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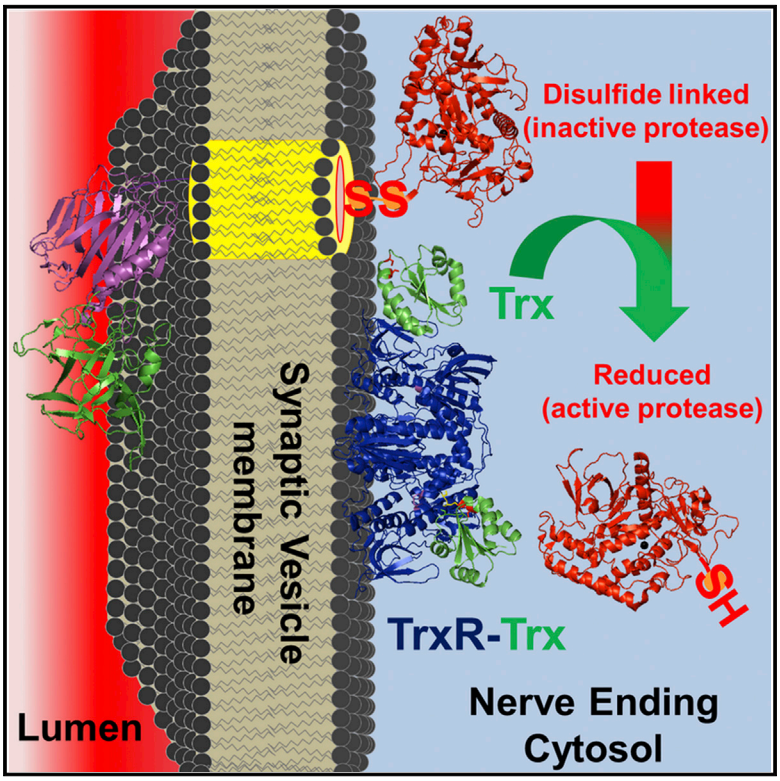
Thioredoxin and Its Reductase Are Present on Synaptic Vesicles, and Their Inhibition Prevents the Paralysis Induced by Botulinum Neurotoxins

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

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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.

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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

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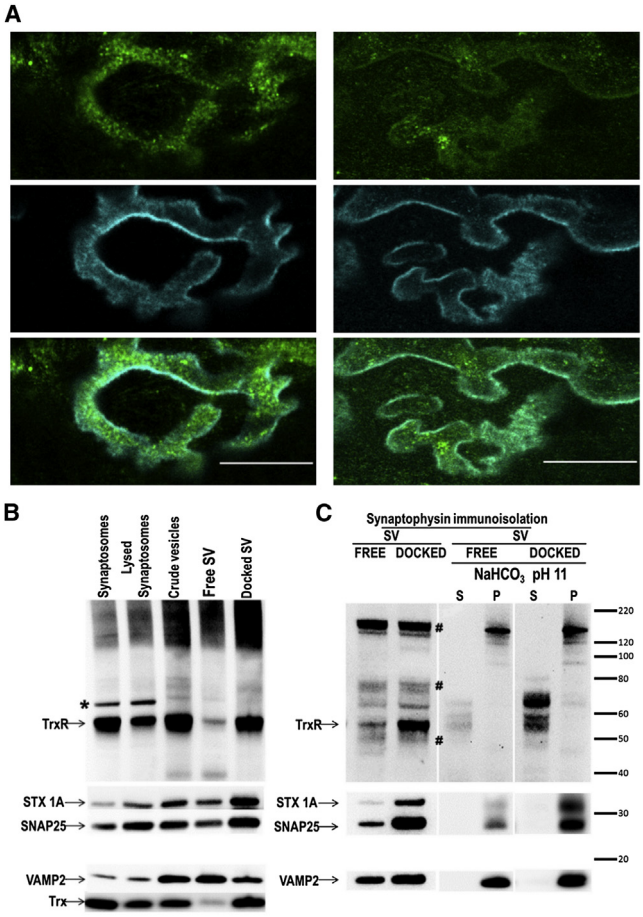

Figure 1. Thioredoxin Reductase and Thioredoxin Are Present in Nerve Terminals and Are Loosely Bound to the Surface of Synaptic Vesicles

(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

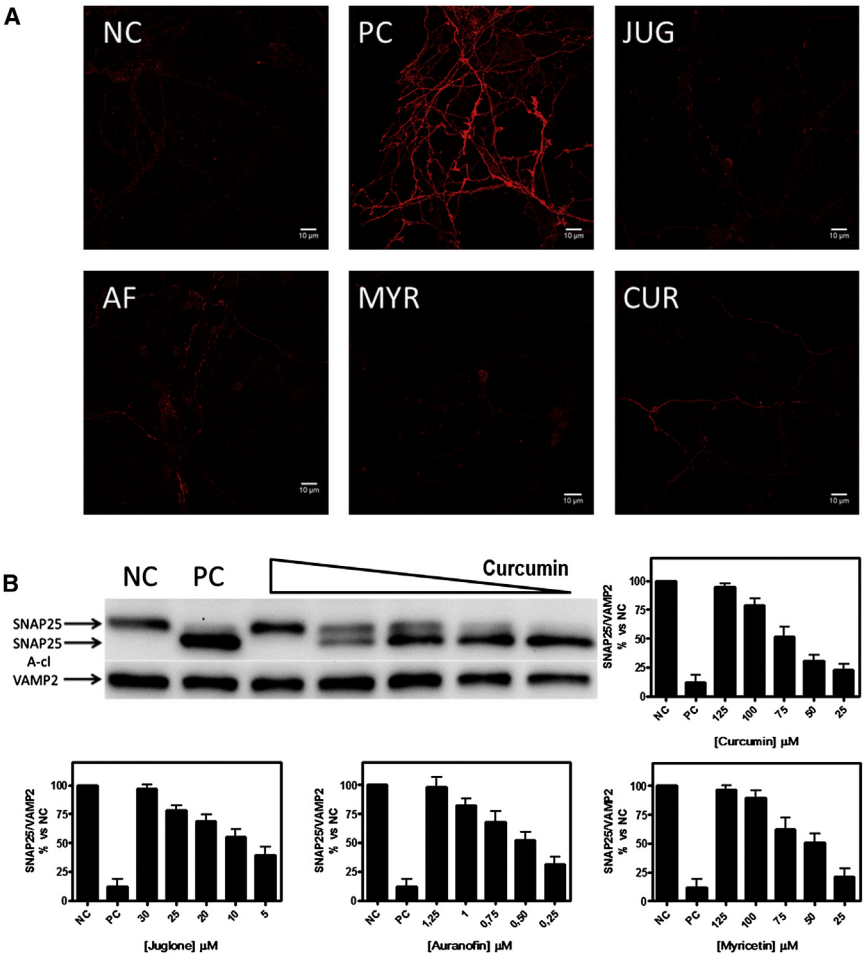

Figure 2. The BoNT/A-Induced Cleavage of SNAP25 Is Prevented in Cerebellar Granular Neurons by Thioredoxin Reductase Inhibitors

(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

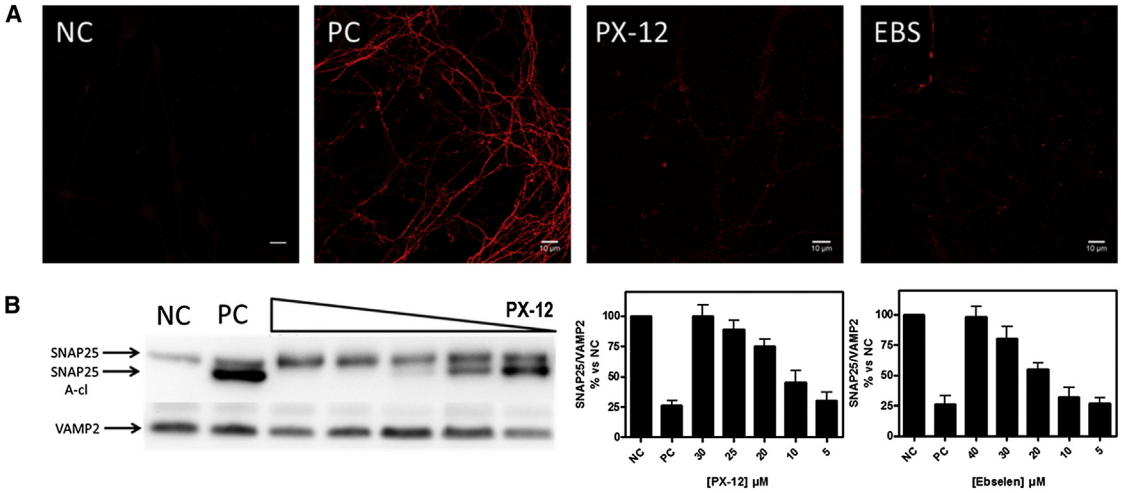

Figure 3. Inhibitors of Thioredoxin Prevent the SNAP25 Cleavage by BoNT/A in Cerebellar Granular Neurons

(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.

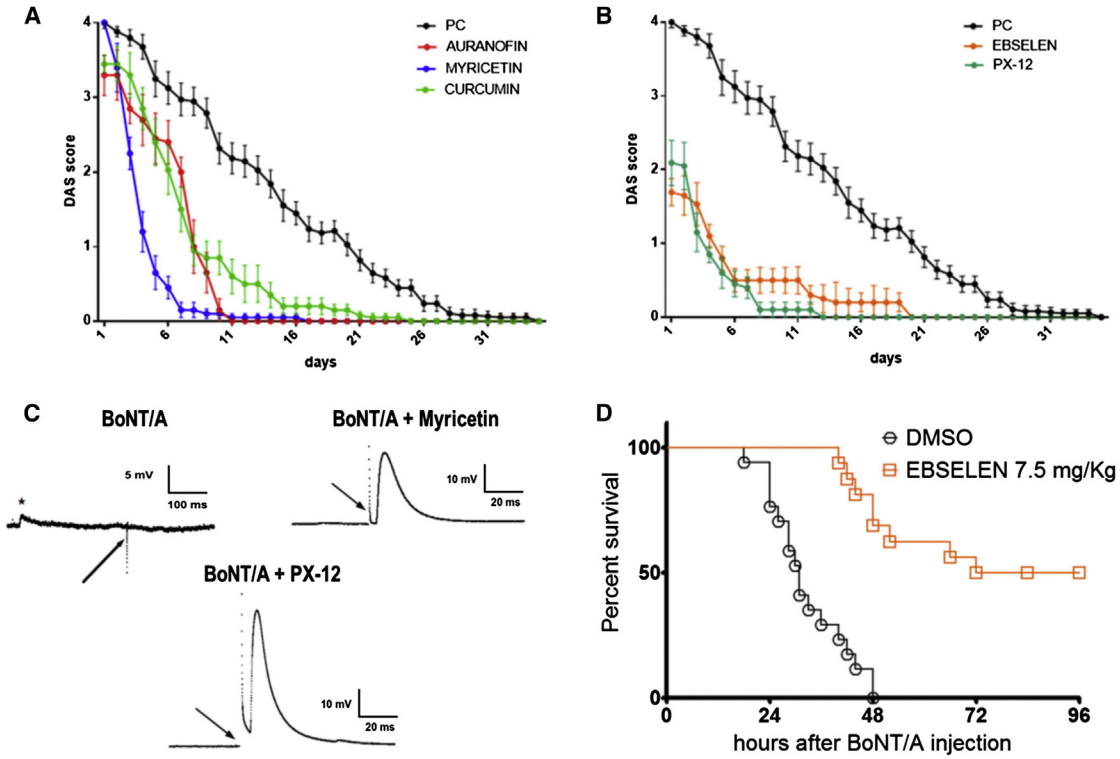

Figure 4. Thioredoxin and Thioredoxin Reductase Inhibitors Largely Prevent the Local Paralysis and Death Induced by BoNT/A

(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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