of endosomal trafficking pathways exploited by multiple toxins and viruses. *Proc Natl Acad Sci USA* **110,** E4904–4912 (2013).
28. Eleopra, R. *et al.* Botulinum neurotoxin serotype D is poorly effective in humans: An *in vivo* electrophysiological study. *Clinical Neurophysiology* **124,** 999–1004 (2013).
29. Jung, M. E., Chamberlain, B. T., Ho, C.-L. C., Gillespie, E. J. & Bradley, K. A. Structure–Activity Relationship of Semicarbazone EGA Furnishes Photoaffinity Inhibitors of Anthrax Toxin Cellular Entry. *ACS Medicinal Chemistry Letters* **5,** 363–367 (2014).
30. Lalli, G. *et al.* Functional characterisation of tetanus and botulinum neurotoxins binding domains. *Journal of Cell Science* **112,** 2715–2724 (1999).
31. Restani, L. *et al.* Botulinum neurotoxins A and E undergo retrograde axonal transport in primary motor neurons. *PLoS pathogens* **8,** e1003087 (2012).
32. Blum, F. C., Chen, C., Kroken, A. R. & Barbieri, J. T. Tetanus Toxin and Botulinum Toxin A Utilize Unique Mechanisms To Enter Neurons of the Central Nervous System. *Infection and Immunity* **80,** 1662–1669 (2012).
33. Kraszewski, K. *et al.* Synaptic vesicle dynamics in living cultured hippocampal neurons visualized with CY3-conjugated antibodies directed against the lumenal domain of synaptotagmin. *The Journal of Neuroscience* **15,** 4328–4342 (1995).
34. Keller, J. E., Cai, F. & Neale, E. A. Uptake of botulinum neurotoxin into cultured neurons. *Biochemistry.* **43,** 526–532 (2004).
35. Simpson, L. L. Ammonium chloride and methylamine hydrochloride antagonize clostridial neurotoxins. *J Pharmacol Exp Ther.* **225,** 546–552 (1983).
36. Pirazzini, M., Rossetto, O., Bolognese, P., Shone, C. C. & Montecucco, C. Double anchorage to the membrane and intact inter-chain disulfide bond are required for the low pH induced entry of tetanus and botulinum neurotoxins into neurons. *Cell Microbiol.* **13,** 1731–1743 (2011).
37. Strotmeier, J. *et al.* Botulinum neurotoxin serotype D attacks neurons via two carbohydrate-binding sites in a ganglioside-dependent manner. *Biochem J.* **431,** 207–216 (2010).
38. Rasetti-Escargueil, C., Liu, Y., Rigsby, P., Jones, R. G. & Sesardic, D. Phrenic nerve-hemidiaphragm as a highly sensitive replacement assay for determination of functional botulinum toxin antibodies. *Toxicon* **57,** 1008–1016 (2011).
39. Smith, T. J., Hill, K. K. & Raphael, B. H. Historical and current perspectives on Clostridium botulinum diversity. *Research in Microbiology* **166,** 290–302 (2015).
40. Montecucco, C. & Rasotto, M. B. On botulinum neurotoxin variability. *MBio* **6** (2015).
41. Fischer, A. *et al.* Bimodal modulation of the botulinum neurotoxin protein-conducting channel. *Proc Natl Acad Sci USA* **106,** 1330–1335 (2009).
42. Montal, M. Redox regulation of botulinum neurotoxin toxicity: therapeutic implications. *Trends Mol Med* **20,** 602–603 (2014).
43. Jähne, S., Rizzoli, S. O. & Helm, M. S. The structure and function of presynaptic endosomes. *Experimental Cell Research* **335,** 172–179 (2015).
44. Cousin, M. A. Synaptic Vesicle Endocytosis and Endosomal Recycling in Central Nerve Terminals: Discrete Trafficking Routes? *Neuroscientist* **21,** 413–423 (2015).
45. Saheki, Y. & De Camilli, P. Synaptic vesicle endocytosis. *Cold Spring Harb Perspect Biol* **4** (2012).
46. Wucherpfennig, T., Wilsch-Bräuninger, M. & González-Gaitán, M. Role of Drosophila Rab5 during endosomal trafficking at the synapse and evoked neurotransmitter release. *The Journal of Cell Biology* **161,** 609–624 (2003).
47. Morgan, J. R., Comstra, H. S., Cohen, M. & Faundez, V. Presynaptic membrane retrieval and endosome biology: defining molecularly heterogeneous synaptic vesicles. *Cold Spring Harb Perspect Biol* **5,** a016915 (2013).
48. de Hoop, M. J. *et al.* The involvement of the small GTP-binding protein Rab5a in neuronal endocytosis. *Neuron* **13,** 11–22 (1994)
49. Pellett, S., Tepp, W. H., Scherf, J. M. & Johnson, E. A. Botulinum Neurotoxins Can Enter Cultured Neurons Independent of Synaptic Vesicle Recycling. *PLoS One* **10,** e0133737 (2015).
50. Keller, J. E., Neale, E. A., Oyler, G. & Adler, M. Persistence of botulinum neurotoxin action in cultured spinal cord cells. *FEBS Lett.* **456,** 137–142 (1999).
51. Eleopra, R., Tugnoli, V., Rossetto, O., De Grandis, D. & Montecucco, C. Different time courses of recovery after poisoning with botulinum neurotoxin serotypes A and E in humans. *Neurosci Lett* **256,** 135–138 (1998).
52. Meunier, F. r. A., Lisk, G., Sesardic, D. & Dolly, J. O. Dynamics of motor nerve terminal remodeling unveiled using SNARE-cleaving botulinum toxins: the extent and duration are dictated by the sites of SNAP-25 truncation. *Molecular and Cellular Neuroscience* **22,** 454–466 (2003).
53. Keller, J. E. Recovery from botulinum neurotoxin poisoning *in vivo*. *Neuroscience* **139,** 629–637 (2006).
54. Diril, M. K., Wienisch, M., Jung, N., Klingauf, J. & Haucke, V. Stonin 2 Is an AP-2-Dependent Endocytic Sorting Adaptor for Synaptotagmin Internalization and Recycling. *Developmental Cell* **10,** 233–244 (2006).
55. Sun, S., Tepp, W. H., Johnson, E. A. & Chapman, E. R. Botulinum neurotoxins B and E translocate at different rates and exhibit divergent responses to GT1b and low pH. *Biochemistry.* **51,** 5655–5662 (2012).
56. Willig, K. I., Rizzoli, S. O., Westphal, V., Jahn, R. & Hell, S. W. STED microscopy reveals that synaptotagmin remains clustered after synaptic vesicle exocytosis. *Nature* **440,** 935–939 (2006).
57. Peng, L., Tepp, W. H., Johnson, E. A. & Dong, M. Botulinum neurotoxin D uses synaptic vesicle protein SV2 and gangliosides as receptors. *PLoS Pathog.* **7,** e1002008 (2011).
58. Simpson, L. The life history of a botulinum toxin molecule. *Toxicon* **68,** 40–59 (2013).
59. Fagan, R. P., McLaughlin, J. B. & Middaugh, J. P. Persistence of botulinum toxin in patients' serum: Alaska, 1959-2007. *The Journal of infectious diseases* **199,** 1029–1031.(2009).
60. Sheth, A. N. *et al.* International outbreak of severe botulism with prolonged toxemia caused by commercial carrot juice. *Clinical infectious diseases: an official publication of the Infectious Diseases Society of America* **47,** 1245–1251 (2008).
61. Koepke, R., Sobel, J. & Arnon, S. S. Global occurrence of infant botulism, 1976-2006. *Pediatrics* **122,** e73–82 (2008).

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Author Contributions

C.M. conceived the project together with M.P., D.A.T., A.M., F.L. and C.P. A.M. performed the chemical synthesis. D.A.T. performed and evaluated *in vitro* experiments. G.Z. performed and evaluated *in vivo* experiments together with M.P., D.A.T. and C.M. C.C.S., T.B. and O.R. produced, purified and tested botulinum neurotoxins, and with S.F. provided advices. O.L. cloned, expressed and purified recombinant proteins. M.P., A.M. and C.M. wrote the paper with contributions of all co-authors.

Additional Information

Supplementary information accompanies this paper at http://www.nature.com/srep

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A Novel Inhibitor Prevents the Peripheral Neuroparalysis of Botulinum Neurotoxins

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Supplementary figures

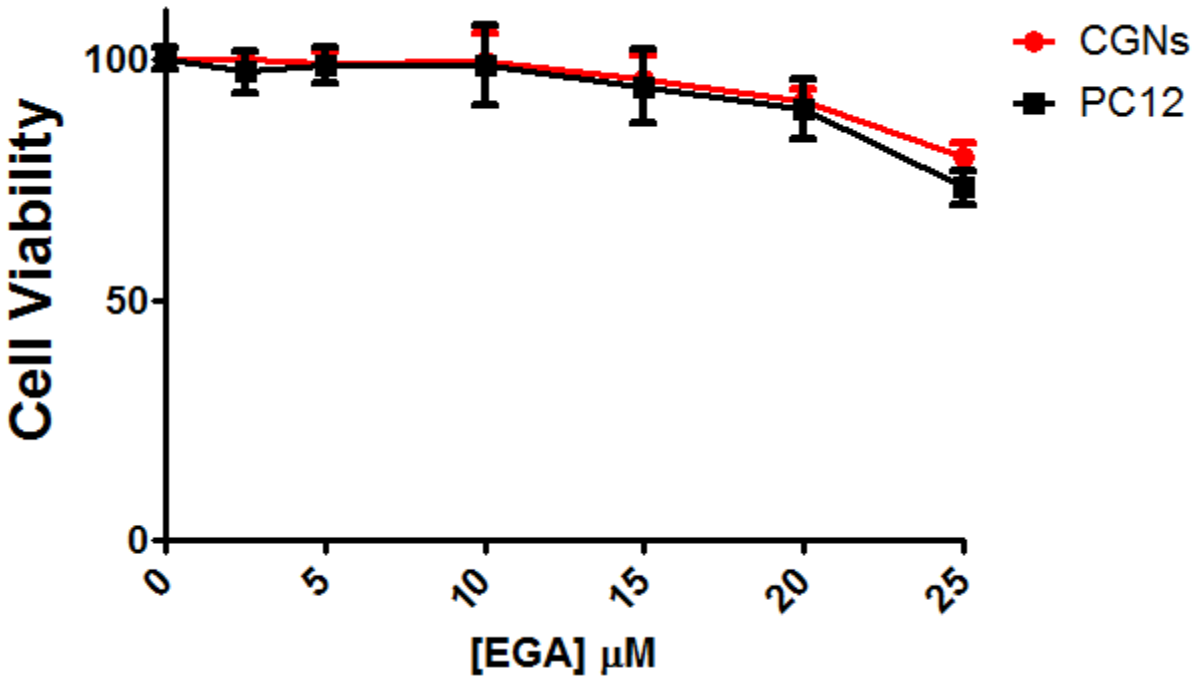

Figure S1. EGA does not affect cell viability of CGNs and PC12. CGNs and PC12 were treated with increasing concentration of EGA ranging from 2.5 to 25 µM or vehicle in culture medium at 37° C. After 24 hours, cell viability has been assayed with a MTS assay. Data are presented as a percentage with respect to cells treated with the vehicle, set as 100%. All data are presented as mean values and error bars indicated the deviation standard obtained from three independent experiments.

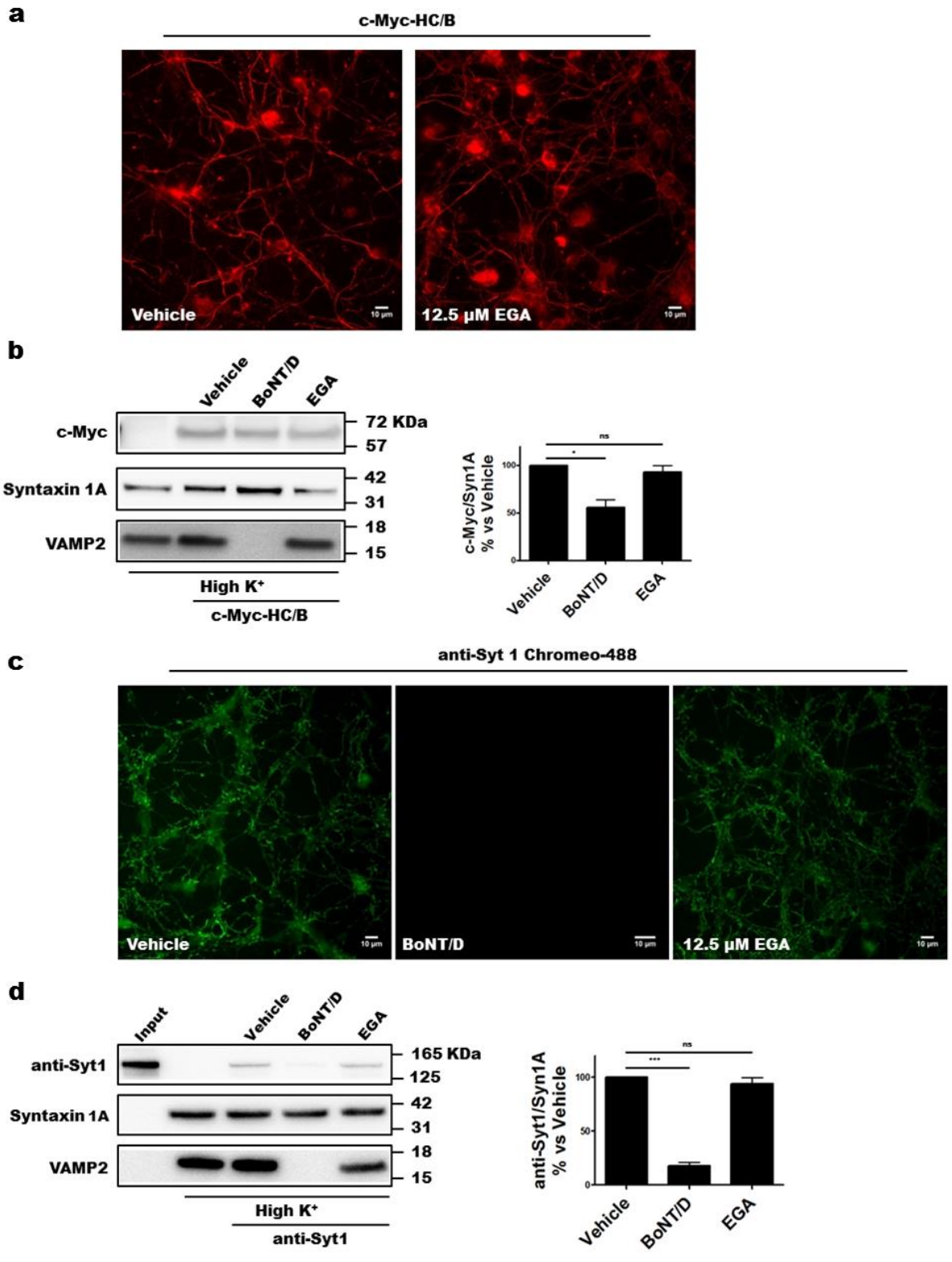
a
c-Myc-HC/B
Vehicle
12.5 µM EGA
b
Vehicle
BoNT/D
EGA
c-Myc
Syntaxin 1A
VAMP2
72 KDa
57
42
31
18
15
High K+
c-Myc-HC/B
c-Myc/Syn1A
% vs Vehicle
ns
*
100
50
0
Vehicle
BoNT/D
EGA
c
anti-Syt 1 Chromeo-488
Vehicle
BoNT/D
12.5 µM EGA
d
Input
Vehicle
BoNT/D
EGA
anti-Syt1
Syntaxin 1A
VAMP2
165 KDa
125
42
31
18
15
High K+
anti-Syt1
anti-Syt1/Syn1A
% vs Vehicle
ns
***
100
50
0
Vehicle
BoNT/D
EGA

Figure S2. EGA does not affect binding and synaptic vesicles dynamics in CGNs. (a) CGNs were treated with EGA (12.5 µM) or vehicle in culture medium at 37° C. After 30 min, 100 nM c-Myc-HC/B was added in high K^+ buffer for 1 h. Neurons were then washed, fixed, permeabilized and stained with a primary antibody specific for the c-Myc epitope. An Alexa Fluor 488 goat anti-mouse secondary antibody was used for detection. These images are representative of two independent sets of experiments. Scale bar, 10 µm. (b) CGNs were treated as in *(a)* with 250 nM of c-Myc-HC/B and then lysed. The c-Myc-HC/B content was estimated with specific antibodies against c-Myc epitope. Syntaxin 1A was used as loading control and VAMP2 to assess BoNT/D cleavage. The amount of c-Myc-HC/B was determined as a ratio to Syntaxin 1A staining taking the value in non-treated cells (vehicle) as 100%. All data are presented as mean values and error bars indicated the standard deviation obtained from two independent experiments (* $p<0.05$; ns – non significant). (c) CGNs were treated with EGA (12.5 µM) or vehicle at 37° C for 30 minutes. Where indicated, neurons were pre-treated with BoNT/D (10 nM) for 30 min. Cells were then incubated for 20 min with an antibody against the lumenal domain of Synaptotagmin-1 conjugated to Chromeo 488 in high K^+ buffer. At the end of the incubation, CGNs were washed twice, fixed and imaged by fluorescence microscopy. These images are representative of three independent sets of experiments. Scale bar, 10 µm. (d) CGNs were treated as in (c), using a non-fluorescent version of the same anti-Synaptotagmin-1 antibody. At the end of the incubation, neurons were washed twice and lysed in non-reducing Laemmli sample buffer. In the upper panel the internalized antibody was detected by immunoblotting, using an anti mouse HRP-conjugated secondary antibody. Blots were then stripped and incubated with specific antibodies against VAMP2 to assess BoNT/D cleavage and against Syntaxin 1A as loading control. In the first lane (input) 50 ng of the anti-Synaptotagmin 1 antibody were loaded as reference. The bottom panel reports the quantification. All data are presented as mean values and error bars indicated the

standard deviation obtained from three independent experiments (*** p<0.0001; ns – non significant).

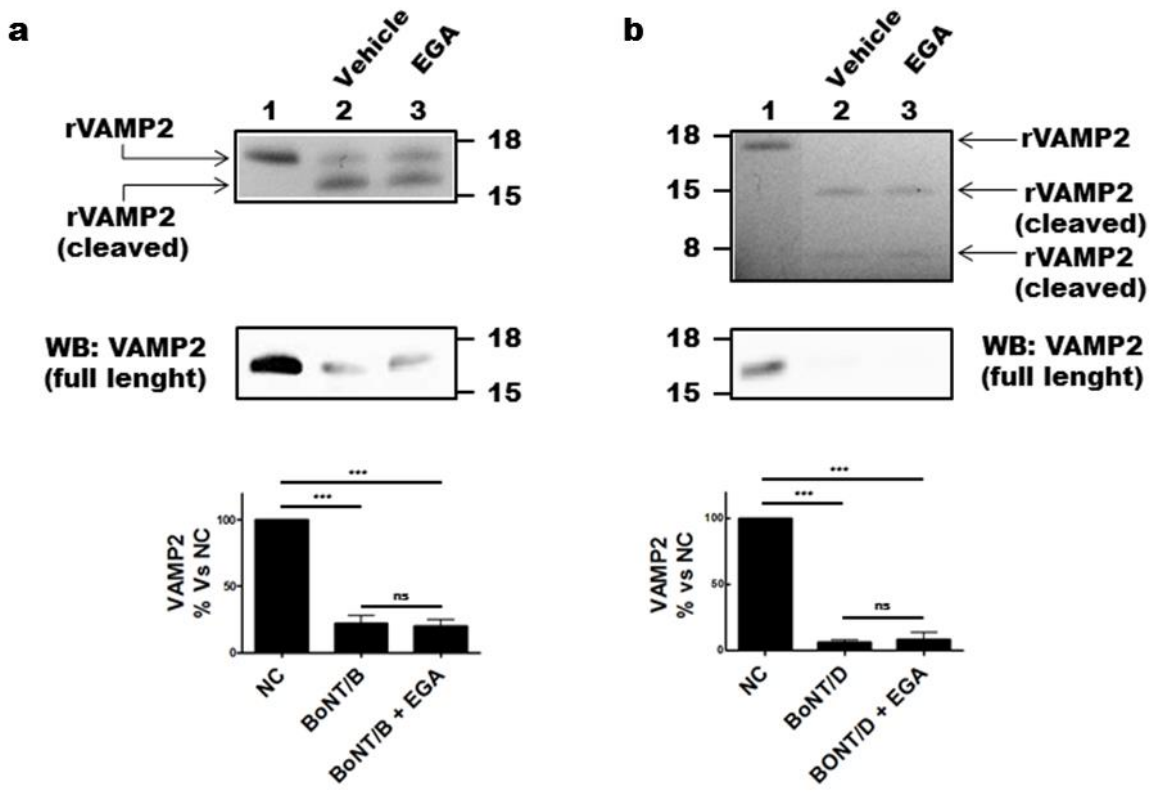

Figure S3. EGA does not affect BoNT/B and /D metalloprotease activity in vitro. (a) BoNT/B (1 µg) or (b) BoNT/D (0.25 µg) was reduced in the presence of EGA (12.5 µM) for 30 min at 37 °C. 1 µg of recombinant VAMP2 (rVAMP2) was then added, the concentration of inhibitor restored and the reaction carried out for 12 hours at 37 °C. VAMP2 cleavage was assessed by SDS-PAGE (top panels) or immunoblotting (middle panels) with an antibody that recognizes full length VAMP2. Lower panels show the densitometry analysis of western blots. All data are presented as mean values and error bars indicated the standard deviation obtained from three independent experiments (*** p<0.0001; ns – non significant).

	BoNT/A		BoNT/B		BoNT/D	
	DMSO	**EGA**	**DMSO**	**EGA**	**DMSO**	**EGA**
Lag phase (minutes)	36.3 ± 3.37	59.6 ± 0.9**	56.3 ± 2.4	82.0 ± 5.3*	29.0 ± 5.1	34.7 ± 2.4^{ns}
$t_{1/2}$ (minutes)	64.0 ± 2.6	146.3 ± 21.5*	99.0 ± 3.8	154.3 ± 11.9*	46.7 ± 4.1	67.7 ± 4.9*
Slope	2.1 ± 0.14	0.5 ± 0.09**	1.3 ± 0.11	0.6 ± 0.01**	2.4 ± 0.16	1.46 ± 0.09**
N	3	3	3	3	3	3

Table S1. Statistical analysis of the phrenic nerve-hemidiaphram twitch model. Given the variability of each muscle contraction, the data from BoNT/A, /B and /D intoxicated muscles treated with EGA or DMSO, were normalized and each single group was compared. The average values±SEM of lag phase, $t_{1/2}$ and slope derive from three independent experiments. Significance was calculated by Student's t test (unpaired, two-side). *P < 0.05; **P < 0.01. Only values below 0.05 were considered significant.

Supplementary experimental procedures

Reagents. BoNT/A was purified as previously described[1,2]. BoNT/B and BoNT/D were produced in *E. coli* as recombinant proteins[3,4]. LysoTracker® Red DND-99 was purchased from ThermoFischer Scientific (L-7528), instead, Bafilomycin A1 (sc-201550) from Santa Cruz Biotechnology. Cytosine β-D-arabinofuranoside hydrochloride (C6645), DNAse I from bovine pancreas (DN25), poly-L-lysine hydrobromide (P1274), solvents and reagents were purchased from Sigma Aldrich, and were used as received.

Chemical Synthesis. Phenyl (2,6-dimethylphenyl)carbamate (**2**): 2,6-dimethylaniline (1.00 g, 8.3 mmol, 1.0 eq) was dissolved in DCM (15 mL) and pyridine (0.73 g, 9.2 mmol, 1.1 eq) and cooled on ice-bath. Phenyl chloroformate (1.43 g, 9.2 mmol, 1.1 eq) in DCM (25 mL) was added dropwise. After addition the flask was brought to room temperature and stirred for 3 h. The reaction mixture was diluted with DCM (50 mL) and washed with 0.5 N HCl (5 × 100 mL). The organic layer was dried over MgSO4 and concentrated. The resulting crude product was purified by flash column chromatography on silica gel (eluent: Petroleum Ether/DCM/Acetone 60:35:5) to afford **2** as a bright white solid (1.92 g, 8.0 mmol, 96%). ^{1}H NMR (500 MHz, DMSO) δ 9.35 (s, 1H, -N*H*-), 7.43 (m, 2H, 2 × Ar-*H*), 7.24 (s, 1H, 1 × Ar-*H*), 7.22 (m, 2H, 2× Ar-*H*), 7.21 (m, 2H, 2 × Ar-*H*), 7.12 (m, 3H, 3 × Ar-*H*), 2.28 (s, 6H, 2 × -C*H3*); ^{13}C NMR (126 MHz, DMSO) δ 152.95, 151.56, 136.01, 134.76, 129.86, 128.38, 127.23, 125.56, 122.20, 18.47; ESI-MS: m/z = 242 = $[M+H]^+$; HRMS (ESI^+): m/z 242.1193 $[M+H]^+$, calculated for $C_{15}H_{16}NO_2$: 242.1181. 2-(4-Bromobenzylidene)-N-(2,6-dimethylphenyl)hydrazinecarboxamide (3, *EGA*): 2 (0.50 g, 2.1 mmol, 1.0 eq) was dissolved in DME (5 mL) and mixed with hydrazine monohydrate (50-60%, 0.13 g, 4.2 mmol, 2.0 eq) at 0°C. After addition the flask was brought to rt and stirred for 24 h. The reaction mixture was concentrated to give a crude white solid that was used

without further purification. This white solid was dissolved in chloroform (20 mL), mixed with 4-bromobenzaldehyde (0.77 g, 4.2 mmol, 2.0 eq) and vigorously stirred 15 h at room temperature. Solvent was removed under reduced pressure and the crude was purified by flash column chromatography on silica gel (eluent: DCM/EtOAc 85:15) to afford EGA (3) as a bright white solid (0.63 g, 1.8 mmol, 88%). ^{1}H NMR (500 MHz, DMSO) δ 10.65 (s, 1H, -N*H*-), 8.58 (s, 1H, -C*H*=N-), 7.88 (s, 1H, -N*H*-), 7.82-7.80 (d, 2H, 2 × Ar-*H*), 7.59-7.57 (d, 2H, 2 × Ar-*H*), 7.09 (m, 3H, 3 × Ar-*H*), 2.20 (s, 6H, 2 × -CH_3). ^{13}C NMR (126 MHz, DMSO) δ 154.20, 138.99, 136.71, 136.02, 134.59, 131.94, 129.24, 128.01, 126.67, 122.70, 18.68. ESI-MS: m/z = 346 = $[M+H]^+$; HRMS (ESI^+): m/z 346.0563 $[M+H]^+$, calcd for $C_{16}H_{17}BrN_3O$: 346.0555. TLCs were run on silica gel supported on plastic (Macherey-Nagel Polygram®SIL G/UV_{254}, silica thickness 0.2 mm) and visualized by UV detection. Flash chromatography was performed on silica gel (Macherey-Nagel 60, 230-400 mesh granulometry (0.063-0.040 mm)) under air pressure. The solvents were analytical or synthetic grade and were used without further purification. ^{1}H NMR spectra were recorded with a Bruker AVII500 spectrometer operating at 500 MHz. Chemical shifts (δ) are given in ppm relative to the signal of the solvent. Mass spectra were performed with a 1100 Series Agilent Technologies system, equipped with binary pump (G1312A) and MSD SL Trap mass spectrometer (G2445D SL) with ESI source. ESI-MS positive spectra of reaction intermediates and the final purified product were obtained from solutions in acetonitrile, eluting with a water:acetonitrile, 1:1 mixture containing 0.1% formic acid. High-resolution mass measurements were obtained using a Mariner ESI-TOF spectrometer (PerSeptive Biosystems). HPLC-MS analysis was used to confirm the purity (> 95%).

Cerebellar Granule Neurons (CGN) cultures. Primary cultures of rat cerebellar granule neurons (CGNs) were prepared from 6- to 8-days-old rats[5]. Cerebella were isolated, mechanically disrupted and then trypsinized in the presence of DNase I. Cells were then

plated into 24 well plates, pre-coated with poly-L-lysine (50 µg/mL), at a cell density of 4 x 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 gentamicin (hereafter indicated as complete culture medium). To arrest growth of non-neuronal cells, cytosine arabinoside (10 µM) was added to the medium 18–24 h after plating.

Botulinum neurotoxin inhibition assay and immunocytochemistry. CGNs at 6–8 DIV were treated for 30 min with 12.5 µM of EGA or vehicle (DMSO) in complete culture medium at 37 °C and 5% CO_2. 0.3 nM BoNT/A or 5 nM BoNT/B or 0.025 pM BoNT/D was added and left for 12 hours at 37 °C and 5% CO_2. Neurons were then washed with PBS, fixed for 10 minutes at RT with 4% paraformaldehyde in PBS, quenched (50 mM NH_4Cl in PBS) for 20 minutes and permeabilized with 5% acetic acid in ethanol for 20 minutes at -20° C. CGNs were then incubated with indicated primary antibodies. BoNT/A cleavage was evaluated following the generation of the cleaved form of SNAP25, whereas the cleavage of BoNT/B and BoNT/D was evaluated following the disappearance of the staining due to a primary antibody recognizing the full-length form of VAMP2 (Synaptic System, 104 211). As internal control (not shown) it was used an anti SV2A (Santa Cruz, [E-8] sc376234) or anti SV2B (Synaptic System, 119 102), respectively. Primary antibodies were detected with Alexa Fluor 488 goat anti-mouse IgG (Life Technologies, A-11001) and Alexa Fluor 555 goat anti-rabbit IgG (Life Technologies, A-21428). Coverslips were mounted using Fluorescent Mounting Medium (Dako) and examined by epifluorescence (Leica CTR6000) microscopy. Images were collected with the same lamp intensity and exposure time.

For immunoblotting experiments, the neurons were treated with the same condition and the neurotoxicity was evaluated following the specific proteolytic activity of the toxin with

specific antibodies against their SNARE protein targets: anti BoNT/A-cleaved SNAP25[6], anti SNAP25 (SMI81: abcam, ab24737), anti VAMP2 (Synaptic System, 104 211) and anti BoNT/B-cleaved form of VAMP2[7]. The staining with anti Na^+/K^+ ATPase (abcam, ab7671) was used as loading control.

Immunoblotting. Cells were directly lysed with reducing Laemmli sample buffer 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 or 12% NuPage gel (Life technologies) and separated by electrophoresis in 1X MES buffer or 1X MOPS (Life technologies), respectively. Proteins were then transferred onto Protran nitrocellulose membranes (Whatman) and saturated for 1 h in PBST (PBS 0.1% Tween20) supplemented with 5% non-fatty milk. Incubation with primary antibodies was performed overnight at 4°C. The membranes were then washed three times with PBST and incubated with secondary HRP-conjugated antibodies (goat anti-mouse IgG, H&L chain specific peroxidase conjugate, Merk Millipore 401215 and goat anti-rabbit IgG, H&L chain specific peroxidase conjugate, Merk Millipore 401393). Finally, membranes were washed twice with PBST and once with PBS; visualization was carried out using Luminata Crescendo (Merck Millipore).

cpV-HC/A and c-Myc-HC/B expression, purification and binding assay. The HC of BoNT/B (nucleotides corresponding to residues 833-1291) with a N-terminus c-Myc tag was cloned into a pRSETa His-tag vector (Novagen) and expressed into BL21pLysS *E.coli* cells. Protein purification was achieved by affinity chromatography with a prepacked HisTrap Ni column (GE Healthcare) and then by size-exclusion chromatography using a Superdex 200, 10/300GL column (GE Healthcare). The purified c-Myc-HC/B fusion protein

were pooled and concentrated using a membrane filter with a cutoff of 30 kDa (Amicon Millipore).

The HC of BoNT/A (nucleotides corresponding residues 876-1296) fused with cpV (Circularly Permuted Venus) at the N-terminus was cloned into a pET28a His-tag vector (Novagen) and expressed in BL21 DE3 *E.coli* cells. Purification of cpV-HC/A fusion protein was achieved as described for c-Myc-HC/B.

In binding and internalization assay, CGNs were treated with EGA 12.5 µM or vehicle (DMSO) in culture medium at 37° C. After 30 minutes, 100 nM cpV-HC/A or c-Myc-HC/B was added in stimulating culture medium (complete culture medium, 57 mM KCl), for 1 hr. Neurons were then washed twice, fixed with 4% paraformaldehyde in PBS and directly imaged for cpV or permeabilized and stained with a primary antibody specific for the c-Myc epitope (Sigma Aldrich, M4439). An Alexa Fluor 488 goat anti-mouse (Life Technologies, A-11001) was used to detect the c-Myc primary antibody.

The same experiment was performed with 250 nM of cpV-HC/A or c-Myc-HC/B but neurons were then lysed and immunoblotted to obtain a quantitative result. Where indicated, neurons were pre-treated with 10 nM of BoNT/D for 30 minutes. Syntaxin 1A (Synaptic System, 110 111) staining was used as loading control, instead, VAMP2 (Synaptic System, 104 211) staining to assess BoNT/D cleavage. cpV-HC/A was detected with an anti-GFP antibody (Cell Signaling, #2956) whereas c-Myc-HC/B was detected with the aforementioned antibody.

Maturation of acidic compartment assay. CGNs at 6–8 DIV were treated for 30 min with the indicated concentrations of EGA or 10 nM Bafilomycin A1 in complete culture medium supplemented with 6.25 mM Hepes at 37 °C and 5% CO_2. 75 nM Lysotracker® Red DND-99 was added for 90 minutes. Cells were then washed with Krebs-Ringer buffer (KRH: 128 mM NaCl, 2.5 mM HEPES, 4.8 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$ and 1.2 mM

K_2HPO_4) and images of living neurons were acquired with a Leica CTR6000 microscope. Fluorescence intensity was quantified using ImageJ software.

In vitro proteolytic activity. 0.25 µg BoNT/A or BoNT/D or 1 µg BoNT/B was incubated in reducing buffer (150 mM NaCl, 10 mM NaH2PO4, 15 mM DTT pH 7.4) in the presence of 12.5 µM of EGA for 30 min at 37 °C or DMSO. Then 1 µg of recombinant GST-SNAP25 or recombinant VAMP2 (1-96) was added to the reduced toxins, the concentration of inhibitor was restored, and the reaction was carried out for 12 hours at 37 °C. SNAP25 or VAMP2 cleavage was assessed by SDS-PAGE or immunoblotting using an anti-SNAP25 A-cleaved form or an anti-VAMP2 (Synaptic System, 104 211).

Low pH induced translocation of BoNT/B and BoNT/D across the plasma membrane. At a glance, 10^4 PC12-SYT N24Q were plated into 12-wells plates and maintained in RPMI supplemented with 10% HS, 5% FBS, 2 mM L-alanyl-L-glutamine (GlutaMAX), 100 U/ml penicillin, 100 µg/ml streptomycin, 250 ng/ml amphotericin B at 37 °C in a humid incubator. After adhesion, cells were incubated with a mixture of ganglioside (50 ug) for 24 hours. Cells were washed twice with culture medium and subsequently incubated with 10 nM of BoNT/B in ice-cooled medium (pH 7.4) and left at 4 °C for 15 minutes. After washing twice with the same cold medium, pre-warmed (37 °C) 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 indicated pH (7.4 or 4.5) with 1 M TRIS-base - was added and left for 10 minutes. Cells where then washed twice and further incubated in normal culture medium (pH 7.4) containing 50 nM Bafilomycin A1 for 24 hours. Where indicated, 12.5 µM EGA was pre-incubated with cells for 30 minutes and was then present in all solutions used along experiment. The same procedure was performed using CGNs, 2.5 pM of BoNT/D and after low pH assay the incubation was prolonged for 24 hr. Where indicated, 12.5 µM EGA was pre-incubated with cells for 30 minutes and was then present in all

solutions used along experiment. The translocation of BoNT/B or BoNT/D was assessed by following the metalloprotease activity against VAMP2 via western blot using an anti-VAMP2 (Synaptic System, 104211). The staining of SNAP25 (SMI81: abcam, ab24737), was used as loading control.

Mouse diaphragm and lethality assay. Muscles were mounted into two chambers filled with 4 ml of oxygenated (95% O_2, 5% CO_2) solution (139 mM NaCl, 12 mM $NaHCO_3$, 4 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM KH_2PO_4 and 11 mM glucose, pH 7.4). The phrenic nerves were stimulated via two ring platinum electrodes with supramaximal stimuli of 3 V amplitude and 0.1 ms pulse duration, with a frequency of 0.1 Hz (Stimulator 6002, Harvard Apparatus, Massachusetts, USA). Muscle contraction was monitored with an isometric transducer (Harvard Apparatus); data was recorded and analysed via an i-WORX 118 system with Labscribe software (Harvard Apparatus). EGA was added directly to the oxygenated solution of one muscle to reach the final concentration of 12.5 µM of and the same volume of vehicle (DMSO) was added to the contralateral one for direct comparison. After 30 minutes of incubation, 10 pM BoNT/A or BoNT/B or 100 pM BoNT/D was added to both preparations and the twitch monitored until complete paralysis was achieved. Graphs show muscle twitching capability over time, reported as percentage with respect to the initial value obtained before toxin addition.

For lethality assay, EGA was dissolved in DMSO as a stock solution (12.5 mg/ml). Mice were conditioned for 3 days, with 12.5 mg/kg EGA or vehicle with intraperitoneal (i.p.) injections b.i.d. (every 12 hours). After the last injection of drug (or vehicle), mice were weighted and i.p. injected with 1 µl/g body weight of BoNT/A, /B or /D prepared as stock solutions (BoNT/A 0.5 pg/µl, BoNT/B 0.9 pg/µl and BoNT/D 0.045 pg/µl in 0.9% NaCl with 0.2% gelatin) roughly corresponding to 2XMLD_{50}. Mice were monitored every 4 hr for 96

hr, after which the experiment was considered ended. Results are displayed as Kaplan-Meyer plots, and analysed with a Mantel-Cox test for statistical significance.

Synaptic vesicles dynamics assay. Experiment was performed as previously described [8]. Briefly, CGNs were conditioned in high K^+ buffer (70 mM NaCl, 2.5 mM HEPES, 57 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$ and 1.2 mM K_2HPO_4) for 10 minutes. Hereafter, 5 µg/ml of Chromeo 488 (Synaptic System, 105 311CR1) was added for 20 min in the same buffer. Cells were then washed twice with PBS and fixed. Internalized antibodies were imaged using a Leica CTR6000 microscope. Where indicated, 10 nM BoNT/D or 12.5 µM of EGA (or DMSO) were pre-incubated in normal culture medium for 30 minutes. The same concentration of EGA is maintained during antibody incubation. In order to have a quantitative result, the same experiment was performed using as a read-out western blot. Accordingly, CGNs were lysed in non-reducing condition, blotted on nitrocellulose membrane saturated for 1 hr in PBST supplemented with 5% non-fatty milk and directly incubated with secondary antibody to detect the internalized anti-Synaptotagmin1 antibody (Synaptic System, 105 101). The staining of VAMP2 (Synaptic System, 104 211) was used to assess BoNT/D cleavage, instead, Syntaxin 1A (Synaptic System, 110 111) was used as loading control.

Viability test. CGNs or PC12-SYT N24Q were seeded in a 96 wells plates at a cell density 10^5 cells per well. Different concentration of EGA, ranging from 0 to 25 µM, were added and left for 24 hours. Neurons were then washed and MTS assay (Promega) performed according to manufacturer indication.

References

1 Schiavo, G. & Montecucco, C. Tetanus and botulism neurotoxins: isolation and assay. *Methods Enzymol* **248**, 643-652. (1995).

2 Shone, C. C. & Tranter, H. S. Growth of clostridia and preparation of their neurotoxins. *Curr Top Microbiol Immunol* **195**, 143-160. (1995).

3 Rummel, A., Mahrhold, S., Bigalke, H. & Binz, T. The HCC-domain of botulinum neurotoxins A and B exhibits a singular ganglioside binding site displaying serotype specific carbohydrate interaction. *Mol Microbiol.* **51**, 631-643. (2004).

4 Bade, S. *et al.* Botulinum neurotoxin type D enables cytosolic delivery of enzymatically active cargo proteins to neurones via unfolded translocation intermediates. *J Neurochem* **91**, 1461-1472. (2004).

5 Rigoni, M. *et al.* Snake presynaptic neurotoxins with phospholipase A2 activity induce punctate swellings of neurites and exocytosis of synaptic vesicles. *J Cell Sci.* **117**, 3561-3570. (2004).

6 Antonucci, F., Rossi, C., Gianfranceschi, L., Rossetto, O. & Caleo, M. Long-distance retrograde effects of botulinum neurotoxin A. *J Neurosci.* **28**, 3689-3696. (2008).

7 Mainardi, M., Pietrasanta, M., Vannini, E., Rossetto, O. & Caleo, M. Tetanus neurotoxin-induced epilepsy in mouse visual cortex. *Epilepsia* **53**, e132-136. (2012).

8 Kraszewski, K. *et al.* Synaptic vesicle dynamics in living cultured hippocampal neurons visualized with CY3-conjugated antibodies directed against the lumenal domain of synaptotagmin. *The Journal of Neuroscience* **15**, 4328-4342 (1995).

3. CONCLUSION

Until now, the simplest explanation for the existence of botulinum neurotoxins is related to their strategic function in increasing the availability of a suitable anaerobic environment necessary to the growth and dissemination of Clostridia, mainly among animals in wildlife[2]. BoNTs are indeed the foremost poisons found in nature and isolated outbreaks of animal botulism can rapidly assume epidemic proportions. Such an efficiency derives from the ability of BoNTs to target specifically the neuromuscular junction, a compartment essential for the survival of their principal hosts, the vertebrates[7]. On the other hand, the parallel evolution of many BoNTs variants remains quite mysterious, especially in light of the fact that their final aim remains the same. An explanation can be found in the many animal species affected by BoNTs and in the many different alimentary chains that lead to botulism in wildlife[2]. It is however remarkable that, beside these genetic heterogeneity and differences, their molecular architecture is very similar with each region of the toxin shaped by evolution to exploit fundamental features and/or events of neuronal physiology[4,7].

Therefore, given the relevant role of the interchain disulphide bond before, during and after translocation, our finding that the Thioredoxin Reductase-Thioredoxin system resides on the cytosolic face of the synaptic vesicle membrane and mediates the detachment of the catalytic L chain by reducing the disulphide on the cytosolic side, could be seen once more as an evolutionary choice exploited by BoNTs to achieve their final goal[105,111,112]. Moreover, my thesis work also emphasize the importance of BoNTs trafficking within nerve terminals. In fact, BoNTs appear to have evolved different ways to invade host neurons, as judged from their kinetics of intoxication[85,130]. Our finding that EGA inhibits the different toxin serotypes at different extent suggest that this molecule (or its derivates) could be useful to further investigate on their different route of entry[84].

In light of the evidence discussed in this thesis, we propose the reduction of the interchain disulphide bond by the thioredoxin reductase-thioredoxin redox system and the different BoNT trafficking within nerve terminals as a new fundamental events of BoNTs intoxication process, consequently recapitulated into five main steps: i) binding to polysialogangliosides for toxin accumulation on the target neurons, ii) interaction with the lumenal domain of an SV protein for its internalization inside different endosomal compartments, iii) acidification of this “Trojan horse” to deliver the catalytically active part across the SV membrane, iv) re-

duction of the interchain disulphide bond by TrxR-Trx to complete the translocation and release the L chain on the cytosolic side, v) L chain-mediated cleavage of SNARE proteins and disruption of SV ability to fuse with the presynaptic plasma membrane[105].

Our results have important implications concerning the possible application of the compounds presented here as therapeutic agents to treat botulism. The most important point is that these inhibitors are active regardless of the serotype causing intoxication. This is the more important in light of the large number of different BoNTs that are being discovered and, is additionally relevant to those cases of botulism caused by Clostridia producing more than one BoNT serotype. Indeed, these drugs can be administered without knowing the BoNT serotype and sub-type, therefore saving the time required for toxin characterization. For this reason, if given soon after diagnosis, these inhibitors might reduce symptoms severity by preventing the entry of circulating BoNT, thus shortening the period of hospitalization and reducing the associated high costs of intensive care. Importantly, since these inhibitors are small molecules which enter the cells, they can affect toxin molecules which have already been taken up by the neurons, but cannot be blocked any longer by anti-BoNT specific antibodies. Clearly, the toxicity of L chains that have already reached the neuronal cytosol cannot be blocked. However, it was shown that in clinical botulism, BoNTs can remain in the blood circulation and/or in interstitial fluids for many days[131]. Henceforth, using these inhibitors, the severity of BoNT intoxication may be substantially mitigated by blocking BoNT molecules that have not yet entered nerve terminals.

Moreover, some of these compounds, having a good record of safety in humans, might have a great potential in the treatment of infant botulism where a continuous release of freshly produced BoNT takes place, which is the case of infant botulism. In this form of the disease, the BoNT producing Clostridia colonize the intestine, owing to the lack of competitive bacterial flora, and release the toxin within the general circulation for long periods of time.

PUBLICATIONS LIST

- **Azarnia Tehran D.*,** Zanetti G.*, Oneda L., Florigio L., Fillo S., Binz T., Clifford C.C., Rossetto O., Montecucco C., Paradisi C., Mattarei A., Pirazzini M. A novel inhibitor prevents the peripheral neuroparalysis of botulinum neurotoxins. **Sci. Rep**., (2015) Dec 16 **co-first authors*

- Zanetti G.*, **Azarnia Tehran D.*,** Pirazzini M., Binz T., Shone C.C., Fillo S., Lista F., Rossetto O., Montecucco C. Inhibition of botulinum neurotoxins interchain disulfide bond reduction prevents the peripheral neuroparalysis of botulism. Biochem Pharmacol, **2015** Oct 5 **co-first authors*

- Giordani F., Fillo S., Anselmo A., Palozzi A.M., Fortunato A., Gentile B., **Azarnia Tehran D.,** Ciammaruconi A., Spagnolo F., Pittiglio V., Anniballi F., Auricchio B., De Medici D., Lista F. Genomic characterization of italian Clostridium botulinum group I strains. Infect Genet Evol, **2015** Sep 1;

- Pirazzini M., **Azarnia Tehran D.,** Leka O., Zanetti G., Rossetto O., Montecucco C. On the translocation of botulinum and tetanus neurotoxins across the membrane of acidic intracellular compartments. Biochim Biophys Acta, Biomembranes **2015** Aug 22;

- Pirazzini M., **Azarnia Tehran D.,** Zanetti G., Lista F., Binz T., Shone C.C., Rossetto O., Montecucco C. The thioredoxin reductase-Thioredoxin redox system cleaves the interchain disulphide bond of botulinum neurotoxins on the cytosolic surface of synaptic vesicles. Toxicon, **2015** Jun 27;

- Pirazzini M.*, **Azarnia Tehran D.*,** Zanetti G., Megighian A., Scorzeto M., Fillo S., Shone C.C., Binz T., Rossetto O., Lista F., Montecucco C. Thioredoxin and its reductase are present on synaptic vesicles, and their inhibition prevents the paralysis induced by botulinum neurotoxins. Cell Rep. **2014** Sep 25; **co-first authors*

REFERENCES

1. Gill DM. Bacterial toxins: a table of lethal amounts. *Microbiological Reviews.* 1982;46(1):86-94.
2. Montecucco C, Rasotto MB. On botulinum neurotoxin variability. *MBio.* 2015;6(1).
3. Pantano S, Montecucco C. The blockade of the neurotransmitter release apparatus by botulinum neurotoxins. *Cell Mol Life Sci.* 2013.
4. Rummel A. The long journey of botulinum neurotoxins into the synapse. *Toxicon.* 2015;107(Pt A):9-24.
5. Carter AT, Peck MW. Genomes, neurotoxins and biology of Clostridium botulinum Group I and Group II. *Res Microbiol.* 2015;166(4):303-317.
6. Smith TJ, Hill KK, Raphael BH. Historical and current perspectives on Clostridium botulinum diversity. *Research in Microbiology.* 2015;166(4):290-302.
7. Rossetto O, Pirazzini M, Montecucco C. Botulinum neurotoxins: genetic, structural and mechanistic insights. *Nat Rev Microbiol.* 2014.
8. Johnson EA, Montecucco C. Botulism. *Handb Clin Neurol.* 2008;91:333-368.
9. Aureli P, Fenicia L, Pasolini B, Gianfranceschi M, McCroskey LM, Hatheway CL. Two cases of type E infant botulism caused by neurotoxigenic Clostridium butyricum in Italy. *J Infect Dis.* 1986;154(2):207-211.
10. Koepke R, Sobel J, Arnon SS. Global occurrence of infant botulism, 1976-2006. *Pediatrics.* 2008;122(1):e73-82.
11. Wenham TN. Botulism: a rare complication of injecting drug use. *Emerg Med J.* 2008;25(1):55-56.
12. Chertow DS, Tan ET, Maslanka SE, et al. Botulism in 4 adults following cosmetic injections with an unlicensed, highly concentrated botulinum preparation. *Jama.* 2006;296(20):2476-2479.
13. Arnon SS, Schechter R, Inglesby TV, et al. Botulinum toxin as a biological weapon: medical and public health management. *JAMA.* 2001;285(8):1059-1070.
14. Cherington M. Clinical spectrum of botulism. *Muscle Nerve.* 1998;21(6):701-710.
15. Centers for Disease Control and Prevention DoHaHS. Possession, use, and transfer of select agents and toxins; biennial review. Final rule. *Fed Regist.* 2012;77(194):61083-61115.
16. Hallett M, Albanese A, Dressler D, et al. Evidence-based review and assessment of botulinum neurotoxin for the treatment of movement disorders. *Toxicon.* 2013;67:94-114.
17. Naumann M, Dressler D, Hallett M, et al. Evidence-based review and assessment of botulinum neurotoxin for the treatment of secretory disorders. *Toxicon.* 2013;67:141-152.
18. Rossetto O, Seveso M, Caccin P, Schiavo G, Montecucco C. Tetanus and botulinum neurotoxins: turning bad guys into good by research. *Toxicon.* 2001;39(1):27-41.
19. Lim EC, Seet RC. Use of botulinum toxin in the neurology clinic. *Nat Rev Neurol.* 2010;6(11):624-636.
20. De Spain Smith L, Sugiyama H. *Botulism: the organism, its toxins, the disease.* Charles C. Thomas Publisher, Limited; 1988.
21. Bentivoglio AR, Del Grande A, Petracca M, Ialongo T, Ricciardi L. Clinical differences between botulinum neurotoxin type A and B. *Toxicon.* 2015;107(Pt A):77-84.
22. Popoff MR, Bouvet P. Genetic characteristics of toxigenic Clostridia and toxin gene evolution. *Toxicon.* 2013.
23. Erbguth FJ. Historical notes on botulism, Clostridium botulinum, botulinum toxin, and the idea of the therapeutic use of the toxin. *Mov Disord.* 2004;19(Suppl 8):S2-6.
24. Moriishi K, Koura M, Fujii N, et al. Molecular cloning of the gene encoding the mosaic neurotoxin, composed of parts of botulinum neurotoxin types C1 and D, and PCR detection of this gene from Clostridium botulinum type C organisms. *Appl Environ Microbiol.* 1996;62(2):662-667.
25. Moriishi K, Koura M, Abe N, et al. Mosaic structures of neurotoxins produced from

Clostridium botulinum types C and D organisms. *Biochim Biophys Acta.* 1996;1307(2):123-126.
26. Kalb SR, Baudys J, Raphael BH, et al. Functional characterization of botulinum neurotoxin serotype H as a hybrid of known serotypes F and A (BoNT F/A). *Anal Chem.* 2015;87(7):3911-3917.
27. Hill KK, Smith TJ. Genetic diversity within Clostridium botulinum serotypes, botulinum neurotoxin gene clusters and toxin subtypes. *Curr Top Microbiol Immunol.* 2013;364:1-20.
28. Giordani F, Fillo S, Anselmo A, et al. Genomic characterization of Italian Clostridium botulinum group I strains. *Infect Genet Evol.* 2015;36:62-71.
29. Fillo S, Giordani F, Anniballi F, et al. Clostridium botulinum group I strain genotyping by 15-locus multilocus variable-number tandem-repeat analysis. *J Clin Microbiol.* 2011;49(12):4252-4263.
30. Smith TJ, Lou J, Geren IN, et al. Sequence Variation within Botulinum Neurotoxin Serotypes Impacts Antibody Binding and Neutralization. *Infection and Immunity.* 2005;73(9):5450-5457.
31. Kalb SR, Baudys J, Webb RP, et al. Discovery of a novel enzymatic cleavage site for botulinum neurotoxin F5. *FEBS Lett.* 2012;586(2):109-115.
32. Henkel JS, Jacobson M, Tepp W, Pier C, Johnson EA, Barbieri JT. Catalytic properties of botulinum neurotoxin subtypes A3 and A4. *Biochemistry.* 2009;48(11):2522-2528.
33. Wang D, Krilich J, Pellett S, et al. Comparison of the catalytic properties of the botulinum neurotoxin subtypes A1 and A5. *Biochim Biophys Acta.* 2013;1834(12):2722-2728.
34. Whitemarsh RC, Tepp WH, Bradshaw M, et al. Characterization of botulinum neurotoxin a subtypes 1 through 5 by investigation of activities in mice, in neuronal cell cultures, and in vitro. *Infect Immun.* 2013;81(10):3894-3902.
35. Kalb SR, Santana WI, Geren IN, et al. Extraction and inhibition of enzymatic activity of botulinum neurotoxins /B1, /B2, /B3, /B4, and /B5 by a panel of monoclonal anti-BoNT/B antibodies. *BMC Biochem.* 2011;12:58.
36. Hutson RA, Collins MD, East AK, Thompson DE. Nucleotide sequence of the gene coding for non-proteolytic Clostridium botulinum type B neurotoxin: comparison with other clostridial neurotoxins. *Curr Microbiol.* 1994;28(2):101-110.
37. Kumaran D, Eswaramoorthy S, Furey W, Navaza J, Sax M, Swaminathan S. Domain organization in Clostridium botulinum neurotoxin type E is unique: its implication in faster translocation. *J Mol Biol.* 2009;386(1):233-245. Epub 2008 Dec 2024.
38. Lacy DB, Tepp W, Cohen AC, DasGupta BR, Stevens RC. Crystal structure of botulinum neurotoxin type A and implications for toxicity. *Nat Struct Biol.* 1998;5(10):898-902.
39. Swaminathan S, Eswaramoorthy S. Structural analysis of the catalytic and binding sites of Clostridium botulinum neurotoxin B. *Nat Struct Biol.* 2000;7(8):693-699.
40. Montecucco C. How do tetanus and botulinum toxins bind to neuronal membranes? *Trends in biochemical sciences.* 1986;11(8):314-317.
41. Rummel A. Double receptor anchorage of botulinum neurotoxins accounts for their exquisite neurospecificity. *Curr Top Microbiol Immunol.* 2013;364:61-90.
42. Binz T, Rummel A. Cell entry strategy of clostridial neurotoxins. *J Neurochem.* 2009;109(6):1584-1595. Epub 2009 Apr 1528.
43. Brunger AT, Rummel A. Receptor and substrate interactions of clostridial neurotoxins. *Toxicon.* 2009;54(5):550-560.
44. Ayyar BV, Aoki KR, Atassi MZ. The C-terminal heavy-chain domain of botulinum neurotoxin a is not the only site that binds neurons, as the N-terminal heavy-chain domain also plays a very active role in toxin-cell binding and interactions. *Infect Immun.* 2015;83(4):1465-1476.
45. Muraro L, Tosatto S, Motterlini L, Rossetto O, Montecucco C. The N-terminal half of the receptor domain of botulinum neurotoxin A binds to microdomains of the plasma membrane. *Biochem Biophys Res Commun.* 2009;380(1):76-80. Epub 2009 Jan 2020.
46. Zhang Y, Varnum SM. The receptor binding domain of botulinum neurotoxin serotype C binds phosphoinositides. *Biochimie.* 2012;94(3):920-923. Epub 2011 Nov 2018.
47. Fischer A, Montal M. Single molecule detection of intermediates during botulinum

neurotoxin translocation across membranes. *Proc Natl Acad Sci U S A.* 2007;104(25):10447-10452. Epub 12007 Jun 10411.
48. Montal M. Botulinum neurotoxin: a marvel of protein design. *Annu Rev Biochem.* 2010;79:591-617.
49. Pirazzini M, Tehran DA, Leka O, Zanetti G, Rossetto O, Montecucco C. On the translocation of botulinum and tetanus neurotoxins across the membrane of acidic intracellular compartments. *Biochim Biophys Acta.* 2015.
50. Matteoli M, Verderio C, Rossetto O, et al. Synaptic vesicle endocytosis mediates the entry of tetanus neurotoxin into hippocampal neurons. *Proc Natl Acad Sci U S A.* 1996;93(23):13310-13315.
51. Ledeen RW, Diebler MF, Wu G, Lu ZH, Varoqui H. Ganglioside composition of subcellular fractions, including pre- and postsynaptic membranes, from Torpedo electric organ. *Neurochem Res.* 1993;18(11):1151-1155.
52. Sonnino S, Mauri L, Chigorno V, Prinetti A. Gangliosides as components of lipid membrane domains. *Glycobiology.* 2007;17(1):1R-13R. Epub 2006 Sep 2018.
53. Rummel A, Mahrhold S, Bigalke H, Binz T. The HCC-domain of botulinum neurotoxins A and B exhibits a singular ganglioside binding site displaying serotype specific carbohydrate interaction. *Mol Microbiol.* 2004;51(3):631-643.
54. Rummel A, Eichner T, Weil T, et al. Identification of the protein receptor binding site of botulinum neurotoxins B and G proves the double-receptor concept. *Proc Natl Acad Sci U S A.* 2007;104(1):359-364. Epub 2006 Dec 2021.
55. Fu Z, Chen C, Barbieri JT, Kim JJ, Baldwin MR. Glycosylated SV2 and gangliosides as dual receptors for botulinum neurotoxin serotype F. *Biochemistry.* 2009;48(24):5631-5641.
56. Berntsson RP, Peng L, Dong M, Stenmark P. Structure of dual receptor binding to botulinum neurotoxin B. *Nat Commun.* 2013;4:2058.
57. Karalewitz AP, Kroken AR, Fu Z, Baldwin MR, Kim JJ, Barbieri JT. Identification of a unique ganglioside binding loop within botulinum neurotoxins C and D-SA. *Biochemistry.* 2010;49(37):8117-8126.
58. Strotmeier J, Gu S, Jutzi S, et al. The biological activity of botulinum neurotoxin type C is dependent upon novel types of ganglioside binding sites. *Mol Microbiol.* 2011;81(1):143-156. doi: 110.1111/j.1365-2958.2011.07682.x. Epub 02011 Jun 07682.
59. Karalewitz AP, Fu Z, Baldwin MR, Kim JJ, Barbieri JT. Botulinum neurotoxin serotype C associates with dual ganglioside receptors to facilitate cell entry. *J Biol Chem.* 2012;287(48):40806-40816. doi: 40810.41074/jbc.M40112.404244. Epub 402012 Oct 404241.
60. Strotmeier J, Lee K, Volker AK, et al. Botulinum neurotoxin serotype D attacks neurons via two carbohydrate-binding sites in a ganglioside-dependent manner. *Biochem J.* 2010;431(2):207-216.
61. Zhang Y, Buchko GW, Qin L, Robinson H, Varnum SM. Crystal structure of the receptor binding domain of the botulinum C-D mosaic neurotoxin reveals potential roles of lysines 1118 and 1136 in membrane interactions. *Biochem Biophys Res Commun.* 2011;404(1):407-412. Epub 2010 Dec 2013.
62. Dong M, Richards DA, Goodnough MC, Tepp WH, Johnson EA, Chapman ER. Synaptotagmins I and II mediate entry of botulinum neurotoxin B into cells. *J Cell Biol.* 2003;162(7):1293-1303. Epub 2003 Sep 1222.
63. Dong M, Tepp WH, Liu H, Johnson EA, Chapman ER. Mechanism of botulinum neurotoxin B and G entry into hippocampal neurons. *J Cell Biol.* 2007;179(7):1511-1522. Epub 2007 Dec 1524.
64. Mahrhold S, Rummel A, Bigalke H, Davletov B, Binz T. The synaptic vesicle protein 2C mediates the uptake of botulinum neurotoxin A into phrenic nerves. *FEBS Lett.* 2006;580(8):2011-2014. Epub 2006 Mar 2017.
65. Nishiki T, Kamata Y, Nemoto Y, et al. Identification of protein receptor for Clostridium botulinum type B neurotoxin in rat brain synaptosomes. *J Biol Chem.* 1994;269(14):10498-

10503.
66. Peng L, Berntsson RP, Tepp WH, et al. Botulinum neurotoxin D-C uses synaptotagmin I and II as receptors, and human synaptotagmin II is not an effective receptor for type B, D-C and G toxins. *J Cell Sci.* 2012;125(Pt 13):3233-3242. doi: 3210.1242/jcs.103564. Epub 102012 Mar 103527.
67. Rummel A, Karnath T, Henke T, Bigalke H, Binz T. Synaptotagmins I and II act as nerve cell receptors for botulinum neurotoxin G. *J Biol Chem.* 2004;279(29):30865-30870. Epub 32004 Apr 30830.
68. Nishiki T, Tokuyama Y, Kamata Y, et al. The high-affinity binding of Clostridium botulinum type B neurotoxin to synaptotagmin II associated with gangliosides GT1b/GD1a. *FEBS Lett.* 1996;378(3):253-257.
69. Chai Q, Arndt JW, Dong M, et al. Structural basis of cell surface receptor recognition by botulinum neurotoxin B. *Nature.* 2006;444(7122):1096-1100. Epub 2006 Dec 1013.
70. Jin R, Rummel A, Binz T, Brunger AT. Botulinum neurotoxin B recognizes its protein receptor with high affinity and specificity. *Nature.* 2006;444(7122):1092-1095. Epub 2006 Dec 1013.
71. Berntsson RP, Peng L, Svensson LM, Dong M, Stenmark P. Crystal Structures of Botulinum Neurotoxin DC in Complex with Its Protein Receptors Synaptotagmin I and II. *Structure.* 2013;21(9):1602-1611.
72. Willjes G, Mahrhold S, Strotmeier J, Eichner T, Rummel A, Binz T. Botulinum neurotoxin G binds synaptotagmin-II in a mode similar to that of serotype B: tyrosine 1186 and lysine 1191 cause its lower affinity. *Biochemistry.* 2013;52(22):3930-3938.
73. Benoit RM, Frey D, Hilbert M, et al. Structural basis for recognition of synaptic vesicle protein 2C by botulinum neurotoxin A. *Nature.* 2014;505(7481):108-111.
74. Dong M, Liu H, Tepp WH, Johnson EA, Janz R, Chapman ER. Glycosylated SV2A and SV2B mediate the entry of botulinum neurotoxin E into neurons. *Mol Biol Cell.* 2008;19(12):5226-5237. Epub 2008 Sep 5224.
75. Dong M, Yeh F, Tepp WH, et al. SV2 is the protein receptor for botulinum neurotoxin A. *Science.* 2006;312(5773):592-596. Epub 2006 Mar 2016.
76. Peng L, Tepp WH, Johnson EA, Dong M. Botulinum neurotoxin D uses synaptic vesicle protein SV2 and gangliosides as receptors. *PLoS Pathog.* 2011;7(3):e1002008. doi: 1002010.1001371/journal.ppat.1002008. Epub 1002011 Mar 1002031.
77. Rummel A, Hafner K, Mahrhold S, et al. Botulinum neurotoxins C, E and F bind gangliosides via a conserved binding site prior to stimulation-dependent uptake with botulinum neurotoxin F utilising the three isoforms of SV2 as second receptor. *J Neurochem.* 2009;110(6):1942-1954. Epub 2009 Jul 1923.
78. Sudhof TC, Rizo J. Synaptic vesicle exocytosis. *Cold Spring Harb Perspect Biol.* 2011;3(12).
79. Sudhof TC. The presynaptic active zone. *Neuron.* 2012;75(1):11-25.
80. Sudhof TC. Neurotransmitter release: the last millisecond in the life of a synaptic vesicle. *Neuron.* 2013;80(3):675-690.
81. Colasante C, Rossetto O, Morbiato L, Pirazzini M, Molgo J, Montecucco C. Botulinum neurotoxin type A is internalized and translocated from small synaptic vesicles at the neuromuscular junction. *Mol Neurobiol.* 2013;48(1):120-127.
82. Harper CB, Martin S, Nguyen TH, et al. Dynamin inhibition blocks botulinum neurotoxin type A endocytosis in neurons and delays botulism. *J Biol Chem.* 2011;286(41):35966-35976. Epub 32011 Aug 35965.
83. Pellett S, Tepp WH, Scherf JM, Johnson EA. Botulinum Neurotoxins Can Enter Cultured Neurons Independent of Synaptic Vesicle Recycling. *PLoS ONE.* 2015;10(7):e0133737.
84. Azarnia Tehran D, Zanetti G, Leka O, et al. A Novel Inhibitor Prevents the Peripheral Neuroparalysis of Botulinum Neurotoxins. *Scientific Reports.* 2015;5:17513.
85. Sun S, Tepp WH, Johnson EA, Chapman ER. Botulinum neurotoxins B and E translocate at different rates and exhibit divergent responses to GT1b and low pH. *Biochemistry.* 2012;51(28):5655-5662. Epub 2012 Jul 5652.
86. Simpson LL, Coffield JA, Bakry N. Inhibition of vacuolar adenosine triphosphatase antagonizes

the effects of clostridial neurotoxins but not phospholipase A2 neurotoxins. *J Pharmacol Exp Ther.* 1994;269(1):256-262.
87. Koriazova LK, Montal M. Translocation of botulinum neurotoxin light chain protease through the heavy chain channel. *Nat Struct Biol.* 2003;10(1):13-18.
88. Fischer A, Montal M. Molecular dissection of botulinum neurotoxin reveals interdomain chaperone function. *Toxicon.* 2013.
89. Galloux M, Vitrac H, Montagner C, et al. Membrane Interaction of botulinum neurotoxin A translocation (T) domain. The belt region is a regulatory loop for membrane interaction. *J Biol Chem.* 2008;283(41):27668-27676.
90. Sheridan RE. Gating and permeability of ion channels produced by botulinum toxin types A and E in PC12 cell membranes. *Toxicon.* 1998;36(5):703-717.
91. Sun S, Suresh S, Liu H, et al. Receptor binding enables botulinum neurotoxin B to sense low pH for translocation channel assembly. *Cell Host Microbe.* 2011;10(3):237-247. doi: 210.1016/j.chom.2011.1006.1012.
92. Fischer A, Nakai Y, Eubanks LM, et al. Bimodal modulation of the botulinum neurotoxin protein-conducting channel. *Proc Natl Acad Sci U S A.* 2009;106(5):1330-1335. Epub 2009 Jan 1321.
93. Fischer A, Montal M. Crucial role of the disulfide bridge between botulinum neurotoxin light and heavy chains in protease translocation across membranes. *J Biol Chem.* 2007;282(40):29604-29611. Epub 22007 Jul 29631.
94. Cai S, Kukreja R, Shoesmith S, Chang TW, Singh BR. Botulinum neurotoxin light chain refolds at endosomal pH for its translocation. *Protein J.* 2006;25(7-8):455-462.
95. Kukreja R, Singh B. Biologically active novel conformational state of botulinum, the most poisonous poison. *J Biol Chem.* 2005;280(47):39346-39352.
96. Eswaramoorthy S, Kumaran D, Keller J, Swaminathan S. Role of Metals in the Biological Activity of Clostridium botulinum Neurotoxins†,‡. *Biochemistry.* 2004;43(8):2209-2216.
97. Fu FN, Busath DD, Singh BR. Spectroscopic analysis of low pH and lipid-induced structural changes in type A botulinum neurotoxin relevant to membrane channel formation and translocation. *Biophys Chem.* 2002;99(1):17-29.
98. Puhar A, Johnson EA, Rossetto O, Montecucco C. Comparison of the pH-induced conformational change of different clostridial neurotoxins. *Biochem Biophys Res Commun.* 2004;319(1):66-71.
99. Montecucco C, Schiavo G, Dasgupta BR. Effect of pH on the interaction of botulinum neurotoxins A, B and E with liposomes. *Biochem J.* 1989;259(1):47-53.
100. 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(11):1731-1743. doi: 1710.1111/j.1462-5822.2011.01654.x. Epub 02011 Aug 01625.
101. Pirazzini M, Henke T, Rossetto O, et al. Neutralisation of specific surface carboxylates speeds up translocation of botulinum neurotoxin type B enzymatic domain. *FEBS Lett.* 2013.
102. Nordera P, Serra MD, Menestrina G. The adsorption of Pseudomonas aeruginosa exotoxin A to phospholipid monolayers is controlled by pH and surface potential. *Biophys J.* 1997;73(3):1468-1478.
103. Schiavo G, Papini E, Genna G, Montecucco C. An intact interchain disulfide bond is required for the neurotoxicity of tetanus toxin. *Infect Immun.* 1990;58(12):4136-4141.
104. Fisher A, Montal M. Characterization of Clostridial botulinum neurotoxin channels in neuroblastoma cells. *Neurotox Res.* 2006;9(2-3):93-100.
105. Pirazzini M, Tehran DA, Zanetti G, et al. The thioredoxin reductase - Thioredoxin redox system cleaves the interchain disulphide bond of botulinum neurotoxins on the cytosolic surface of synaptic vesicles. *Toxicon.* 2015;107(Pt A):32-36.
106. Arner ES, Holmgren A. Physiological functions of thioredoxin and thioredoxin reductase. *Eur J Biochem.* 2000;267(20):6102-6109.
107. Hanschmann EM, Godoy JR, Berndt C, Hudemann C, Lillig CH. Thioredoxins, glutaredoxins,

and peroxiredoxins-molecular mechanisms and health significance: from cofactors to antioxidants to redox signaling. *Antioxid Redox Signal.* 2013;19(13):1539-1605.
108. Holmgren A, Lu J. Thioredoxin and thioredoxin reductase: current research with special reference to human disease. *Biochem Biophys Res Commun.* 2010;396(1):120-124.
109. Powis G, Kirkpatrick DL. Thioredoxin signaling as a target for cancer therapy. *Curr Opin Pharmacol.* 2007;7(4):392-397.
110. Pirazzini M, Bordin F, Rossetto O, Shone CC, 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 Lett.* 2013;587(2):150-155.
111. Pirazzini M, Azarnia Tehran D, Zanetti G, et al. Thioredoxin and its reductase are present on synaptic vesicles, and their inhibition prevents the paralysis induced by botulinum neurotoxins. *Cell Rep.* 2014;8(6):1870-1878.
112. Zanetti G, Azarnia Tehran D, Pirazzini M, et al. Inhibition of botulinum neurotoxins interchain disulfide bond reduction prevents the peripheral neuroparalysis of botulism. *Biochem Pharmacol.* 2015.
113. Ratts R, Zeng H, Berg EA, et al. The cytosolic entry of diphtheria toxin catalytic domain requires a host cell cytosolic translocation factor complex. *J Cell Biol.* 2003;160(7):1139-1150.
114. Schiavo G, Santucci A, Dasgupta BR, et al. Botulinum neurotoxins serotypes A and E cleave SNAP-25 at distinct COOH-terminal peptide bonds. *FEBS Lett.* 1993;335(1):99-103.
115. Blasi J, Chapman ER, Link E, et al. Botulinum neurotoxin A selectively cleaves the synaptic protein SNAP-25. *Nature.* 1993;365(6442):160-163.
116. Schiavo G, Benfenati F, Poulain B, et al. Tetanus and botulinum-B neurotoxins block neurotransmitter release by proteolytic cleavage of synaptobrevin. *Nature.* 1992;359(6398):832-835.
117. Schiavo G, Shone CC, Rossetto O, Alexander FC, Montecucco C. Botulinum neurotoxin serotype F is a zinc endopeptidase specific for VAMP/synaptobrevin. *J Biol Chem.* 1993;268(16):11516-11519.
118. Schiavo G, Rossetto O, Catsicas S, et al. Identification of the nerve terminal targets of botulinum neurotoxin serotypes A, D, and E. *J Biol Chem.* 1993;268(32):23784-23787.
119. Sutton RB, Fasshauer D, Jahn R, Brunger AT. Crystal structure of a SNARE complex involved in synaptic exocytosis at 2.4 A resolution. *Nature.* 1998;395(6700):347-353.
120. Schiavo G, Matteoli M, Montecucco C. Neurotoxins affecting neuroexocytosis. *Physiol Rev.* 2000;80(2):717-766.
121. Binz T. Clostridial neurotoxin light chains: devices for SNARE cleavage mediated blockade of neurotransmission. *Curr Top Microbiol Immunol.* 2013;364:139-157.
122. Binz T, Blasi J, Yamasaki S, et al. Proteolysis of SNAP-25 by types E and A botulinal neurotoxins. *J Biol Chem.* 1994;269(3):1617-1620.
123. Rossetto O, Schiavo G, Montecucco C, et al. SNARE motif and neurotoxins. *Nature.* 1994;372(6505):415-416.
124. Yamasaki S, Hu Y, Binz T, et al. Synaptobrevin/vesicle-associated membrane protein (VAMP) of Aplysia californica: structure and proteolysis by tetanus toxin and botulinal neurotoxins type D and F. *Proc Natl Acad Sci U S A.* 1994;91(11):4688-4692.
125. Sikorra S, Henke T, Galli T, Binz T. Substrate recognition mechanism of VAMP/synaptobrevin-cleaving clostridial neurotoxins. *J Biol Chem.* 2008;283(30):21145-21152. Epub 22008 May 21129.
126. Binz T, Sikorra S, Mahrhold S. Clostridial neurotoxins: mechanism of SNARE cleavage and outlook on potential substrate specificity reengineering. *Toxins (Basel).* 2010;2(4):665-682.
127. Breidenbach MA, Brunger AT. Substrate recognition strategy for botulinum neurotoxin serotype A. *Nature.* 2004;432(7019):925-929. Epub 2004 Dec 2012.
128. Mazuet C, Ezan E, Volland H, Popoff MR, Becher F. Toxin detection in patients' sera by mass spectrometry during two outbreaks of type A Botulism in France. *J Clin Microbiol.* 2012;50(12):4091-4094.
129. Webb RP, Smith TJ, Wright P, Brown J, Smith LA. Production of catalytically inactive BoNT/A1

holoprotein and comparison with BoNT/A1 subunit vaccines against toxin subtypes A1, A2, and A3. *Vaccine.* 2009;27(33):4490-4497.
130. Ruthel G, Burnett JC, Nuss JE, et al. Post-Intoxication Inhibition of Botulinum Neurotoxin Serotype A within Neurons by Small-Molecule, Non-Peptidic Inhibitors. *Toxins (Basel).* 2011;3(3):207-217. Epub 2011 Mar 2015.
131. Simpson L. The life history of a botulinum toxin molecule. *Toxicon.* 2013;68:40-59.