Hsp90 is involved in the entry of clostridial neurotoxins into the cytosol of nerve terminals

toxins

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Hsp90 Facilitates the Folding of Clostridial Neurotoxins Enzymatic Domain into Host Cells Cytosol and Guarantees Their Neurotoxicity

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Abstract: Botulinum and tetanus neurotoxins are the most toxic substances known and form the growing family of clostridial neurotoxins (CNTs). They are composed of a metalloprotease light chain (L), linked via a disulfide bond to a heavy chain (H). H mediates binding to nerve terminals and the membrane translocation of L into the cytosol where their substrates, the three SNARE proteins, are localized. L translocation is accompanied by unfolding and L has to be reduced and reacquire the native fold to exert its neurotoxicity. The Thioredoxin reductase-Thioredoxin system is responsible for the reduction, but it is unknown whether the refolding of L is spontaneous or aided by host chaperones. Here we report that geldanamycin, a specific inhibitor of Hsp90, hampers the refolding of L after membrane translocation and completely prevents the cleavage of SNAREs. We also found that the effect of geldanamycin strongly synergises with that of PX-12, an inhibitor of thioredoxin, suggesting that the processes of L chain refolding and interchain disulphide reduction are strictly coupled. Indeed we found that Hsp90 and the Thioredoxin reductase-Thioredoxin system physically interact on synaptic vesicle where they orchestrate a chaperone-redox machinery which is exploited by CNTs to deliver their catalytic part in the cytosol.

Keywords: botulinum neurotoxins; tetanus neurotoxin; heat shock protein 90; geldanamycin; PX-12; neuroexocytosis; synaptic vesicles;

1. Introduction

The seven serotypes of botulinum neurotoxins (BoNTs), which include a very large number of sub-types [1], form with tetanus neurotoxin (TeNT) the family of clostridial neurotoxins (CNTs) [2]. BoNTs are the etiological agents of botulism, a neuroparalytic syndrome whose major symptom is an extended flaccid paralysis [3] whilst TeNT causes tetanus, a disease characterised by spastic paralysis which still is a major cause of death in non-vaccinated populations [4]. BoNTs and TeNT are very similar toxins, both from a structural and biochemical point of view, and their opposite clinical manifestations are due to their divergent journey inside the host [5, 6]. Indeed, the main BoNTs site of action is the neuromuscular junction where they block the release of acetylcholine, whilst TeNT reaches the spinal cord via retroaxonal transport and prevents the release of inhibitory neurotransmitters [4, 6, 7].

CNTs are the deadliest substances known with lethal doses ranging in the order of few ng/kg [8]. Such a toxicity is ascribed to potency and specificity, two properties that are the final result of an efficient molecular architecture evolutionary shaped to exploit essential processes of vertebrates nervous system physiology [9]. CNTs structure is composed by a catalytic light chain (L, 50 kDa) that is disulphide-linked to a heavy chain (H, 100 kDa), which is responsible for neurospecific binding and delivery of L into nerve terminals [10-12]. The C-terminal part of the H chain (HC) mediates the binding to peripheral nerve terminals [2, 13-15] and the ensuing trafficking within specific vesicles which provide either the tropism of BoNTs for the neuromuscular junction [16] or the retrograde axonal transport of TeNT toward the spinal cord [4, 7, 17]. The N-terminal part of H (HN) mediates the translocation of L across the membrane of the endocytic organelles [18-22]. L is a metalloprotease which specifically cleaves one of the SNARE proteins that form the core of the neuroexocytosis nanomachine [23, 24]: the L of BoNT/A and /E targets SNAP-25 [25-28], that of BoNT/B, /D, /F, /G and TeNT removes the largest part of the cytosolic domain of VAMP-1/2 [27, 29-32] whilst the L chain of BoNT/C is the sole having more than one substrates, i.e. SNAP-25 and some isoforms of Syntaxins [33, 34].

CNT's mechanism of nerve intoxication consists of 5 main steps [35] i) binding to nerve terminals, ii) internalisation within endocytic compartments, iii) low-pH induced translocation of L, iv) cytosolic reduction of the interchain disulphide bond and v) proteolysis of SNAREs. Among these several steps, the molecular mechanism underlying the translocation of the enzymatic domain into neuronal cytosol is the least understood [22], though it is long known that the process is initiated when the organelles exploited for internalization becomes acidic [36, 37]. HN and L undergo a concerted structural change that mediates the passage of the enzymatic domain across the

membrane with the formation of an ion-conducting channel [2, 21, 22, 38]. It is not clear if channel formation is a prerequisite or a consequence of translocation [22], but it is reported that L has to undergo partial unfolding in order to cross the membrane, otherwise the translocation is abortive [20]. In fact, cargo proteins, which do not unfold at low pH attached to the amino terminus of BoNTs, drastically reduce the delivery of the L into cell cytosol [39]. As a result of its unfolding, once it has reached the cytosolic face of the membrane, the L chain has to reacquire the native structure in order to cleave its substrates. It is presently unknown whether this process is spontaneous or it is assisted by host chaperone proteins, which normally reside on the organelle membrane [40].

The involvement of host chaperones such as heat shock protein 90 (Hsp90), peptidyl prolyl *cis*/*trans* isomerases (PPIases) and protein disulphide isomerase (DPI) in the entry of several plant and bacterial exotoxins into the cytosol has been widely documented [41-48].

Recently, we found that the Thioredoxin Reductase-thioredoxin system (TrxR-Trx) is responsible for the cytosolic reduction of BoNTs and TeNT interchain disulphide bond [35, 49-51], a molecular event, which is necessary to terminate the translocation step [20, 21, 38] and enable the metalloprotease activity [26, 27, 31]. Since TrxR and Hsp90 were previously found to be fundamental in mediating both the refolding and the release of diphtheria toxin catalytic subunit [42], in the present study, we have tested the possible involvement of this cytosolic chaperone also in the uptake of CNTs enzymatic domain inside neurons. Using geldanamycin (GA), a specific and potent inhibitor of Hsp90, we found that this drug prevents CNTs toxicity in primary cell culture. We demonstrate that GA interferes with the translocation process, most likely by inhibiting the cytosolic refolding of L, but not with the other essential steps of CNTs mechanism of intoxication. Moreover, by blocking at the same time Hsp90 and the TrxR-Trx system, we observed a strong synergistic inhibitory effect indicating a combined action of these systems in the entry of the L chain into the cytosol.

2. Results

2.1. Hsp90 pharmacological inhibition protects primary neurons from CNTs intoxication

The involvement of Hsp90 in the uptake of several bacterial toxins was suggested by pharmacological studies using specific inhibitors [41]. The most specific one, geldanamycin (GA) is a benzoquinone antibiotic that binds the ATP-binding pocket of Hsp90 thereby inhibiting its chaperone activity thus allowing the degradation of its client proteins [52]. To test the possible involvement of Hsp90 in CNTs entry, we treated primary cerebellar granule neurons (CGNs) either with BoNTs or TeNT in the presence of GA and we evaluated their toxicity by using two complementary read-outs: Western blotting (WB) and immunofluorescence (IF) (Figure 1-2). We

started with BoNT/A, which is the most common cause of human botulism [3] and the one almost invariably used in human therapy [53-55]. As expected, when BoNT/A is added to neurons, it cleaves SNAP-25, as assessed by the appearance in WB of its truncated form (SNAP-25$_A$), detected with a specific antibody (Figure 1A, lane PC). The same result is evident also via IF analysis (Figure 1B, middle panel). It is worth to note that the amount of BoNT/A used causes the almost complete cleavage of SNAP-25, as determined with an antibody that recognizes both the intact and the cleaved form of SNAP-25 (Figure S1). Whatever the read-out used, addition of GA significantly protects neurons from BoNT/A. The inhibition is concentration-dependent and complete at 12.5 μM, as indicated by the absence of SNAP-25$_A$ in WB (Figure 1A and Figure S1) and IF (Figure 1B, compare middle with right panel). As Hsp90 is involved in multiple aspects of cell physiology [56], GA is highly toxic. Accordingly, the experiment was conducted by pretreating CGNs with GA for 30 minutes and concluded within 4 hours after toxin addition (see M&M) to avoid the toxicity of the drug that develops with longer incubation times (not shown); under the experimental conditions used here cell viability was not significantly affected (Figure S2).

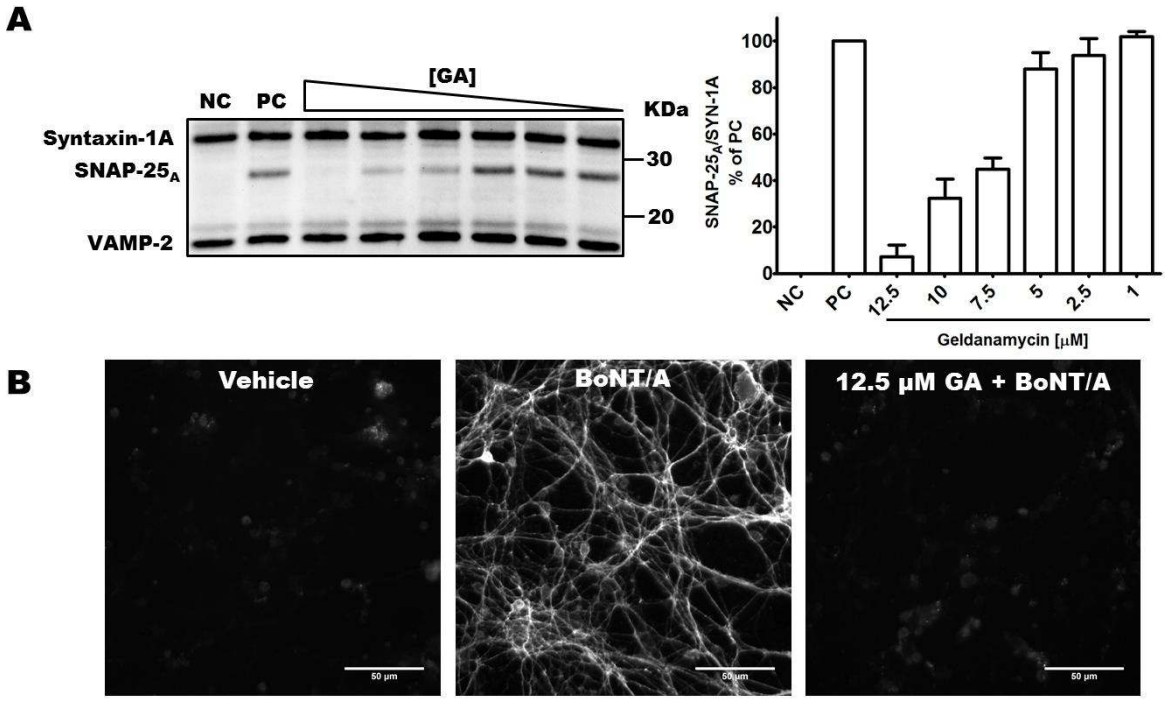

Figure 1. Geldanamycin abolishes SNAP-25 cleavage in neurons treated with BoNT/A. (A) CGNs were pretreated for 30 minutes with the indicated concentrations of GA at 37 °C; then BoNT/A was added (2.5 nM final concentration) and the incubation prolonged for 3.5 hours. Thereafter, neurons were lysed and the cleavage of SNAP-25 estimated with a specific antibody against the BoNT/A truncated form. VAMP-2 and Syntaxin 1A were used as loading controls. A typical immunoblot is reported on the left, while the right panel shows the quantification of cleaved SNAP-25 as a ratio to Syntaxin 1A, taking the value in non-treated cells (NC) as 100%. SD values derive from at least three independent experiments performed in triplicates. (B) Neurons were treated as described in A, but the experiment was stopped by fixation and the presence of SNAP-25$_A$ evaluated via IF, using an

125 antibody specific for the BoNT/A truncated form. All the images are representative of three
126 independent experiments.

127 We extended our investigations to other members of CNTs family, the serotype D of BoNT
128 (BoNT/D), a major cause of animal botulism [57] and TeNT, which is the sole responsible of tetanus.
129 We choose these CNTs because, at variance from BoNT/A which acts on SNAP-25, they cleave
130 VAMP-1/2 and at two different peptide bonds [27, 31]. Figures 2A and 2C show that these two toxins
131 cause the complete disappearance of VAMP-2 staining and that the addition of GA caused a
132 remarkable and concentration-dependent protection from the intoxication (Figure 2A and Figure
133 2C). Consistently, IF analysis (Figure 2B and Figure 2D) shows that these two neurotoxins cause in
134 CGNs a clear decrease of VAMP-2 (middle panels) that is prevented by treating neurons with GA
135 (right panels).

136 Taken together, these data clearly indicate that, despite the different protein receptors and
137 intracellular substrates, the inhibition of Hsp90 prevents the entry of enzymatically active L chains
138 of different CNTs into the host cell cytosol and the ensuing cleavage of their respective cytosolic
139 substrates.

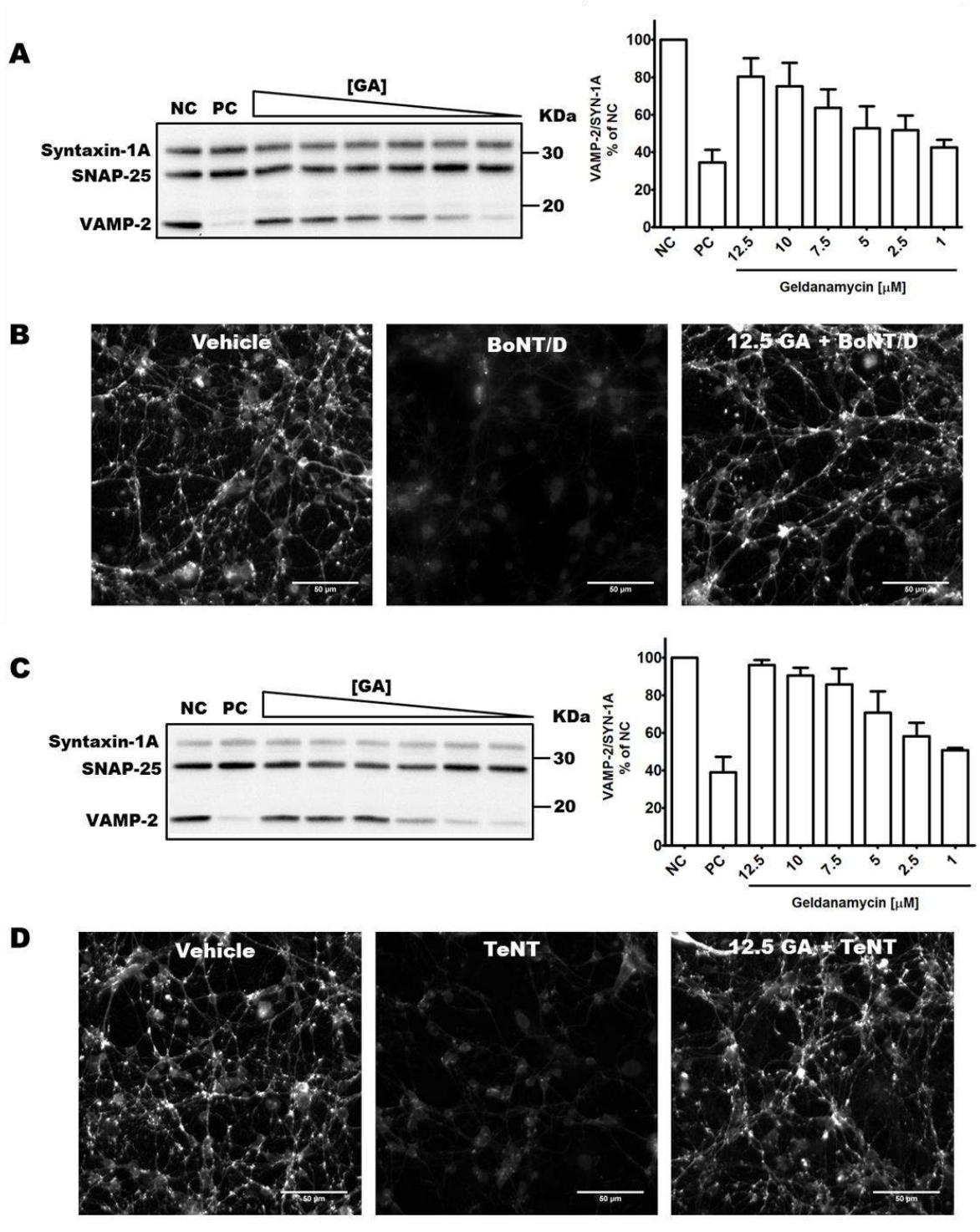

Figure 2. Geldanamycin abolishes the cleavage of VAMP-2 in neurons intoxicated with BoNT/D and TeNT. CGNs were treated like in Figure 1 using BoNT/D (A-B, 0.01 nM final concentration) or TeNT (C-D, 0.5 nM final concentration). BoNT/D was added as a pulse of 15 minutes after which culture medium with the same concentration of GA was restored for further 3.5 hours. Thereafter, neurons were lysed and the VAMP-2 content estimated with an antibody, which recognizes the intact form of the protein. SNAP-25 and Syntaxin 1A were used as loading controls. Panels on the left reports typical immunoblots while panels on the right show the quantification of residual VAMP-2 reported as a ratio to Syntaxin 1A, taking the value of non-treated cells (NC) as 100%. SD values derive from three independent experiments performed at least in triplicates. (B-D) CGNs were treated as in A and B but the experiment was stopped by fixation and evaluated by immunofluorescence using an

antibody, which detect only the intact form of VAMP-2. All the images are representative of three independent sets of experiments.

The involvement of molecular chaperones different from Hsp90 in membrane translocation of several exotoxins was previously suggested [44, 46, 58, 59]. Accordingly, we took advantage by the existence of specific inhibitors such as cyclosporine A (CsA) which specifically targets Cyclophilin A [60], and VER-155008, a general inhibitor of the ATPase activity of Hsp70s [61] to test the possible role in CNTs toxicity of these chaperones. We also synthetized an Acridizinium derivative, dubbed "compound 2", which potently inhibits Hsc70 [59], the constitutively expressed isoform of Hsp70. Remarkably, none of these molecules impacted on the cleavage of SNAP-25 by BoNT/A (Figure S3), or of VAMP-2 by BoNT/D and TeNT (not shown), strongly suggesting the primary role of Hsp90 in supporting CNTs toxicity.

2.2. Geldanamycin inhibits the L chain refolding after its pH-dependent membrane translocation

The remarkable inhibitory activity of GA on BoNT/A, BoNT/D and TeNT cleavage of their SNARE substrates called for a study of the effect of this drug on the various steps of their mechanism of action in order to identify which of them is affected.

First, we investigated whether the inhibition of Hsp90 prevents the binding of CNTs to CGNs plasma membrane and their subsequent internalization into endosomal compartments. Neurons were pretreated either with the vehicle or with GA at a concentration causing the complete inhibition of BoNT/A activity. CGNs were then incubated for 30 minutes with the BoNT/A binding domain fused to circularly permutated Venus (cpV-HC/A), used as reporter for WB analysis. Figure 3A shows that binding and internalization of cPV-HC/A were not affected by GA. The same experiment was repeated using as a reporter an antibody against the lumenal portion of the SV integral membrane protein synaptotagmin-1, to check synaptic vesicles (SVs) dynamics [62] upon GA treatment. Figure S4 shows that in control condition the antibody is incorporated through SVs cycle and can be detected by WB (lane vehicle). On the contrary, when treated with BoNT/D, which prevents the assembly of a functional SNARE complex and therefore blocks SVs dynamics, CGNs do not take up the antibody (lane BoNT/D). In GA-treated neurons the amount of internalized antibody was comparable to that of vehicle-treated neurons (lane GA), suggesting that the trafficking of the organelles used by BoNT/A [16, 63, 64], TeNT [65] and possibly BoNT/D for internalization within nerve terminals is not altered by the drug. These two complementary approaches therefore indicate that Hsp90 inhibition does not affect toxin binding and endocytosis in cultured neurons.

Next, we investigated the effect of GA on endosomes acidification, which is strictly required for the membrane translocation of all CNTs [2, 66]. We used Lysotracker Red DND-99, a sensitive dye

which labels and tracks acidic organelles in living cells. Figure 3B shows that GA does not interfere with organelle acidification at variance from bafilomycin A1, which is a specific inhibitor of the vacuolar H^+-ATPases [67], and is used here as positive control.

Finally, Figure 3C shows that GA does not interfere with the metalloproteolytic activity of CNTs on SNAREs, using BoNT/D cleavage on VAMP-2 as an example. Similar results were obtained with BoNT/A and TeNT using their respective substrates (data not shown).

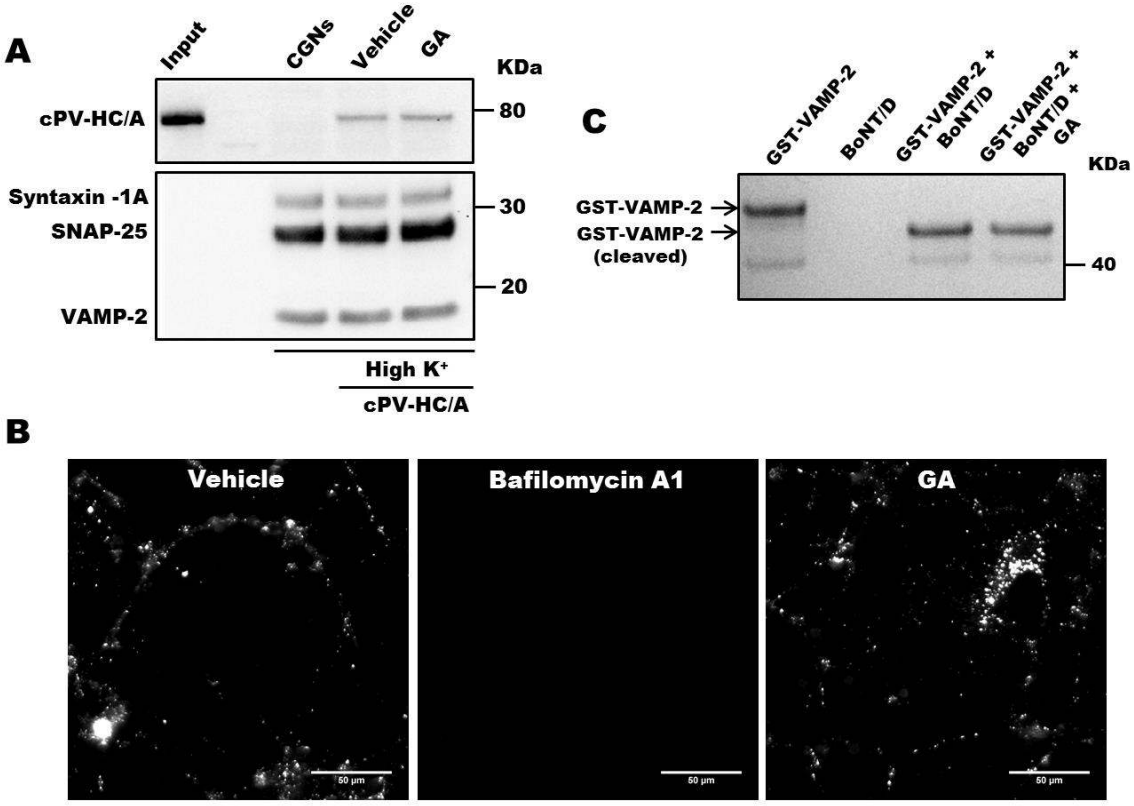

Figure 3. Geldanamycin has no effect on CNTs binding, endosome acidification and *in vitro* metalloprotease activity. (A) CGNs were pretreated with geldanamycin (GA, 12.5 μM) or vehicle (DMSO) at 37 °C for 30 minute after which, cpV-HC/A (250 nM final concentration) was added in high K^+ buffer for further 30 minutes. Neurons were then washed, lysed and the cpV-HC/A amount was estimated by WB with an antibody against GFP. Syntaxin 1A, SNAP-25 and VAMP-2 are used as loading controls. In the first lane (input) 50 ng of the cpV-HC/A were loaded as reference. The immunoblot is representative of three independent sets of experiments. (B) CGNs were treated with vehicle (DMSO) or 12.5 μM of GA or 50 nM bafilomycin A1 for 30 minutes at 37 °C. Then, Lysotracker Red was added for 1 hour and neurons were imaged by fluorescence microscopy. Images are representative of two independent experiments. (C) BoNT/D (100 ng) was reduced with 10 mM DTT in the presence of 12.5 μM GA or vehicle (DMSO) for 30 minutes at 37 °C before adding 1 μg of GST-VAMP-2. The reaction was carried out for 4 hours at 37 °C. VAMP-2 cleavage was assessed by SDS-PAGE. A representative image is shown.

Taken together the previous results reinforce the suggestion that Hsp90 might be involved in assisting and promoting the metalloprotease L chain to reacquire its enzymatically active form after its translocation into the cytosol. If this is the case, GA is expected to impact directly on the translocation process. To test this idea, we took advantage of an intoxication method, dubbed "pH

jump", which induces the entry of L from the plasma membrane [68-70]. As shown in Figure 4, by lowering the pH to 4.5, BoNT/D L chain is productively translocated across the plasma membrane of CGNs and delivered into the cytosol in its active form, as indicated by the cleavage of VAMP-2 (lane "4.5"). The assay is amenable to manipulation, indeed the delivery of the L chain, and the ensuing cleavage of VAMP-2, can be prevented by PX-12 (lane PX-12), a known inhibitor of Trx [71] which blocks the release of the metalloprotease in the cytosol by interfering with the reduction of the interchain disulphide bridge [49, 50]. Accordingly, the absence of VAMP-2 cleavage upon treatment with GA (lane GA), suggests that this drug directly impacts on the translocation, likely on the L chain refolding.

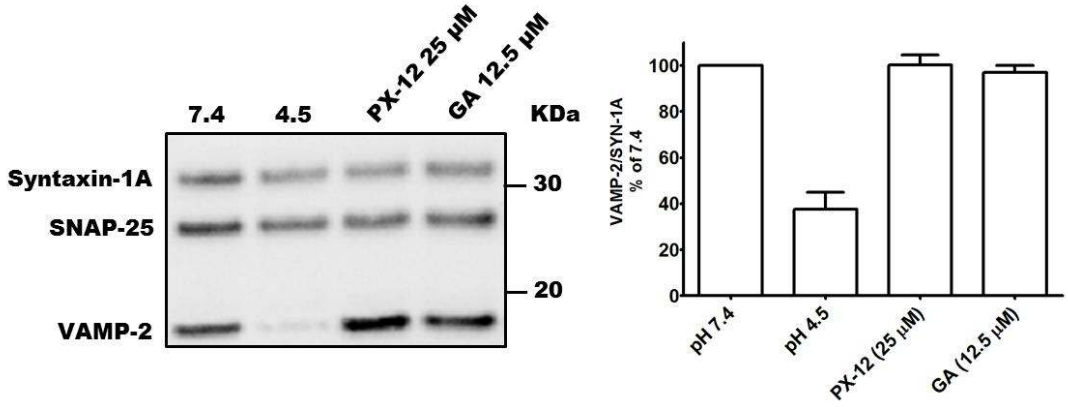

Figure 4. PX-12 and geldanamycin inhibit L translocation across the membrane. CGNs were treated with 25 μM of PX-12 or 12. 5 μM GA for 30 min at 37 °C. Thereafter, BoNT/D (200 pM) was added in the cold for 15 minutes. Neurons were then washed and incubated for 10 min with medium A buffered at indicated pH at 37 °C supplemented with inhibitors or vehicle. Then, cells were washed and incubated for 3.25 hours in complete medium containing 50 nM bafilomycin. The translocation of BoNT/D was assessed by monitoring the cleavage of VAMP-2, determined via Western blot as a ratio to Syntaxin 1A which served as internal control, taking the value at pH 7.4 as 100%. SD values derive from three independent experiments performed in triplicates.

2.3. Hsp90 and Trx-TrxR are present on synaptic vesicles and are essential to the release of active L chain in the cytosol

The effect of PX-12 indicates the essential role of the Thioredoxin-Thioredoxin reductase system in reducing the interchain disulfide bond as a prerequisite for the intracellular activity of the L chain [2, 50]. This redox system was previously suggested to form with Hsp90 a complex that plays an essential role in the entry of diphtheria toxin into the cytosol [42]. As the CNTs are similarly composed of a catalytic domain disulphide-linked to a subunit mediating binding and translocation, we reasoned that the two processes of disulfide reduction and cytosolic refolding may be coupled and that Hsp90 may act in concert with the Trx-TrxR system to enable the catalytic activity of the L chain in the cytosol. If this is the case, the addition at the same time of GA and of PX-12 should

produce a synergistic effect, whilst if the two systems act in a non-concerted way, the simultaneous presence of the two inhibitors should give an additive effect. This possibility was tested with the pH jump assay, in order to test the synergism of the drugs on the translocation step in a direct way. CGNs were treated with either PX-12 or GA or with their combination at concentrations, which do not cause by themselves any inhibition (2.5 μM each; Figure S5). Figure 5 shows that a very high level of inhibition is achieved in this way, strongly supporting the possibility of a concerted action of Hsp90 and TrxR-Trx in enabling the proteolytic activity of the L chain.

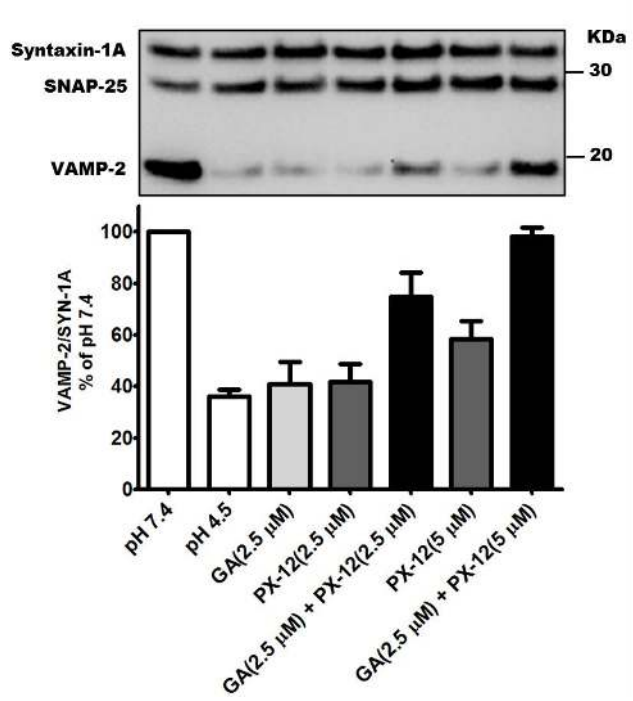

Figure 5. PX-12 synergizes the effect of Geldanamycin in inhibiting the delivery of BoNT/D L into the cytosol. CGNs were treated like in Figure 4 with the indicated concentration of PX-12, GA, or their combination. The translocation of L was assessed by monitoring the cleavage of VAMP-2, determined via WB as a ratio to Syntaxin1A, which served as internal control, tacking the value at pH 7.4 as 100%. SD values derive from two independent experiments performed in triplicates.

This result prompted us to assay for a physical interaction between Hsp90 and TrxR-Trx on synaptic vesicles, the organelle wherefrom BoNT/A translocates its L chain (Colasante et al., 2013). Figure 6A shows that rat brain synaptosomes contain both Hsp90 and Trx-TrxR and that both proteins are also present on isolated SVs. Intriguingly, the relative amount of Hsp90 increases in the fraction of docked SVs, as it is the case for Trx and TrxR [50], opening the possibility of their direct interaction on the cytosolic face of SV. Starting from crude SVs, i.e. a pool of free and docked SVs, we immunoprecipitated Hsp90 and assayed by WB for the presence of Trx and TrxR. Figure 6B shows that the three proteins physically interact and precipitate together with Rab3a which was previously reported to co-immunoprecipitate with Hsp90 [72].

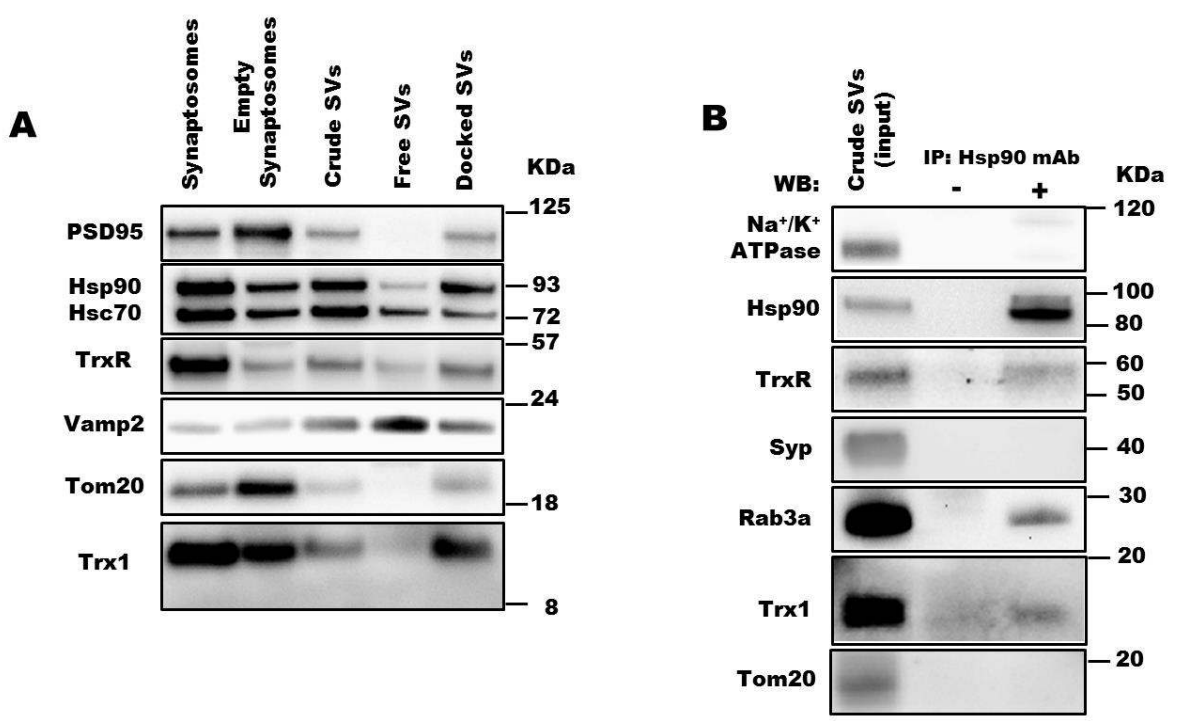

Figure 6. Hsp90 interacts with TrxR-Trx system on synaptic vesicles. (A) reports the relative amount of indicated proteins contained within 10 μg of rat brain synaptosomes and their sub-fractions: PSD95 (post synaptic density protein 95), Hsp90 (Heat shock protein 90), Hsc70 (Heat shock cognate protein 70), TrxR (Thioredoxin reductase), VAMP-2 (synaptobrevin-2), Tom20 (translocase of outer membrane) and Trx1 (Thioredoxin 1). (B) Hsp90-interacting proteins were recovered by immunoprecipitation. A crude synaptic vesicles fraction was isolated from 2 mg of rat brain synaptosomes and incubated with Protein G-decorated beads coupled (or not as a control) to a monoclonal antibody specific for Hsp90. After over-night incubation at 4 °C beads were washed and Hsp90 interactors were processed for WB to assess the presence of TrxR and Trx1. The staining for Na^+/K^+ ATPase (Sodium-Potassium pump), Syp (synaptophysin) or Tom20 were used to exclude contaminations of plasma membrane, SVs and mitochondria respectively, Rab3a (Ras-related protein 3a) staining was used as a positive control for known interactors [72]. In the first lane (input), 5 μg of crude synaptic vesicles fraction were loaded as reference.

3. Discussion

The results reported here are part of a large effort of this laboratory aimed at the identification of small-molecules inhibitors (SMIs) capable of preventing the neurotoxicity of BoNTs regardless of their different sequences and immunological properties [35, 73]. In fact, the growing number of BoNTs calls for an increase effort in the research of pan-inhibitors, i.e. molecules whose action does not depend on toxins specific antigenicity as it is the case of serotype-based treatments and vaccines [74, 75]. So far, research on SMIs was aimed at inhibiting the proteolysis of SNAREs, as such strategy can in principle be effective after the L chain has entered into nerve terminal cytosol. However, the recognition and binding of the L chains to their substrates requires extended interactions involving the active site and a number of exosites [23, 76-81] and this fact undermined these efforts. We

pursued a different approach based on the knowledge about the steps of the mechanism of neuron intoxication that are shared by all BoNT serotypes [2, 35].

Here we show that geldanamycin, a specific inhibitor of a major heath shock protein, Hsp90, strongly prevents the activity of three prototypes of CNTs on neuronal cultures. Together with previous reports [42, 43, 45], this finding indicates that Hsp90 assists the refolding of the active chains of several bacterial exotoxins with intracellular targets that have to translocate across the membrane in an unfolded conformation.

It may appear surprising that Hsp90 assists the entry of so many different toxins. However, Hsp90 is not a "folding catalyst", i.e. it does not control the rate of protein folding in a direct way such as DPI or PPI, rather it recognizes unfolded intermediates with secondary structure avoiding their aggregation [82]. Interestingly, Hsp90 has a preference for positively charged substrates [83], making the catalytic chain of CNTs an ideal substrate during translocation, as the low pH induces L to acquire a positive charge as a result of carboxylates neutralization [22, 69, 84]. Accordingly, the interaction of Hsp90 with the three different L chain tested here, is not based, probably, on specific protein-protein interactions involving defined interfaces, instead it may be due to a hydrophobic binding activity of Hsp90 that prevents the improper folding or aggregation of the unfolded L chain emerging from the membrane in the cytosol.

We also investigated the involvement other chaperons, and in particular of Hsc70, the constitutively expressed isoform of Hsp70 that together with CSP (cysteine string protein) and SGT (small glutamine-rich tetratricopeptide repeat-containing protein) takes part to a tripartite chaperone machine residing on SV [87]. However, the pharmacological inhibition of this protein did not interfere with toxin activity, underscoring the primary role of Hsp90 in CNTs entry. We believe that this choice is strictly connected to the concomitant presence of Hsp90 and the thioredoxin system on synaptic vesicles [50, 85, 86], the organelle wherefrom the L chain is expected to translocate [2], and to their physical interaction, reported here for the first time. This idea is reinforced by the second relevant finding of the present paper, i.e. the synergistic effect of GA and PX-12. This result parallel the one found at the level of early endosomes for diphtheria toxin [42] where it was shown that Hsp90 and TrxR (likely together with Trx) orchestrate a translocation complex mediating the entry of the enzymatic subunit of diphtheria toxin in the cytosol. Moreover it was recently found that Auranofin, the most potent inhibitor of TrxR identified so far, blocks the entry of diphtheria toxin, as GA does [88]. Taken together, these results suggest that DT and CNTs have evolved a mode of membrane translocation that uses the activities of Hsp90 and TrxR-Trx. We did not investigate the physiological role of this couple, however, it was previously reported that in nerve terminals Hsp90 interacts with αGDI and this interaction coordinates neurotransmitter release [89] by regulating the extraction of Rab3A from SVs [72]. Considering that the association of αGDI to

Rab proteins is strongly dependent on its redox state [90, 91], the role of Trx-TrxR redox system within the chaperone complex may be that of managing the many cysteines present on αGDI structure.

4. Materials and Methods

4.1 Materials

BoNT/A and TeNT were purified as previously described [92, 93]. BoNT/D was produced in *E. coli* by recombinant methods [39, 94]. LysoTracker® Red DND-99 was purchased from Thermo Fischer Scientific (L-7528), Bafilomycin A1 (sc-201550) and PX-12 (sc-358518) from Santa Cruz Biotechnology, Cytosine β-D-arabinofuranoside hydrochloride (C6645), DNAse I (DN25), poly-L-lysine hydrobromide (P1274), Cyclosporin A (30024) and VER-155008 (SML0271) from Sigma Aldrich. Geldanamycin (GA) was purchased from Santa Cruz (sc-200617) and to prevent loss of activity, a fresh stock solution of 12.5 mM in DMSO was prepared for each experiment. Syntaxin 1A (110 111), VAMP-2 (104 211) and Rab3a (107 111) antibodies were purchased from Synaptic System. Anti-synaptophysin (clone Sy38) was from Dako, anti-thioredoxin reductase 1 (07-613) was from Merck Millipore, anti-thioredoxin 1 (clone EPR6111) was from GeneTex, anti-PSD95 (P-246) was from Sigma Aldrich, anti-Hsp90 (610418) was from BD Biosciences, anti-tom20 (Fl-145) was from Santa Cruz. SNAP-25 (SMI81,ab24737), Na^+/K^+ ATPase (ab7671) antibodies were purchased from Abcam. The antibody specific for SNAP-25_A (SNAP-25_{1-197}) was produced in our laboratory and used as previously reported [70]. Secondary antibody were from Merk Millipore (HRP-conjugated) and from Life Technologies (Alexa Fluor-conjugated).

The pyrrolidine-substituted acridizinium derivative (compound 2) has been synthesized as described in the literature [95, 96] purified by preparative HPLC (purity > 98 %) and fully characterized by 1H-NMR, 13C-NMR and HRMS.

4.2. Cerebellar Granule Neurons (CGNs) cultures and botulinum neurotoxin inhibition assay

Primary cultures of rat cerebellar granule neurons (CGNs) were prepared from 6- to 8-days-old rats [97]. 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 3.5 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 (complete culture medium). To arrest growth of non-neuronal cells, cytosine arabinoside (10 μM) was added to the medium 18–24 h after plating. CGNs at 6-8 days in vitro were treated for 30 min with the indicated concentration of GA in complete culture medium at 37 °C and 5% CO_2.

Thereafter, 2.5 nM BoNT/A or 0.5 nM TeNT in complete medium was added and left for 3.5 hours at 37 °C and 5% CO_2. In the case of BoNT/D, owing to its potency, the toxin was added as pulse of 15 minutes at a concentration of 0.01 nM. The neuronal culture was then washed and the culture medium with the same concentration of inhibitor was restored for 195 min.

4.3. Immunoblotting and immunocytochemistry

For immunoblotting analysis, cells were directly lysed with reducing Laemmli sample buffer containing protease inhibitors (complete Mini EDTA-free, Roche). Equal amounts of protein were loaded onto a 4-12% NuPage gel or 12% NuPage gel (Life technologies) and separated by electrophoresis in MES or MOPS buffer (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. Finally, membranes were washed three times with PBST and twice with PBS. Visualization was carried out using Luminata Crescendo (Merck Millipore).

For immunocytochemistry analysis, after the treatment, CGNs were 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. BoNT/A cleavage was evaluated following the generation of $SNAP\text{-}25_A$, whereas the cleavage of TeNT and BoNT/D was evaluated with an antibody recognizing the full-length form of VAMP-2. Primary antibodies were detected with secondary Alexa Fluor-conjugated antibodies. Coverslips were mounted using Fluorescent Mounting Medium (Dako, S3023) and examined by epifluorescence (Leica CTR6000) microscopy.

4.4. cpV-HC/A binding and internalization assay

The HC of BoNT/A (nucleotides corresponding to 876-1296) with a N-terminal fused with cpV (Circularly Permutated Venus) at the N-terminus was cloned into a pET28a His-tag vector (Novagen) and expressed into BL21 (DE3) *E. coli* cells. Protein purification was carried out 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). Purified HC/A was dialyzed into 20 mM TRIS, 200 mM NaCl, 40% glycerol (pH 7.6). For the assay, CGNs were treated with GA 12.5 μM or vehicle (DMSO) in culture medium at 37 °C. After 30 minutes, 250 nM cpV-HC/A was added and the concentration of KCl increased at 57 mM. After 30 min, neurons were

393 washed twice with PBS, lysed and immunoblotted. cpV-HC/A was detected with an anti-GFP
394 antibody (Cell Signaling, #2956).

395 *4.5. Synaptic vesicles dynamics assay*

396 Experiment was performed as previously described [62]. CGNs were treated with vehicle (DMSO)
397 or 12.5 μM of GA or 10 nM BoNT/D for 30 min at 37 °C. 5 μg/ml of an anti Synaptotagmin-1 antibody
398 (recognizing the lumenal domain of the protein, Synaptic System, 105-101) was added, with the
399 concentration of KCl adjusted to 57 mM. After 30 minutes, CGNs were lysed in non-reducing
400 condition and processed for WB. Membrane was then saturated for 1 hour in PBST supplemented
401 with 5% non-fatty milk and directly incubated with secondary antibody.

402 *4.6. Maturation of acidic compartment assay*

403 CGNs at 6–8 DIV were treated for 30 min with vehicle (DMSO) or 12.5 μM GA or 10 nM bafilomycin
404 A1 in complete culture medium supplemented with 6.25 mM HEPES at 37 °C and 5% CO_2. 75 nM
405 Lysotracker® Red DND-99 was added for 60 minutes. Cells were then washed with Krebs-Ringer
406 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
407 K_2HPO_4) and images of living neurons were acquired with a Leica CTR6000 microscope.

408 *4.7. In vitro proteolytic activity*

409 0.1 μg BoNT/D or BoNT/A or TeNT was incubated in reducing buffer (150 mM NaCl, 10 mM
410 NaH_2PO_4, 15 mM DTT pH 7.4) in the presence of 12.5 μM GA for 30 min at 37 °C or vehicle (DMSO).
411 Then 1 μg of recombinant GST-VAMP-2 (1-96), for BoNT/D or TeNT, or 1 μg of recombinant
412 GST-SNAP-25, for BoNT/A, was added to the reduced toxins, the concentration of inhibitor or
413 vehicle was restored, and the reaction was carried out for 4 hours at 37 °C. VAMP-2 cleavage was
414 assessed by SDS-PAGE.

415 *4.8. Low pH induced translocation of BoNT/D across the plasma membrane*

416 CGNs at 6–8 DIV were treated with vehicle (DMSO) of different concentrations of GA or PX-12, or
417 their combination for 30 min at 37 °C, 5% CO_2. Neurons were washed twice with cold culture
418 medium and subsequently incubated with 200 pM of BoNT/D in ice-cooled medium (pH 7.4) and
419 left at 4 °C for 15 minutes. After washing twice with the same cold medium, pre-warmed (37 °C)
420 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,
421 5.6 mM glucose, 10 mM NH_4Cl), adjusted at pH 7.4 or 4.5, was added for 10 minutes in the presence
422 of vehicle or of inhibitors (alone or in combination). Cells where then washed twice and further

incubated, for 185 minutes, in normal culture medium (pH 7.4) supplemented with 50 nM Bafilomycin A1 and containing inhibitors.

4.9. Purification of synaptic vesicles and immunoprecipitation

Synaptosomes and synaptic vesicles were isolated from cerebral cortices as previously described [50], with minor changes of the classical procedure [98]. Namely, 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 SV) and those sedimenting at 800-1000 mM (docked SV) were collected and pelleted by centrifugation in a 70Ti rotor. These vesicle fractions were re-suspended in SV buffer (4 mM HEPES, 300 mM glycine, pH 7.4 supplemented with complete protease inhibitors, EDTA-free). For immunoblotting analysis, protein concentration was determined with BCA test (Pierce BCA protein assay, Thermo Scientific) and 10 μg of different fractions were used for SDS-PAGE under reducing conditions.

Immunoprecipitation of Hsp90 was carried out as previously reported [99]: 2 mg of rat brain synaptosomes were re-suspended in buffer B (20 mM HEPES, 140 mM NaCl, 5 mM KCl, 5 mM $NaHCO_3$, 1.2 mM Na_2HPO_4, 1 mM $MgCl_2$ and 10 mM glucose, pH 7.4) and subjected to osmotic shock using H_2O in presence of protease inhibitors for 30 minutes at 4 °C. Sample were subjected to three passages though 18 G needles and three through 27 G needles and then centrifuged for 5 min at 10000 g. The resulting supernatant was centrifuged for 1 hour at 80000 r.p.m. to yield a crude synaptic vesicle fraction in the pellet and a cytosolic fraction in the supernatant [100]. The pellet fraction was re-suspended in buffer B and incubated overnight at 4 °C with G protein-decorated Dynabeads (Life Technology) coupled to anti-Hsp90 antibody, preventively washed in PBS 0.1% BSA. Beads were then extensively washed twice with a buffer containing 20 mM TRIS, 80 mM NaCl and 1% Triton X-100, pH 7.4 and twice with PBS. Hsp90 interactors were eluted by addition of 0.2 M glycine (pH 2.6). Samples were then added to non-reducing loaded sample buffer and subjected to SDS-PAGE. Proteins were then labeled with specific antibodies.

4.10. 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 GA, ranging from 0 to 20 μM, were added and left for 4 hours. Neurons were then washed and CellTiter 96® AQueous (Promega) performed according to manufacturer indication.

Supplementary Materials: The following are available online at www.mdpi.com/link, Figure S1: title, Table S1: title, Video S1: title.

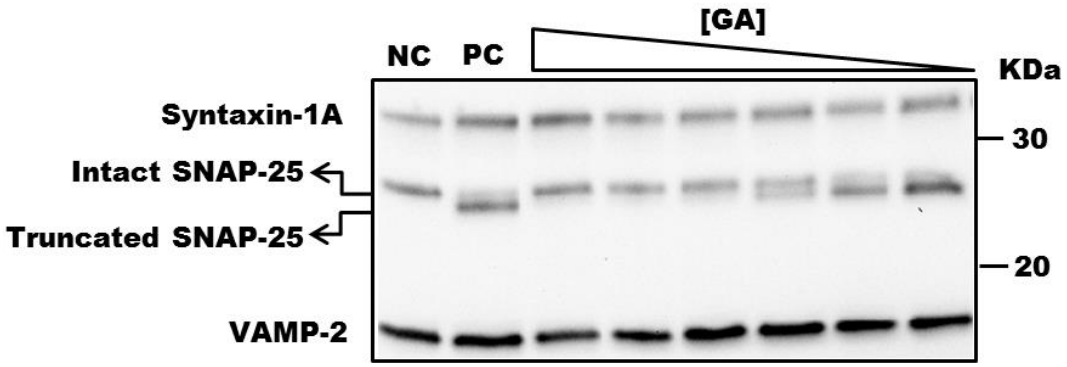

Figure S1. Hsp90 inhibition prevents BoNT/A-mediated cleavage of SNAP-25 in CGNs. Experiment was conducted as in Figure 1, thereafter, neurons were lysed and SNAP-25 cleavage was estimated with an antibody that recognizes both the intact and truncated form; Syntaxin 1A and VAMP2 were used as loading control. A typical immunoblot is reported (NC, only vehicle; PC, only BoNT/A; the six following lanes refer to samples pretreated with different GA concentrations: 12.5, 10, 7.5, 5, 2.5, 1 μM).

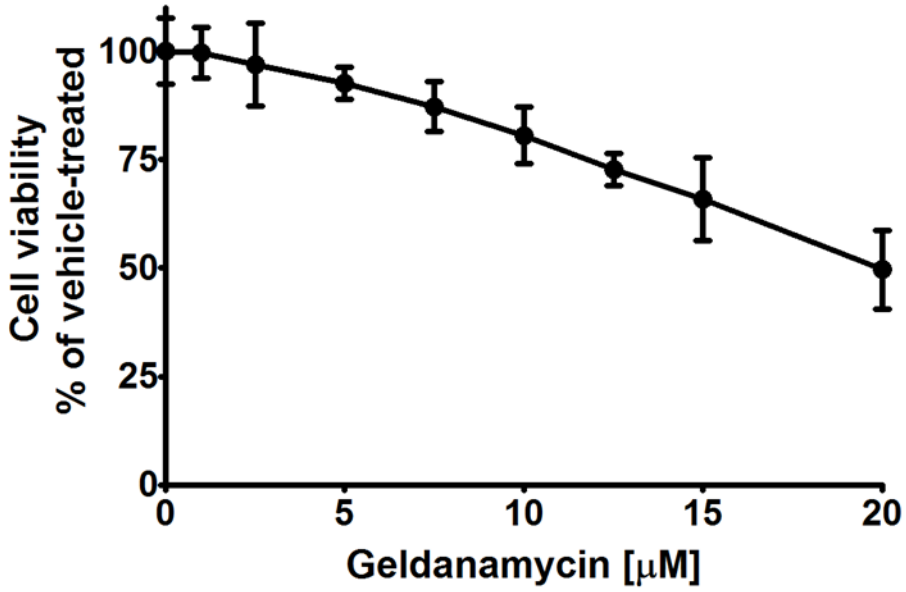

Figure S2. Neuron viability upon geldanamycin treatment. CGNs were treated with increasing concentration of geldanamycin ranging from 2.5 to 20 μM or vehicle (DMSO) in culture medium 37 °C for 4 hours. Cell viability was then assayed with a MTS assay. Data are presented as a percentage with respect to non-treated cells. All data are presented as mean values and error bars indicate the deviation standard obtained from two independent experiments.

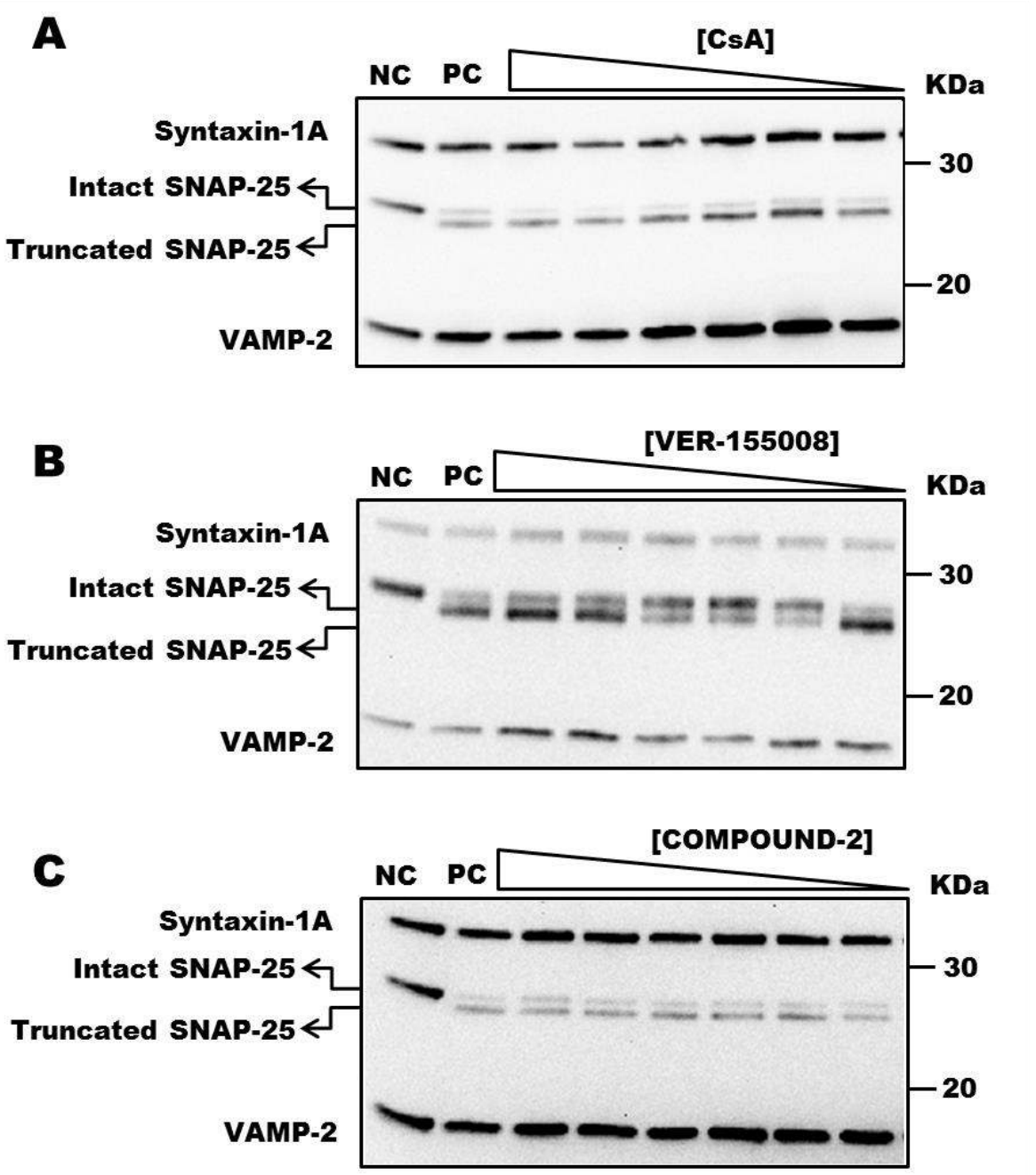

Figure S3. Cyclosporine A, VER-155008 and compound-2 does not prevent BoNT/A intoxication in CGNs. Neurons were treated like in Figure 1 but using different inhibitors: (A) cyslosporine A, (B) VER-155008 or (C) compound-2. Thereafter, CGNs were lysed and immunoblotted staining with anti-SNARE antibodies. Typical WB are shown. NC, only vehicle; PC, only BoNT/A; the six following lanes refer to samples treated with toxin plus different inhibitor concentrations: 50, 40, 30, 20, 10, 5 μM for CsA and VER-155008; 5, 2.5, 1, 0.5, 0.25, 0.1 μM for compound-2.

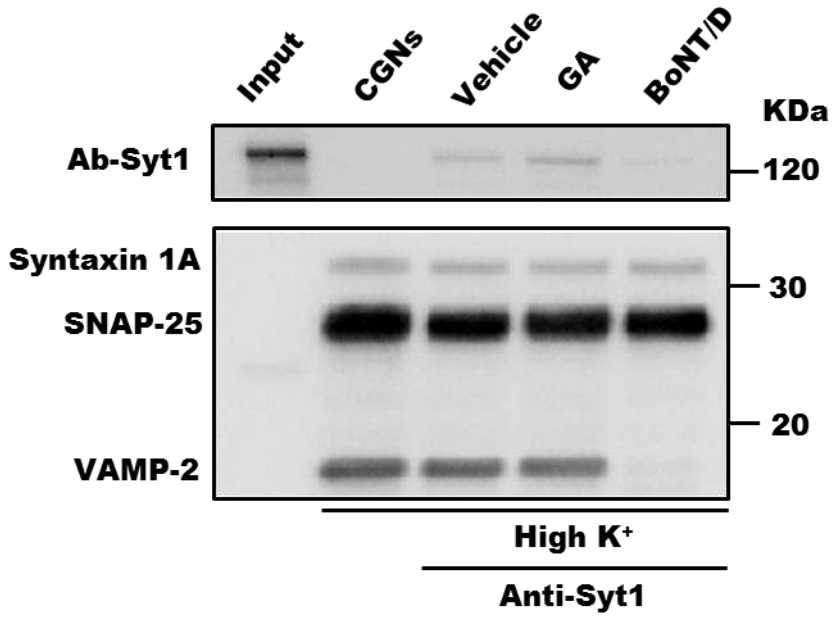

Figure S4. Geldanamycin does not affect synaptic vesicles dynamics. (B) CGNs were treated as in Figure 3A but using as reporter of SVs dynamics an antibody against the lumenal domain of Synaptotagmin-1. Where indicated, BoNT/D was added before starting GA treatment. The internalized antibody was detected by WB, using directly a secondary antibody. Syntaxin 1A and SNAP-25 were considered as loading controls, instead, VAMP-2 staining was used to assess BoNT/D cleavage. In the first lane (input) 50 ng of the anti-Synaptotagmin 1 antibody were loaded as reference. The immunoblot is representative of three independent sets of experiments.

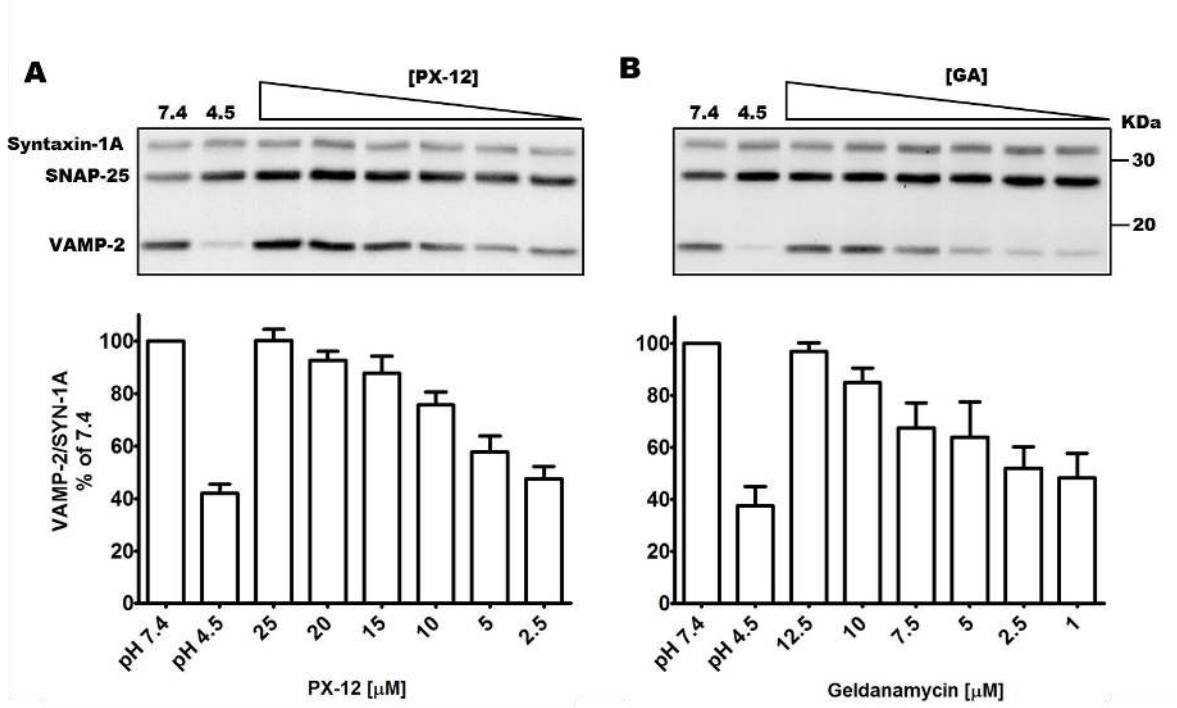

Figure S5. PX-12 and geldanamycin inhibit the translocation of BoNT/D L chain across the plasma membrane in a concentration dependent manner. CGNs were like in Figure 4 using the indicated concentrations of PX-12 (A) or GA (B). The translocation of BoNT/D was assessed by monitoring the cleavage of VAMP-2, determined via WB (top panels). Bottom panels show the amount of intact VAMP-2 reported as a ratio to Syntaxin 1A which served as loading control, taking the value in pH 7.4 treated-cells as 100%. All data are presented as mean values with SD values arising from three independent experiments.

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Conflicts of Interest: The authors declare no conflict of interest.

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