

1 Article

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

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

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

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## 35 1. Introduction

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

46 CNTs are the deadliest substances known with lethal doses ranging in the order of few ng/kg  
47 [8]. Such a toxicity is ascribed to potency and specificity, two properties that are the final result of an  
48 efficient molecular architecture evolutionary shaped to exploit essential processes of vertebrates  
49 nervous system physiology [9]. CNTs structure is composed by a catalytic light chain (L, 50 kDa)  
50 that is disulphide-linked to a heavy chain (H, 100 kDa), which is responsible for neurospecific  
51 binding and delivery of L into nerve terminals [10-12]. The C-terminal part of the H chain (HC)  
52 mediates the binding to peripheral nerve terminals [2, 13-15] and the ensuing trafficking within  
53 specific vesicles which provide either the tropism of BoNTs for the neuromuscular junction [16] or  
54 the retrograde axonal transport of TeNT toward the spinal cord [4, 7, 17]. The N-terminal part of H  
55 (HN) mediates the translocation of L across the membrane of the endocytic organelles [18-22]. L is a  
56 metalloprotease which specifically cleaves one of the SNARE proteins that form the core of the  
57 neuroexocytosis nanomachine [23, 24]: the L of BoNT/A and /E targets SNAP-25 [25-28], that of  
58 BoNT/B, /D, /F, /G and TeNT removes the largest part of the cytosolic domain of VAMP-1/2 [27,  
59 29-32] whilst the L chain of BoNT/C is the sole having more than one substrates, i.e. SNAP-25 and  
60 some isoforms of Syntaxins [33, 34].

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

68 membrane with the formation of an ion-conducting channel [2, 21, 22, 38]. It is not clear if channel  
69 formation is a prerequisite or a consequence of translocation [22], but it is reported that L has to  
70 undergo partial unfolding in order to cross the membrane, otherwise the translocation is abortive  
71 [20]. In fact, cargo proteins, which do not unfold at low pH attached to the amino terminus of  
72 BoNTs, drastically reduce the delivery of the L into cell cytosol [39]. As a result of its unfolding, once  
73 it has reached the cytosolic face of the membrane, the L chain has to reacquire the native structure in  
74 order to cleave its substrates. It is presently unknown whether this process is spontaneous or it is  
75 assisted by host chaperone proteins, which normally reside on the organelle membrane [40].

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

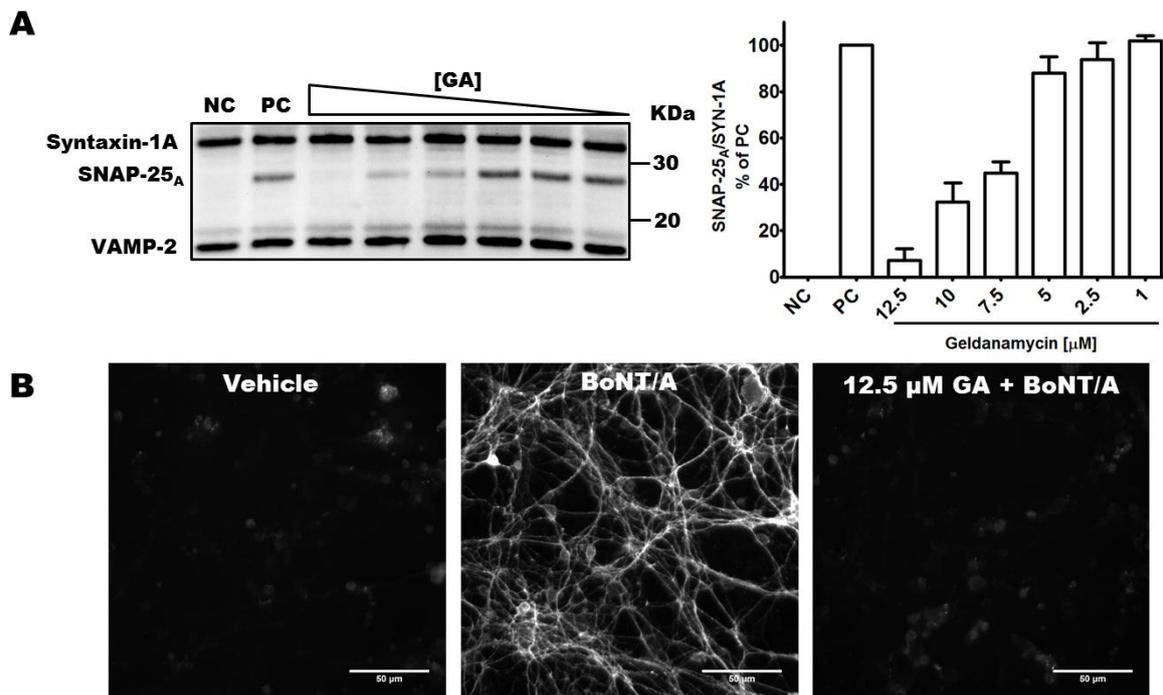
79 Recently, we found that the Thioredoxin Reductase-thioredoxin system (TrxR-Trx) is  
80 responsible for the cytosolic reduction of BoNTs and TeNT interchain disulphide bond [35, 49-51], a  
81 molecular event, which is necessary to terminate the translocation step [20, 21, 38] and enable the  
82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

## 92 **2. Results**

### 93 *2.1. Hsp90 pharmacological inhibition protects primary neurons from CNTs intoxication*

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

101 started with BoNT/A, which is the most common cause of human botulism [3] and the one almost  
 102 invariably used in human therapy [53-55]. As expected, when BoNT/A is added to neurons, it  
 103 cleaves SNAP-25, as assessed by the appearance in WB of its truncated form (SNAP-25<sub>A</sub>), detected  
 104 with a specific antibody (Figure 1A, lane PC). The same result is evident also via IF analysis (Figure  
 105 1B, middle panel). It is worth to note that the amount of BoNT/A used causes the almost complete  
 106 cleavage of SNAP-25, as determined with an antibody that recognizes both the intact and the  
 107 cleaved form of SNAP-25 (Figure S1). Whatever the read-out used, addition of GA significantly  
 108 protects neurons from BoNT/A. The inhibition is concentration-dependent and complete at 12.5 μM,  
 109 as indicated by the absence of SNAP-25<sub>A</sub> in WB (Figure 1A and Figure S1) and IF (Figure 1B,  
 110 compare middle with right panel). As Hsp90 is involved in multiple aspects of cell physiology [56],  
 111 GA is highly toxic. Accordingly, the experiment was conducted by pretreating CGNs with GA for 30  
 112 minutes and concluded within 4 hours after toxin addition (see M&M) to avoid the toxicity of the  
 113 drug that develops with longer incubation times (not shown); under the experimental conditions  
 114 used here cell viability was not significantly affected (Figure S2).

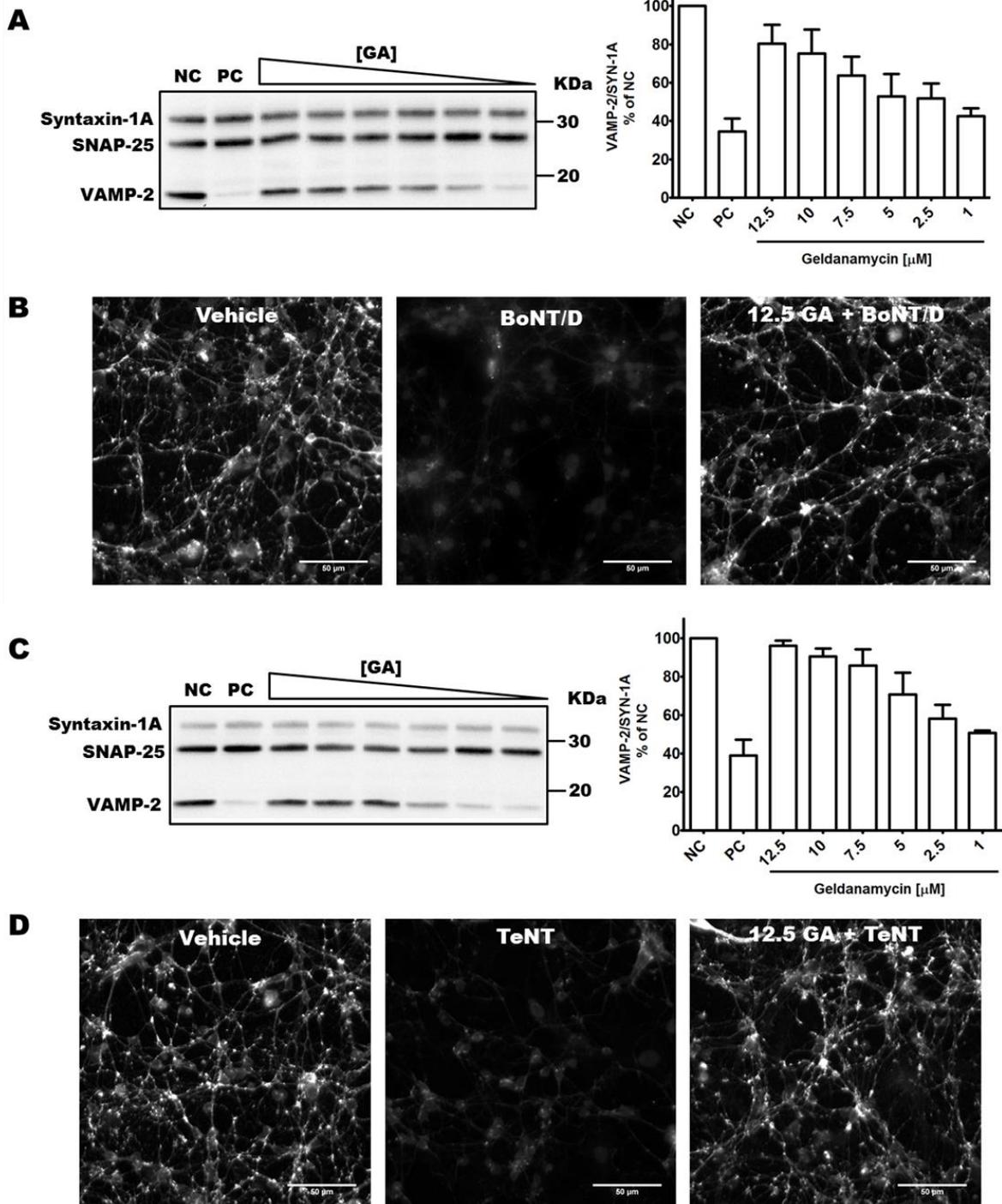


115 **Figure 1.** Geldanamycin abolishes SNAP-25 cleavage in neurons treated with BoNT/A. (A) CGNs  
 116 were pretreated for 30 minutes with the indicated concentrations of GA at 37 °C; then BoNT/A was  
 117 added (2.5 nM final concentration) and the incubation prolonged for 3.5 hours. Thereafter, neurons  
 118 were lysed and the cleavage of SNAP-25 estimated with a specific antibody against the BoNT/A  
 119 truncated form. VAMP-2 and Syntaxin 1A were used as loading controls. A typical immunoblot is  
 120 reported on the left, while the right panel shows the quantification of cleaved SNAP-25 as a ratio to  
 121 Syntaxin 1A, taking the value in non-treated cells (NC) as 100%. SD values derive from at least three  
 122 independent experiments performed in triplicates. (B) Neurons were treated as described in A, but  
 123 the experiment was stopped by fixation and the presence of SNAP-25<sub>A</sub> evaluated via IF, using an  
 124

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.



140

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

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

153 The involvement of molecular chaperones different from Hsp90 in membrane translocation of  
154 several exotoxins was previously suggested [44, 46, 58, 59]. Accordingly, we took advantage by the  
155 existence of specific inhibitors such as cyclosporine A (CsA) which specifically targets Cyclophilin A  
156 [60], and VER-155008, a general inhibitor of the ATPase activity of Hsp70s [61] to test the possible  
157 role in CNTs toxicity of these chaperones. We also synthesized an Acridizinium derivative, dubbed  
158 “compound 2”, which potently inhibits Hsc70 [59], the constitutively expressed isoform of Hsp70.  
159 Remarkably, none of these molecules impacted on the cleavage of SNAP-25 by BoNT/A (Figure S3),  
160 or of VAMP-2 by BoNT/D and TeNT (not shown), strongly suggesting the primary role of Hsp90 in  
161 supporting CNTs toxicity.

## 162 2.2. Geldanamycin inhibits the L chain refolding after its pH-dependent membrane translocation

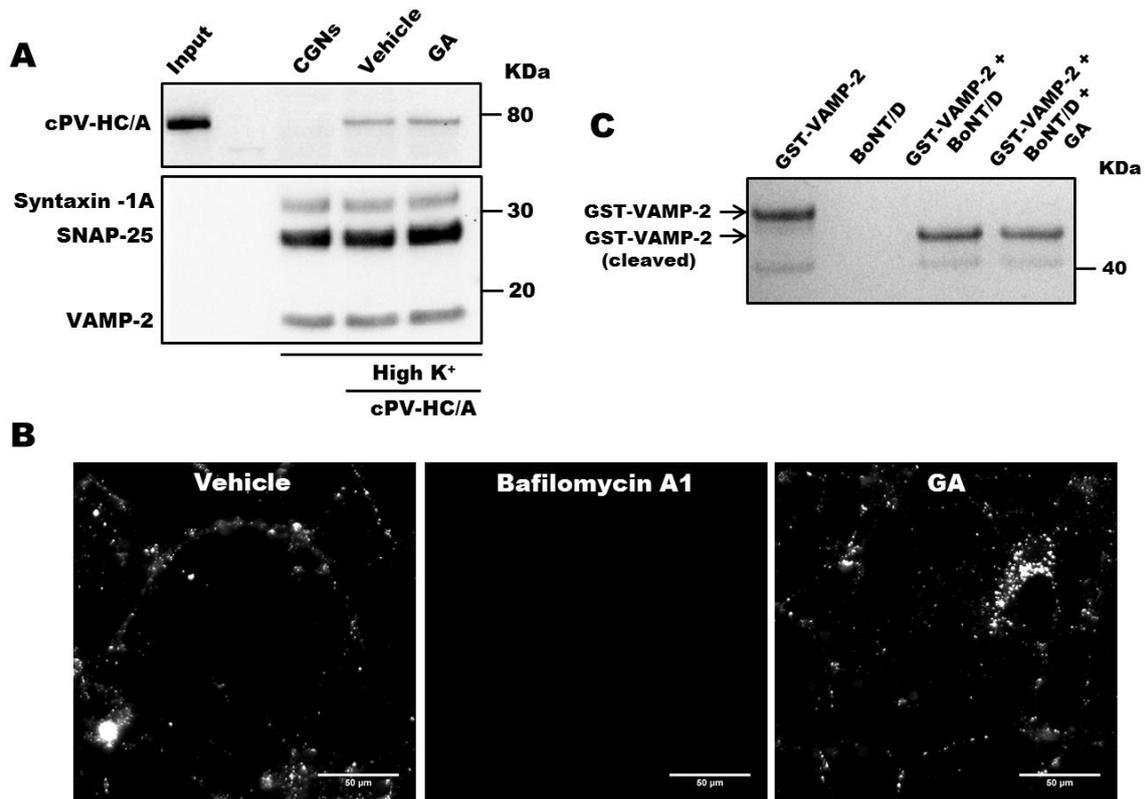
163 The remarkable inhibitory activity of GA on BoNT/A, BoNT/D and TeNT cleavage of their  
164 SNARE substrates called for a study of the effect of this drug on the various steps of their  
165 mechanism of action in order to identify which of them is affected.

166 First, we investigated whether the inhibition of Hsp90 prevents the binding of CNTs to CGNs  
167 plasma membrane and their subsequent internalization into endosomal compartments. Neurons  
168 were pretreated either with the vehicle or with GA at a concentration causing the complete  
169 inhibition of BoNT/A activity. CGNs were then incubated for 30 minutes with the BoNT/A binding  
170 domain fused to circularly permuted Venus (cpV-HC/A), used as reporter for WB analysis. Figure  
171 3A shows that binding and internalization of cpV-HC/A were not affected by GA. The same  
172 experiment was repeated using as a reporter an antibody against the luminal portion of the SV  
173 integral membrane protein synaptotagmin-1, to check synaptic vesicles (SVs) dynamics [62] upon  
174 GA treatment. Figure S4 shows that in control condition the antibody is incorporated through SVs  
175 cycle and can be detected by WB (lane vehicle). On the contrary, when treated with BoNT/D, which  
176 prevents the assembly of a functional SNARE complex and therefore blocks SVs dynamics, CGNs do  
177 not take up the antibody (lane BoNT/D). In GA-treated neurons the amount of internalized antibody  
178 was comparable to that of vehicle-treated neurons (lane GA), suggesting that the trafficking of the  
179 organelles used by BoNT/A [16, 63, 64], TeNT [65] and possibly BoNT/D for internalization within  
180 nerve terminals is not altered by the drug. These two complementary approaches therefore indicate  
181 that Hsp90 inhibition does not affect toxin binding and endocytosis in cultured neurons.

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

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

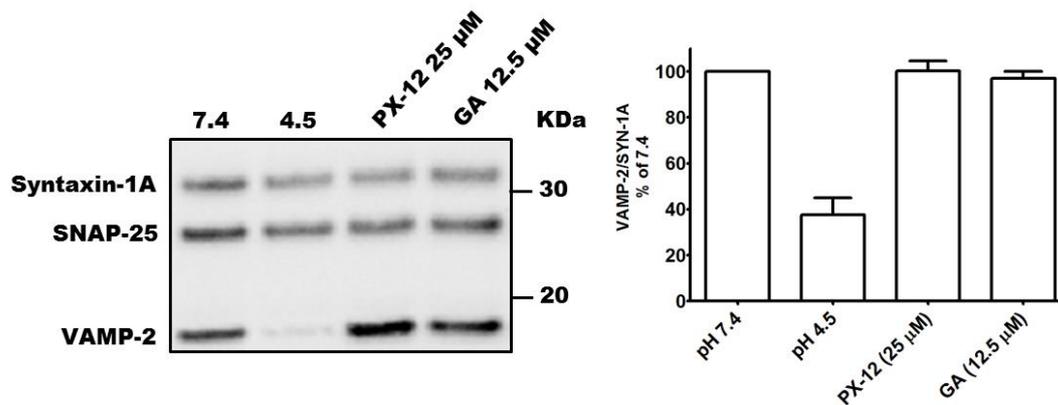
187 Finally, Figure 3C shows that GA does not interfere with the metalloproteolytic activity of  
 188 CNTs on SNAREs, using BoNT/D cleavage on VAMP-2 as an example. Similar results were obtained  
 189 with BoNT/A and TeNT using their respective substrates (data not shown).



190  
 191 **Figure 3.** Geldanamycin has no effect on CNTs binding, endosome acidification and *in vitro*  
 192 metalloprotease activity. (A) CGNs were pretreated with geldanamycin (GA, 12.5 μM) or vehicle  
 193 (DMSO) at 37 °C for 30 minute after which, cpV-HC/A (250 nM final concentration) was added in  
 194 high K<sup>+</sup> buffer for further 30 minutes. Neurons were then washed, lysed and the cpV-HC/A amount  
 195 was estimated by WB with an antibody against GFP. Syntaxin 1A, SNAP-25 and VAMP-2 are used  
 196 as loading controls. In the first lane (input) 50 ng of the cpV-HC/A were loaded as reference. The  
 197 immunoblot is representative of three independent sets of experiments. (B) CGNs were treated with  
 198 vehicle (DMSO) or 12.5 μM of GA or 50 nM bafilomycin A1 for 30 minutes at 37 °C. Then,  
 199 Lysotracker Red was added for 1 hour and neurons were imaged by fluorescence microscopy.  
 200 Images are representative of two independent experiments. (C) BoNT/D (100 ng) was reduced with  
 201 10 mM DTT in the presence of 12.5 μM GA or vehicle (DMSO) for 30 minutes at 37 °C before adding  
 202 1 μg of GST-VAMP-2. The reaction was carried out for 4 hours at 37 °C. VAMP-2 cleavage was  
 203 assessed by SDS-PAGE. A representative image is shown.

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

208 jump”, which induces the entry of L from the plasma membrane [68-70]. As shown in Figure 4, by  
 209 lowering the pH to 4.5, BoNT/D L chain is productively translocated across the plasma membrane of  
 210 CGNs and delivered into the cytosol in its active form, as indicated by the cleavage of VAMP-2 (lane  
 211 “4.5”). The assay is amenable to manipulation, indeed the delivery of the L chain, and the ensuing  
 212 cleavage of VAMP-2, can be prevented by PX-12 (lane PX-12), a known inhibitor of Trx [71] which  
 213 blocks the release of the metalloprotease in the cytosol by interfering with the reduction of the  
 214 interchain disulphide bridge [49, 50]. Accordingly, the absence of VAMP-2 cleavage upon treatment  
 215 with GA (lane GA), suggests that this drug directly impacts on the translocation, likely on the L  
 216 chain refolding.

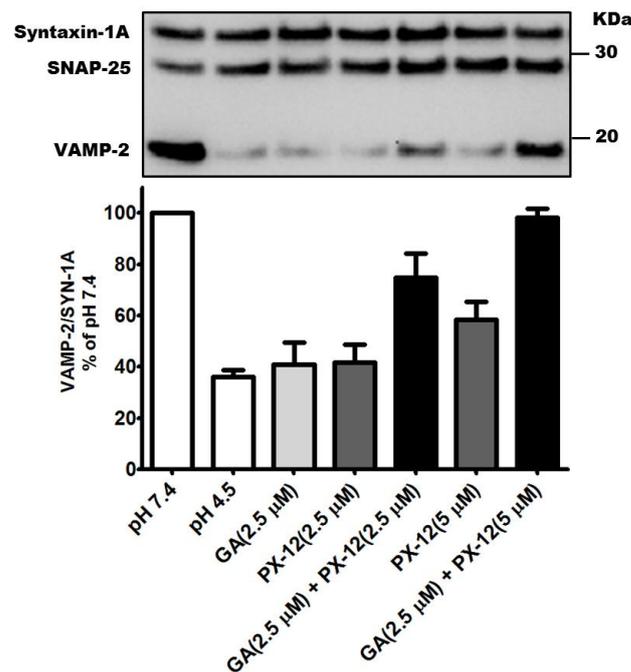


217  
 218 **Figure 4.** PX-12 and geldanamycin inhibit L translocation across the membrane. CGNs were treated  
 219 with 25 μM of PX-12 or 12.5 μM GA for 30 min at 37 °C. Thereafter, BoNT/D (200 pM) was added in  
 220 the cold for 15 minutes. Neurons were then washed and incubated for 10 min with medium A  
 221 buffered at indicated pH at 37 °C supplemented with inhibitors or vehicle. Then, cells were washed  
 222 and incubated for 3.25 hours in complete medium containing 50 nM bafilomycin. The translocation  
 223 of BoNT/D was assessed by monitoring the cleavage of VAMP-2, determined via Western blot as a  
 224 ratio to Syntaxin 1A which served as internal control, taking the value at pH 7.4 as 100%. SD values  
 225 derive from three independent experiments performed in triplicates.

226 *2.3. Hsp90 and Trx-TrxR are present on synaptic vesicles and are essential to the release of active L chain in the*  
 227 *cytosol*

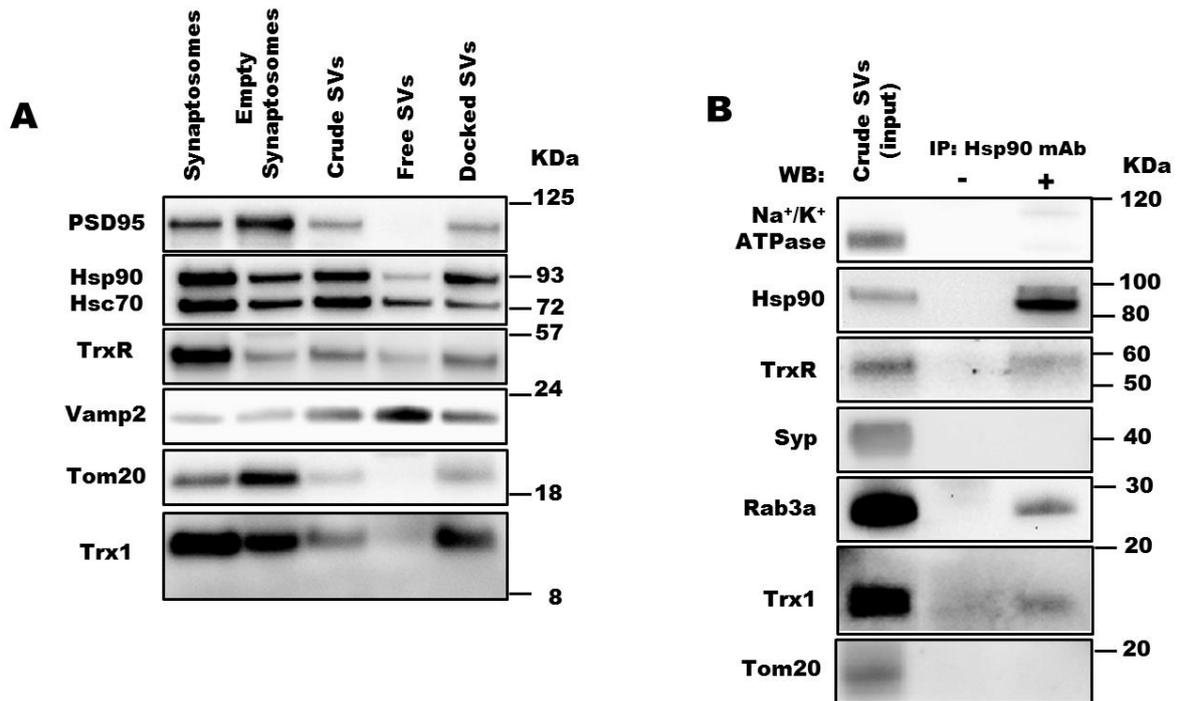
228 The effect of PX-12 indicates the essential role of the Thioredoxin-Thioredoxin reductase system in  
 229 reducing the interchain disulfide bond as a prerequisite for the intracellular activity of the L chain [2,  
 230 50]. This redox system was previously suggested to form with Hsp90 a complex that plays an  
 231 essential role in the entry of diphtheria toxin into the cytosol [42]. As the CNTs are similarly  
 232 composed of a catalytic domain disulphide-linked to a subunit mediating binding and translocation,  
 233 we reasoned that the two processes of disulfide reduction and cytosolic refolding may be coupled  
 234 and that Hsp90 may act in concert with the Trx-TrxR system to enable the catalytic activity of the L  
 235 chain in the cytosol. If this is the case, the addition at the same time of GA and of PX-12 should

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



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

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



270

271 **Figure 6.** Hsp90 interacts with TrxR-Trx system on synaptic vesicles. (A) reports the relative amount  
 272 of indicated proteins contained within 10 µg of rat brain synaptosomes and their sub-fractions:  
 273 PSD95 (post synaptic density protein 95), Hsp90 (Heat shock protein 90), Hsc70 (Heat shock cognate  
 274 protein 70), TrxR (Thioredoxin reductase), VAMP-2 (synaptobrevin-2), Tom20 (translocase of outer  
 275 membrane) and Trx1 (Thioredoxin 1). (B) Hsp90-interacting proteins were recovered by  
 276 immunoprecipitation. A crude synaptic vesicles fraction was isolated from 2 mg of rat brain  
 277 synaptosomes and incubated with Protein G-decorated beads coupled (or not as a control) to a  
 278 monoclonal antibody specific for Hsp90. After over-night incubation at 4 °C beads were washed and  
 279 Hsp90 interactors were processed for WB to assess the presence of TrxR and Trx1. The staining for  
 280 Na<sup>+</sup>/K<sup>+</sup> ATPase (Sodium-Potassium pump), Syp (synaptophysin) or Tom20 were used to exclude  
 281 contaminations of plasma membrane, SVs and mitochondria respectively, Rab3a (Ras-related  
 282 protein 3a) staining was used as a positive control for known interactors [72]. In the first lane (input),  
 283 5 µg of crude synaptic vesicles fraction were loaded as reference.

### 284 3. Discussion

285 The results reported here are part of a large effort of this laboratory aimed at the identification  
 286 of small-molecules inhibitors (SMIs) capable of preventing the neurotoxicity of BoNTs regardless of  
 287 their different sequences and immunological properties [35, 73]. In fact, the growing number of  
 288 BoNTs calls for an increase effort in the research of pan-inhibitors, i.e. molecules whose action does  
 289 not depend on toxins specific antigenicity as it is the case of serotype-based treatments and vaccines  
 290 [74, 75]. So far, research on SMIs was aimed at inhibiting the proteolysis of SNAREs, as such strategy  
 291 can in principle be effective after the L chain has entered into nerve terminal cytosol. However, the  
 292 recognition and binding of the L chains to their substrates requires extended interactions involving  
 293 the active site and a number of exosites [23, 76-81] and this fact undermined these efforts. We

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

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

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

311 We also investigated the involvement other chaperons, and in particular of Hsc70, the  
312 constitutively expressed isoform of Hsp70 that together with CSP (cysteine string protein) and SGT  
313 (small glutamine-rich tetratricopeptide repeat-containing protein) takes part to a tripartite  
314 chaperone machine residing on SV [87]. However, the pharmacological inhibition of this protein did  
315 not interfere with toxin activity, underscoring the primary role of Hsp90 in CNTs entry. We believe  
316 that this choice is strictly connected to the concomitant presence of Hsp90 and the thioredoxin  
317 system on synaptic vesicles [50, 85, 86], the organelle wherefrom the L chain is expected to  
318 translocate [2], and to their physical interaction, reported here for the first time. This idea is  
319 reinforced by the second relevant finding of the present paper, i.e. the synergistic effect of GA and  
320 PX-12. This result parallel the one found at the level of early endosomes for diphtheria toxin [42]  
321 where it was shown that Hsp90 and TrxR (likely together with Trx) orchestrate a translocation  
322 complex mediating the entry of the enzymatic subunit of diphtheria toxin in the cytosol. Moreover it  
323 was recently found that Auranofin, the most potent inhibitor of TrxR identified so far, blocks the  
324 entry of diphtheria toxin, as GA does [88]. Taken together, these results suggest that DT and CNTs  
325 have evolved a mode of membrane translocation that uses the activities of Hsp90 and TrxR-Trx. We  
326 did not investigate the physiological role of this couple, however, it was previously reported that in  
327 nerve terminals Hsp90 interacts with  $\alpha$ GDI and this interaction coordinates neurotransmitter release  
328 [89] by regulating the extraction of Rab3A from SVs [72]. Considering that the association of  $\alpha$ GDI to

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

## 332 4. Materials and Methods

### 333 4.1 Materials

334 BoNT/A and TeNT were purified as previously described [92, 93]. BoNT/D was produced in *E. coli*  
335 by recombinant methods [39, 94]. LysoTracker® Red DND-99 was purchased from Thermo Fischer  
336 Scientific (L-7528), Bafilomycin A1 (sc-201550) and PX-12 (sc-358518) from Santa Cruz  
337 Biotechnology, Cytosine  $\beta$ -D-arabinofuranoside hydrochloride (C6645), DNase I (DN25),  
338 poly-L-lysine hydrobromide (P1274), Cyclosporin A (30024) and VER-155008 (SML0271) from Sigma  
339 Aldrich. Geldanamycin (GA) was purchased from Santa Cruz (sc-200617) and to prevent loss of  
340 activity, a fresh stock solution of 12.5 mM in DMSO was prepared for each experiment. Syntaxin 1A  
341 (110 111), VAMP-2 (104 211) and Rab3a (107 111) antibodies were purchased from Synaptic System.  
342 Anti-synaptophysin (clone Sy38) was from Dako, anti-thioredoxin reductase 1 (07-613) was from  
343 Merck Millipore, anti-thioredoxin 1 (clone EPR6111) was from GeneTex, anti-PSD95 (P-246) was  
344 from Sigma Aldrich, anti-Hsp90 (610418) was from BD Biosciences, anti-tom20 (Fl-145) was from  
345 Santa Cruz. SNAP-25 (SMI81,ab24737), Na<sup>+</sup>/K<sup>+</sup> ATPase (ab7671) antibodies were purchased from  
346 Abcam. The antibody specific for SNAP-25<sub>A</sub> (SNAP-25<sub>1-197</sub>) was produced in our laboratory and used  
347 as previously reported [70]. Secondary antibody were from Merck Millipore (HRP-conjugated) and  
348 from Life Technologies (Alexa Fluor-conjugated).

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

### 352 4.2. Cerebellar Granule Neurons (CGNs) cultures and botulinum neurotoxin inhibition assay

353 Primary cultures of rat cerebellar granule neurons (CGNs) were prepared from 6- to 8-days-old rats  
354 [97]. Cerebella were isolated, mechanically disrupted and then trypsinized in the presence of DNase  
355 I. Cells were then plated into 24 well plates, pre-coated with poly-L-lysine (50  $\mu$ g/mL), at a cell  
356 density of  $3.5 \times 10^5$  cells per well. Cultures were maintained at 37 °C, 5% CO<sub>2</sub>, 95% humidity in BME  
357 supplemented with 10% fetal bovine serum, 25 mM KCl, 2 mM glutamine and 50  $\mu$ g/mL gentamicin  
358 (complete culture medium). To arrest growth of non-neuronal cells, cytosine arabinoside (10  $\mu$ M)  
359 was added to the medium 18–24 h after plating. CGNs at 6-8 days in vitro were treated for 30 min  
360 with the indicated concentration of GA in complete culture medium at 37 °C and 5% CO<sub>2</sub>.

361 Thereafter, 2.5 nM BoNT/A or 0.5 nM TeNT in complete medium was added and left for 3.5 hours at  
362 37 °C and 5% CO<sub>2</sub>. In the case of BoNT/D, owing to its potency, the toxin was added as pulse of 15  
363 minutes at a concentration of 0.01 nM. The neuronal culture was then washed and the culture  
364 medium with the same concentration of inhibitor was restored for 195 min.

#### 365 4.3. Immunoblotting and immunocytochemistry

366 For immunoblotting analysis, cells were directly lysed with reducing Laemmli sample buffer  
367 containing protease inhibitors (complete Mini EDTA-free, Roche). Equal amounts of protein were  
368 loaded onto a 4-12% NuPage gel or 12% NuPage gel (Life technologies) and separated by  
369 electrophoresis in MES or MOPS buffer (Life technologies), respectively. Proteins were then  
370 transferred onto Protran nitrocellulose membranes (Whatman) and saturated for 1 h in PBST (PBS  
371 0.1% Tween20) supplemented with 5% non-fatty milk. Incubation with primary antibodies was  
372 performed overnight at 4°C. The membranes were then washed three times with PBST and  
373 incubated with secondary HRP-conjugated antibodies. Finally, membranes were washed three times  
374 with PBST and twice with PBS. Visualization was carried out using Luminata Crescendo (Merck  
375 Millipore).

376 For immunocytochemistry analysis, after the treatment, CGNs were washed with PBS, fixed for 10  
377 minutes at RT with 4% paraformaldehyde in PBS, quenched (50 mM NH<sub>4</sub>Cl in PBS) for 20 minutes  
378 and permeabilized with 5% acetic acid in ethanol for 20 minutes at -20 °C. BoNT/A cleavage was  
379 evaluated following the generation of SNAP-25<sub>A</sub>, whereas the cleavage of TeNT and BoNT/D was  
380 evaluated with an antibody recognizing the full-length form of VAMP-2. Primary antibodies were  
381 detected with secondary Alexa Fluor-conjugated antibodies. Coverslips were mounted using  
382 Fluorescent Mounting Medium (Dako, S3023) and examined by epifluorescence (Leica CTR6000)  
383 microscopy.

#### 384 4.4. cpV-HC/A binding and internalization assay

385 The HC of BoNT/A (nucleotides corresponding to 876-1296) with a N-terminal fused with cpV  
386 (Circularly Permutated Venus) at the N-terminus was cloned into a pET28a His-tag vector  
387 (Novagen) and expressed into BL21 (DE3) *E. coli* cells. Protein purification was carried out by affinity  
388 chromatography with a prepacked HisTrap Ni column (GE Healthcare) and then by size-exclusion  
389 chromatography using a Superdex 200, 10/300GL column (GE Healthcare). Purified HC/A was  
390 dialyzed into 20 mM TRIS, 200 mM NaCl, 40% glycerol (pH 7.6). For the assay, CGNs were treated  
391 with GA 12.5 μM or vehicle (DMSO) in culture medium at 37 °C. After 30 minutes, 250 nM  
392 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  $\mu$ M of GA or 10 nM BoNT/D for 30 min at 37 °C. 5  $\mu$ g/ml of an anti Synaptotagmin-1 antibody  
398 (recognizing the luminal 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  $\mu$ M GA or 10 nM bafilomycin  
404 A1 in complete culture medium supplemented with 6.25 mM HEPES at 37 °C and 5% CO<sub>2</sub>. 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<sub>2</sub>, 1.2 mM MgSO<sub>4</sub> and 1.2 mM  
407 K<sub>2</sub>HPO<sub>4</sub>) and images of living neurons were acquired with a Leica CTR6000 microscope.

#### 408 4.7. In vitro proteolytic activity

409 0.1  $\mu$ g BoNT/D or BoNT/A or TeNT was incubated in reducing buffer (150 mM NaCl, 10 mM  
410 NaH<sub>2</sub>PO<sub>4</sub>, 15 mM DTT pH 7.4) in the presence of 12.5  $\mu$ M GA for 30 min at 37 °C or vehicle (DMSO).  
411 Then 1  $\mu$ g of recombinant GST-VAMP-2 (1-96), for BoNT/D or TeNT, or 1  $\mu$ g 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<sub>2</sub>. 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<sub>2</sub>, 1.5 mM CaCl<sub>2</sub>, 5 mM NaP<sub>i</sub>, 5 mM citric acid,  
421 5.6 mM glucose, 10 mM NH<sub>4</sub>Cl), 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 were then washed twice and further

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

#### 425 4.9. Purification of synaptic vesicles and immunoprecipitation

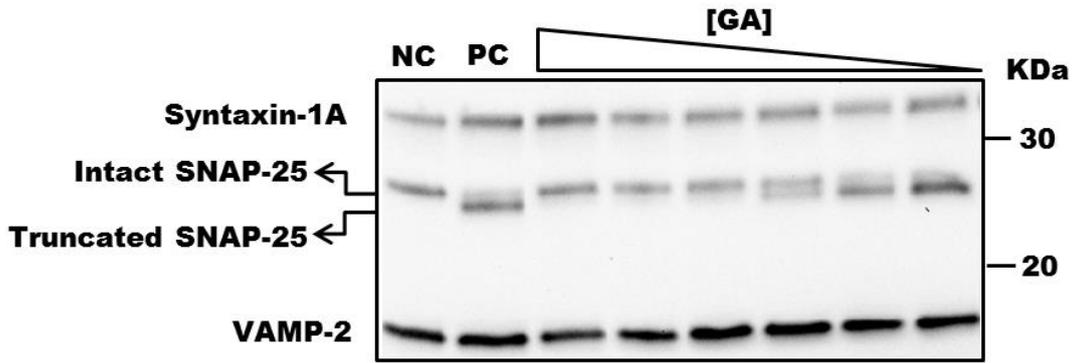
426 Synaptosomes and synaptic vesicles were isolated from cerebral cortices as previously described  
427 [50], with minor changes of the classical procedure [98]. Namely, after differential centrifugations,  
428 crude synaptic vesicles were separated through a continuous sucrose gradient (0.25-1.5 M sucrose, 4  
429 mM HEPES, pH 7.3) in a Beckmann XL-80 ultracentrifuge for 5 hr with a SW28 rotor. Vesicles  
430 sedimenting at about 300-400 mM sucrose (free SV) and those sedimenting at 800-1000 mM (docked  
431 SV) were collected and pelleted by centrifugation in a 70Ti rotor. These vesicle fractions were  
432 re-suspended in SV buffer (4 mM HEPES, 300 mM glycine, pH 7.4 supplemented with complete  
433 protease inhibitors, EDTA-free). For immunoblotting analysis, protein concentration was  
434 determined with BCA test (Pierce BCA protein assay, Thermo Scientific) and 10 µg of different  
435 fractions were used for SDS-PAGE under reducing conditions.

436 Immunoprecipitation of Hsp90 was carried out as previously reported [99]: 2 mg of rat brain  
437 synaptosomes were re-suspended in buffer B (20 mM HEPES, 140 mM NaCl, 5 mM KCl, 5 mM  
438 NaHCO<sub>3</sub>, 1.2 mM Na<sub>2</sub>HPO<sub>4</sub>, 1 mM MgCl<sub>2</sub> and 10 mM glucose, pH 7.4) and subjected to osmotic  
439 shock using H<sub>2</sub>O in presence of protease inhibitors for 30 minutes at 4 °C. Sample were subjected to  
440 three passages though 18 G needles and three through 27 G needles and then centrifuged for 5 min  
441 at 10000 g. The resulting supernatant was centrifuged for 1 hour at 80000 r.p.m. to yield a crude  
442 synaptic vesicle fraction in the pellet and a cytosolic fraction in the supernatant [100]. The pellet  
443 fraction was re-suspended in buffer B and incubated overnight at 4 °C with G protein-decorated  
444 Dynabeads (Life Technology) coupled to anti-Hsp90 antibody, preventively washed in PBS 0.1%  
445 BSA. Beads were then extensively washed twice with a buffer containing 20 mM TRIS, 80 mM NaCl  
446 and 1% Triton X-100, pH 7.4 and twice with PBS. Hsp90 interactors were eluted by addition of 0.2 M  
447 glycine (pH 2.6). Samples were then added to non-reducing loaded sample buffer and subjected to  
448 SDS-PAGE. Proteins were then labeled with specific antibodies.

#### 449 4.10. Viability test

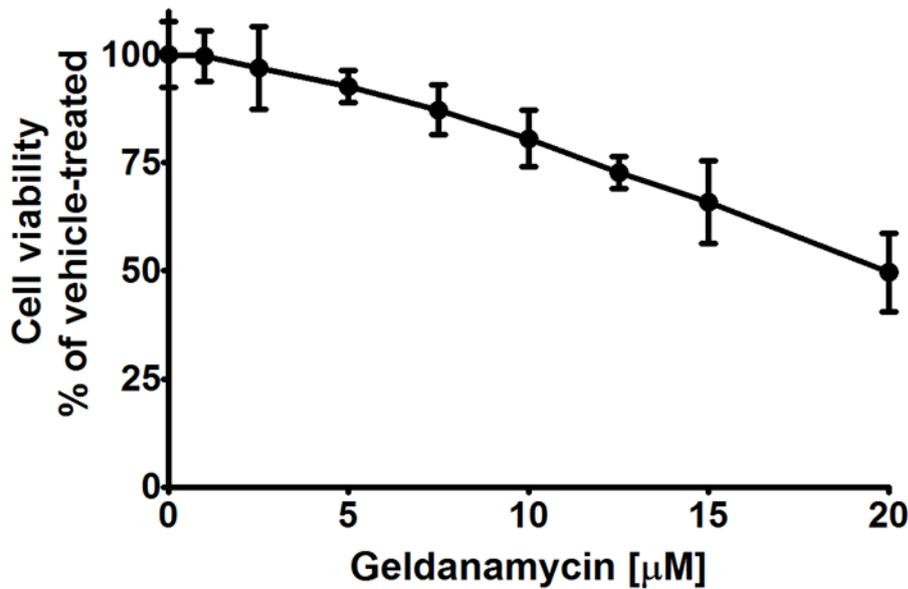
450 CGNs were seeded in a 96 wells plates at a cell density 10<sup>5</sup> cells per well. After 6 div, different  
451 concentration of GA, ranging from 0 to 20 µM, were added and left for 4 hours. Neurons were then  
452 washed and CellTiter 96® AQueous (Promega) performed according to manufacturer indication.

453 **Supplementary Materials:** The following are available online at [www.mdpi.com/link](http://www.mdpi.com/link), Figure S1: title, Table S1:  
454 title, Video S1: title.



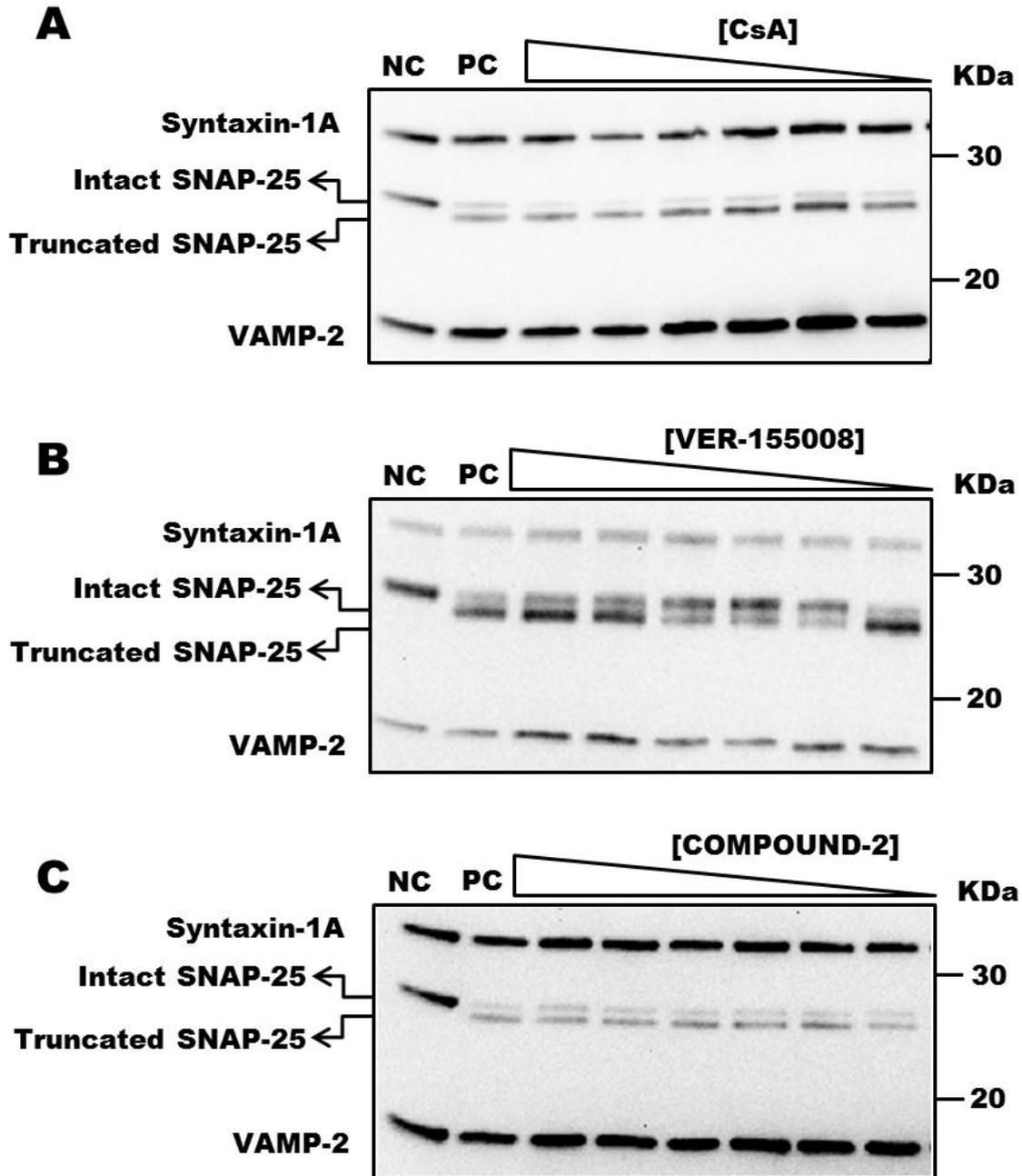
455

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



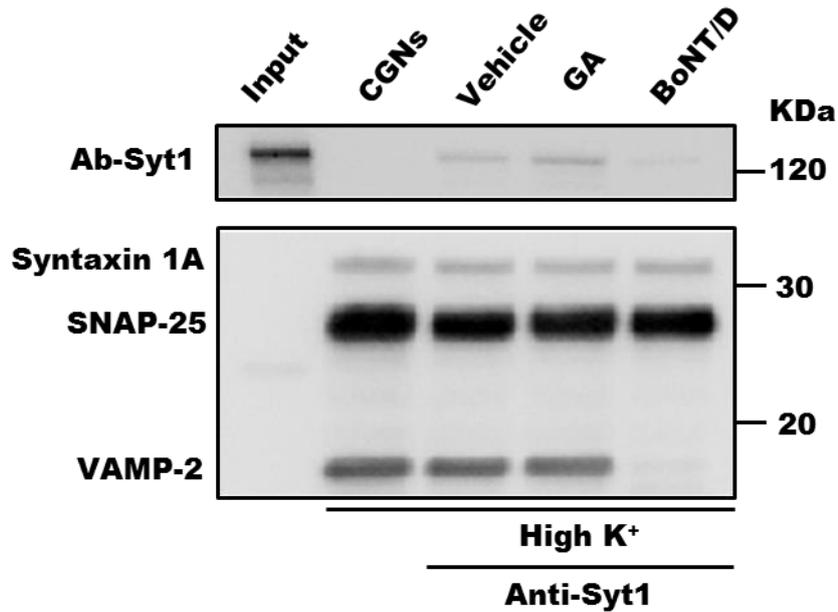
462

463 **Figure S2.** Neuron viability upon geldanamycin treatment. CGNs were treated with increasing  
 464 concentration of geldanamycin ranging from 2.5 to 20  $\mu\text{M}$  or vehicle (DMSO) in culture medium 37  
 465  $^{\circ}\text{C}$  for 4 hours. Cell viability was then assayed with a MTS assay. Data are presented as a percentage  
 466 with respect to non-treated cells. All data are presented as mean values and error bars indicate the  
 467 deviation standard obtained from two independent experiments.

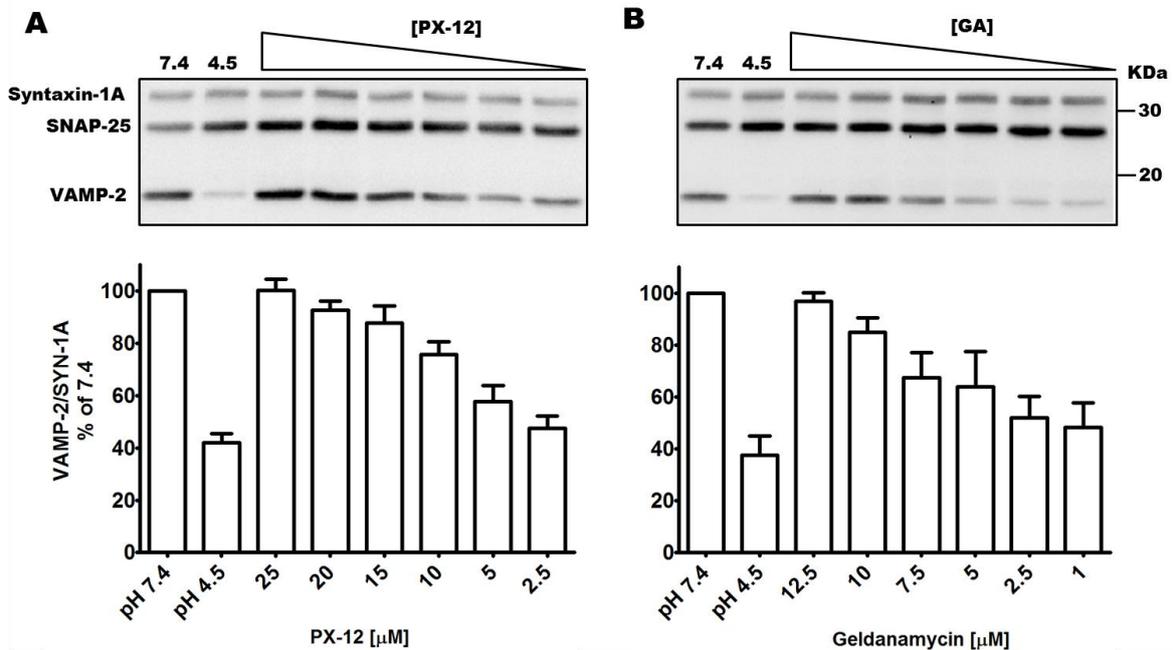


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**Figure S3.** Cyclosporine A, VER-155008 and compound-2 does not prevent BoNT/A intoxication in CGNs. Neurons were treated like in Figure 1 but using different inhibitors: (A) cyclosporine A, (B) VER-155008 or (C) compound-2. Thereafter, CGNs were 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  $\mu$ M for CsA and VER-155008; 5, 2.5, 1, 0.5, 0.25, 0.1  $\mu$ M for compound-2.



475  
 476 **Figure S4.** Geldanamycin does not affect synaptic vesicles dynamics. (B) CGNs were treated as in  
 477 Figure 3A but using as reporter of SVs dynamics an antibody against the luminal domain of  
 478 Synaptotagmin-1. Where indicated, BoNT/D was added before starting GA treatment. The  
 479 internalized antibody was detected by WB, using directly a secondary antibody. Syntaxin 1A and  
 480 SNAP-25 were considered as loading controls, instead, VAMP-2 staining was used to assess BoNT/D  
 481 cleavage. In the first lane (input) 50 ng of the anti-Synaptotagmin 1 antibody were loaded as  
 482 reference. The immunoblot is representative of three independent sets of experiments.



483  
 484 **Figure S5.** PX-12 and geldanamycin inhibit the translocation of BoNT/D L chain across the plasma  
 485 membrane in a concentration dependent manner. CGNs were like in Figure 4 using the indicated  
 486 concentrations of PX-12 (A) or GA (B). The translocation of BoNT/D was assessed by monitoring the  
 487 cleavage of VAMP-2, determined via WB (top panels). Bottom panels show the amount of intact  
 488 VAMP-2 reported as a ratio to Syntaxin 1A which served as loading control, taking the value in pH  
 489 7.4 treated-cells as 100%. All data are presented as mean values with SD values arising from three  
 490 independent experiments.

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495

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497 evaluated experiments under the supervision of M.P., O.R. and C.M. T.B. and O.R. purified and tested  
498 botulinum neurotoxins. O.L. cloned, expressed and purified cPV-HC/A. D.A.T., M.P. and C.M. wrote the paper  
499 with contributions of all co-authors.

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

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