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## Characterization of a highly neutralizing single monoclonal antibody to botulinum neurotoxin type A

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33 mechanism of TA12 and emphasizes on the potential of using single mAbs for the treatment of  
34 botulism type A.

35

36 **Key Words**

37 Botulinum neurotoxin; Botulism; Monoclonal antibody; Synaptic vesicle protein 2; GT1b,  
38 Mass spectrometry; Hydrogen-Deuterium eXchange; Epitope mapping

39

40 **Running title**

41 Potent neutralization of BoNT/A1 with a single mAb

42

43 **Nonstandard abbreviations**

44 BoNT, botulinum neurotoxin

45 L, light chain

46 H, heavy chain

47 H<sub>C</sub>, half C-terminal H chain

48 H<sub>N</sub>, half N-terminal H chain

49 SV2C, synaptic vesicle protein 2C

50 gSV2C, glycosylated SV2C

51 SNARE, soluble *N*-ethylmaleimide-sensitive-factor attachment protein receptor

52 mAb, monoclonal antibody

53 HDX-MS, Hydrogen/Deuterium eXchange Mass Spectrometry

54 TCEP, Tris(2-carboxylethyl)phosphine

55 EIA, enzyme immunoassay

56 LD, luminal domain

57 BSA, bovine serum albumin

58 AMC, anti-mouse Fc

59 TGY, trypticase-glucose-yeast extract

60 VHH, variable domain of heavy chain

61 MLD, mouse lethal dose

62

## 63 INTRODUCTION

64 Botulism is a rare but severe disease that results from botulinum neurotoxin (BoNT)  
65 intoxication. Naturally acquired botulism can develop in three main forms: foodborne BoNT  
66 intoxication, intestinal colonization by BoNT-producing clostridium (infant botulism and more  
67 rarely botulism by intestinal colonization in adults), or wound botulism. Among the seven  
68 different BoNT toxinotypes, BoNT/A is the most potent toxin with a lethal dose for human  
69 estimated at ~1 ng/kg by parenteral route (1, 2). BoNTs and specially the BoNT/A toxinotype  
70 are therefore classified by the Centers for Diseases Control and Prevention (CDC) among the  
71 six major bioterrorism agents (category A) (3). BoNT/A is a ~1296 residues long protein that  
72 is proteolytically processed into a light (L) and a heavy chain (H) linked by a single disulfide  
73 bond. The L chain contains the intracellularly active enzymatic site that specifically cleaves  
74 neuronal members of the *N*-ethylmaleimide sensitive factor attachment protein receptors  
75 (SNAREs) complex required for the release of acetylcholine. The C-terminal H chain (H<sub>C</sub>)  
76 recognizes both the intraluminal loop of the synaptic vesicle glycoprotein 2C (SV2C) and  
77 GT1b/GD1a gangliosides as functional dual receptors on neuronal cells. Finally, the N-terminal  
78 H domain (H<sub>N</sub>) is involved in the translocation of the L chain from endocytic vesicles into the  
79 cytosol of neuronal cell targets (4-11).

80 Antitoxin serotherapies are currently the only specific and available treatments for  
81 botulism. The clinical benefit and effectiveness of such therapies remain however linked to  
82 their use at the early onset of the disease *only*. The equine antitoxin therapy composed of  
83 polyclonal IgG antibodies is the most available treatment although it is often associated to  
84 hypersensitivity reactions including serum sickness and cardiac arrest (12, 13). Human  
85 botulism-immune globulins from vaccinated donors have been developed notably for the  
86 treatment of infant botulism (14). The first one—BabyBIG, is supplied by the California  
87 Department of Public Health, but with limited stock. This formulation has been recently  
88 discontinued and will be replaced with a new formulation currently under clinical development  
89 (15). Recently, the safety of a new cocktail composed of three humanized mAbs (XOMA 3B)  
90 engineered to neutralize BoNT/A subclasses has been evaluated and confirmed in healthy  
91 volunteers (16). The European AntiBotABE Program has allowed the isolation of recombinant  
92 humanized antibodies neutralizing BoNT/A, B and E. Protective antibody combinations against  
93 BoNT/A and BoNT/B showed cross-neutralization of both BoNT/A1 and A2 as well as  
94 BoNT/B1 and B2, respectively (17).

95 Monoclonal antibodies (mAbs) represent therefore a promising alternative strategy to treat  
96 botulism with the advantage of being better tolerated by patients and produced in a more

97 reproducible and safer way than animal hyperimmune serum. Various murine or humanized  
98 mAbs raised against whole BoNTs or BoNT subdomains have been evaluated for their  
99 neutralizing potency and their possible therapeutic use (review in (17, 18)). Most often, efficient  
100 toxin neutralization requires a combination of several mAbs. For example, the association of  
101 three mAbs has been reported to neutralize BoNT/A, BoNT/E, or BoNT/F, respectively (19-  
102 21). Recently, a single anti-BoNT/A tri-epitopic mAb with equivalent binding and  
103 neutralization potency than three distinct mAbs has been generated (22). The use of a single  
104 mAb as alternative therapeutic antitoxin agent appears therefore promising and offers the  
105 advantage to simplify the process of development and to reduce the cost of production.

106 We previously reported a potent neutralizing murine mAb (named TA12) generated against  
107 the C-terminal domain of the BoNT/A1 heavy chain (H<sub>C</sub>A1) (23, 24). In this study, we focused  
108 on the structural characterization of the TA12:H<sub>C</sub>A1 complex by Hydrogen/Deuterium  
109 eXchange Mass Spectrometry (HDX-MS) approach combined with site directed mutagenesis  
110 and functional assays (25, 26). We report that TA12 targets a conformational epitope on H<sub>C</sub>A1  
111 located at the junction between the H<sub>CC</sub> and the H<sub>CN</sub> subdomains. The TA12 epitope overlaps  
112 with both the SV2C cell receptor and the GT1b binding sites, hence perturbing the dual-receptor  
113 binding process required for the entry of BoNT/A1 into neuronal cells.

114

## 115 **MATERIALS AND METHODS**

### 116 **Biological reagents**

117 mAb TA12 was prepared and affinity purified on protein A beads (Prosep-A High capacity,  
118 Millipore, Billerica, USA) as previously described (27).

119 Recombinant H<sub>C</sub> of BoNT/A1 (H<sub>C</sub>A1), corresponding to the receptor binding domain of  
120 BoNT/A1 was prepared as previously described (28). DNAs encoding H<sub>C</sub> of BoNT/A subtypes  
121 were PCR amplified with primers and *C. botulinum* genomic DNA (**Extended Table 1**) and  
122 cloned into pET28a. Recombinant H<sub>C</sub> containing N-terminal 6xHis-tag were produced and  
123 purified as previously described (28). Primers used to generate H<sub>C</sub>A1 mutants are indicated in  
124 **Extended Table 2**.

125 cDNA encoding the human intraluminal SV2C fragment L4 (amino acid 454 to 579,  
126 hereafter named SV2C-LD) was PCR amplified as previously described (29). Briefly, DNA  
127 encoding the amino acids 454 to 579 of human SV2C was PCR amplified with primers P1415  
128 (5'- GGATCCTTCCCTGATGTCATTAAACCTCTG-3') and P1416 (5'-  
129 GAATTCCTAGTAGGCACTATAGTCATCATCAAA-3') adding *Bam*HI and *Eco*RI  
130 restriction sites, respectively, and was cloned into pGEX-2T at the corresponding restriction

131 sites. The construction was verified by DNA sequencing. The fusion protein (41 kDa) contains  
132 N-terminal GST and C-terminal SV2C-LD linked by Gly-Ser resulting from the *Bam*HI site.  
133 The recombinant plasmid was cloned into *E. coli* BL21 DE3 and the resulting bacterial strain  
134 was grown in LB medium containing ampicillin (100 µg/ml). The culture was induced with  
135 IPTG (0.2 mM) and further grown at 18°C for 18 h. The bacterial cells were washed with  
136 distilled water, suspended in 50 mM Tris, 150 mM NaCl, 10 mM MgCl<sub>2</sub>, 1 mM dithiothreitol,  
137 0.1% Tween20 containing complete protease inhibitor cocktail EDTA free (Roche, France),  
138 and lysed by ultrasonication. The lysate was incubated with Glutathione-Sepharose-4B beads  
139 (GE Healthcare) for 1 h at 4°C. The beads were washed three times with the same buffer and a  
140 50% suspension in the same buffer was used for the pull-down assays.

141 For HDX-MS experiments, 6His-tag-SV2C-LD was used. The PCR amplified DNA  
142 encoding SV2C-LD was cloned into pET28 vector at the *Bam*HI-*Eco*RI site. The recombinant  
143 N-terminal 6His-tag-SV2C-LD protein was produced in *E. coli* BL21 DE3 and purified on a  
144 Talon metal affinity matrix (Clontech) equilibrated in 10 mM Tris, 150 mM NaCl, 10 mM  
145 imidazole, 10 mM TCEP, pH 7.5. The recombinant protein was eluted with the same buffer  
146 supplemented with 100 mM imidazole and dialyzed against the same buffer without imidazole.  
147

#### 148 **Affinity determination of mAb TA12**

149 The affinities of mAb TA12 for different recombinant HcBoNT isoforms were determined  
150 by Bio-layer Interferometry using the ForteBio system (Pall Laboratory). mAb TA12 prepared  
151 at 10 µg/mL in EIA (enzyme immunoassay) buffer (0.1 M phosphate buffer, pH 7.4, 0.15 M  
152 NaCl, 0.1% BSA, and 0.01% sodium azide) + 0.02% Tween 20 (Sigma) was dispensed in 96-  
153 well microplates at a volume of 200 µL per well. The same concentration occupied 8 vertical  
154 wells. In other wells, recombinant HcBoNT proteins were dispensed at 8 titrated concentrations  
155 (between 100 and 0 nM depending on the protein). A glycine (Sigma, pH [1.4]) regeneration  
156 solution and EIA buffer + 0.02% Tween 20 for baseline stabilization and neutralization was  
157 also prepared. The plate was agitated at 1000 rpm over the entire course of the experiment.  
158 Prior to the binding measurements, the anti-mouse Fc (AMC) sensors tips were hydrated in EIA  
159 buffer + 0.02% tween 20. The sensor tips were then transferred to the EIA buffer + 0.02%  
160 Tween 20 for the baseline, then for 300 sec into wells containing mAb for loading step. After  
161 baseline step in EIA buffer + 0.02% Tween 20 for 60 sec, the binding kinetics were measured  
162 by dipping the mAb-coated sensors into the wells containing recombinant HcBoNT at varying  
163 concentrations. The binding interactions were monitoring over a 900 sec associated period and  
164 followed by a 900 sec dissociation period in the wells containing EIA buffer +0.02 % Tween

165 20. Between each binding cycle, the AMC sensors tips were regenerated with wells containing  
166 glycine and neutralized in the EIA buffer + 0.02% tween 20. The equilibrium dissociation  
167 constant ( $K_D$ ) was calculated using the ratio between the dissociation rate constant ( $k_{off}$ ) and  
168 the association rate constant ( $k_{on}$ ), obtained with a global Langmuir 1:1 fit (Octet Data Analysis  
169 software, vHT.10).

170

### 171 **GST pull down assay**

172 Recombinant SV2C-LD immobilized on glutathione-Sepharose-4B matrix (GE healthcare,  
173 France) (30  $\mu$ L of 50% bead suspension) was incubated for 90 min at 4°C with H<sub>C</sub>A1 solutions  
174 prepared with or without mAb TA12 in 50 mM Tris buffer, 150 mM NaCl, 10 mM MgCl<sub>2</sub>, 2  
175 mM dithiothreitol, 0.1% Tween 20, pH 7.5, supplemented with antiproteases (protease inhibitor  
176 cocktail EDTA free, Roche Diagnostics, Germany) (final volume 400  $\mu$ L). H<sub>C</sub>A1:TA12  
177 solutions were pre-incubated for 15 min at room temperature before use. Beads were collected  
178 by centrifugation and washed three times with the same buffer. Washed pellets were suspended  
179 in SDS/2-mercaptoethanol sample buffer for 2 min at 95°C and analyzed by SDS-PAGE.  
180 Proteins were stained by Coomassie blue.

181

### 182 **Ganglioside binding assay**

183 Binding of H<sub>C</sub>A1 to the GT1b ganglioside was assessed in a solid phase assay as  
184 previously described (30-32). Briefly, bovine brain GT1b (Calbiochem, France) stored in  
185 dimethyl sulfoxide (20 mg/mL) at -20°C was diluted in methanol and applied overnight to 96-  
186 well plates (Corning-Costar, France) at 2  $\mu$ g per well. Methanol was evaporated at room  
187 temperature and the plates were washed three times with phosphate-buffered saline containing  
188 0.05 % Tween 20 (Sigma, France) (PBST). Plates were blocked with 5% non-fat skim milk in  
189 PBST for 1 h at room temperature and washed with PBST. H<sub>C</sub>A1 was then dispensed (10  
190 nM/well in PBS) in the control wells and incubated for 2 h at 4°C. For competition with mAb  
191 TA12, H<sub>C</sub>A1 (10 nM) was incubated with various concentrations of TA12 in 100  $\mu$ L PBS for  
192 15 min at room temperature prior being applied to GT1b coated wells for 2 h at 4°C. After  
193 washing with PBST, bound H<sub>C</sub>A1 was detected with rabbit anti-H<sub>C</sub>A1 (1:10,000) (29) and horse  
194 radish peroxidase (HRP) goat anti-rabbit immunoglobulin IgG (1:3,000) (Invitrogen, France).  
195 Ortho phenyldiamine (Sigma, France) was used as substrate (1 mg/mL in citrate buffer pH  
196 4.5 containing H<sub>2</sub>O<sub>2</sub>), and the reaction was stopped with HCl 3 M (50  $\mu$ L/well). The extinction  
197 was measured at 490 nm and 650 nm as reference.

198

**199 Neutralizing activity**

200 BoNTs were prepared from *C. botulinum* cultures in TGY (trypticase-glucose-yeast  
201 extract) medium for four days under anaerobic conditions at 37°C as previously described (33).  
202 Briefly, the cultures were acidified at pH 3.5 with sulfuric acid, centrifuged at 10,000 x g for  
203 15 min at 4°C, washed with distilled water, and the BoNT complexes were extracted three times  
204 with 0.2 M phosphate buffer pH 6.3. BoNT complexes were precipitated with 65% ammonium  
205 sulfate saturation and then dialyzed against 50 mM phosphate buffer, pH 6.3 containing 0.2%  
206 gelatin (PB-G).

207 Mouse protection assay was performed with male Swiss mice (Charles River) weighing  
208 20-22 g as previously described (34). Variable amounts of TA12 were incubated with 5  
209 estimated mouse 50% lethal doses of either BoNT/A1, A2, A3, A5 or A7 in PB-G for 30 min  
210 at room temperature. The mixtures (0.5 mL) were then injected intraperitoneally into mice with  
211 control mice receiving BoNT alone. Mice were observed and any death was recorded every day  
212 during 4 days.

213

**214 Ethic statements**

215 All experiments were performed in accordance with French and European Community  
216 guidelines for laboratory animal handling. The protocols of experiments were approved by  
217 Institut Pasteur CETEA (Comité d'Ethique en Expérimentation Animale) with the agreement  
218 of laboratory animal use (N° 2013-0116) and by the Ministère de l'Education Nationale et de  
219 l'Enseignement Supérieur with the agreement 02026.02.

220

**221 HDX-MS experiments**

222 A summary of the HDX data containing the main experimental details is provided in  
223 **Extended Table 3** (35). Prior to initiating the labeling, the quality of each protein was assessed  
224 by SDS-PAGE and intact mass analysis (**Extended Figure 1**).

225

226 **Sample preparation.** For epitope mapping, H<sub>C</sub>A1 was labeled in both the presence and absence  
227 of the mouse mAb TA12. The TA12: H<sub>C</sub>A1 complex was formed by mixing 3 µL of H<sub>C</sub>A1 (29  
228 µM in buffer A: 10 mM Tris, 150 mM NaCl, pH 7.4) with 4.5 µL of mAb TA12 (27 µM in  
229 buffer A). Control samples (unbound H<sub>C</sub>A1) were prepared in parallel by replacing TA12 with  
230 buffer A. After 1 h incubation at 20°C, the labeling was initiated by adding 67.5 µL of



231 deuterated buffer (10 mM Tris, 150 mM NaCl, pD 7.4; D<sub>2</sub>O/H<sub>2</sub>O ratio: 90/10%). Assuming a  
232 K<sub>D</sub> value of ~20 pM (24) (and this study), ~100% of H<sub>C</sub>A1 remains bound to TA12 during  
233 hydrogen exchange. Continuous labeling was performed at 20°C for t = 0.16, 1, 5, 10, 30, 60  
234 and 120 min. Aliquots of 10 μL (*i.e.*, 11.6 pmoles of H<sub>C</sub>A1) were removed and quenched upon  
235 mixing with 50 μL of an ice cold solution of 2% formic acid, 4M urea to decrease the pH to 2.5  
236 (Final D<sub>2</sub>O/H<sub>2</sub>O ratio: 15/85%). Quenched samples were immediately snap frozen in liquid  
237 nitrogen and stored at -80°C until MS acquisition (less than 3 days).

238 An identical procedure was used to map the SV2C-LD interaction site on H<sub>C</sub>A1. The  
239 SV2C-LD: H<sub>C</sub>A1 complex was formed by mixing 3 μL of H<sub>C</sub>A1 (29 μM in buffer A) with 4.5  
240 μL of SV2C-LD (22 μM in buffer A supplemented with 10 mM TCEP). The labeling was  
241 performed in the presence of 1 mM TCEP at 20°C. Assuming a K<sub>D</sub> value of 28 nM (6), 90.5%  
242 of H<sub>C</sub>A1 remains bound to SV2C-LD during labeling.

243 Undeuterated H<sub>C</sub>A1 samples were obtained following the same experimental procedure. A  
244 fully deuterated H<sub>C</sub>A1 control was prepared in 10 mM Tris buffer, 150 mM NaCl, 8 M urea-  
245 d<sub>4</sub>, pD 7.4 (D<sub>2</sub>O/H<sub>2</sub>O ratio: 90/10%), incubated overnight at 20°C and processed as described  
246 above. All samples were prepared in triplicate for each time point and condition (independent  
247 technical replicate).

248  
249 **Data acquisition.** Quenched samples were rapidly thawed and injected onto a nanoACQUITY  
250 UPLC M-Class system (Waters Corporation, Milford, MA) equipped with a HDX manager  
251 maintained at 0°C to minimize back-exchange. Labeled samples (9.7 pmol of H<sub>C</sub>A1 either alone  
252 or with 13.5/11 pmol of TA12/SV2C-LD) were digested using an in-house packed cartridge  
253 (2.0 x 20 mm, 63 μL bed volume) of immobilized pepsin beads (Thermo Scientific, Rockford,  
254 IL) for 2 min at 20°C. Peptides were directly trapped and desalted onto a C18 Trap column  
255 (VanGuard BEH 1.7 μm, 2.1 x 5 mm, Waters Corporation, Milford, MA) at a flow rate of 100  
256 μL/min (0.15% formic acid) and separated by a 10 min linear gradient of 5-40% acetonitrile at  
257 40 μL/min using an ACQUITY UPLC BEH C18 analytical column (1.7 μm, 1 × 100 mm,  
258 Waters Corporation, Milford, MA). After each run, the pepsin column was manually cleaned  
259 with two consecutive injections of 1% formic acid, 5% acetonitrile, 1.5 M guanidinium  
260 chloride, pH 1.7. Blank injections were performed between each run to confirm the absence of  
261 carry-over. Mass spectra were acquired in resolution and positive mode (*m/z* 50-2000) on a  
262 Synapt G2-Si HDMS mass spectrometer (Waters Corporation, Milford, MA) equipped with a  
263 standard ESI source and lock-mass correction. Peptic peptides were identified in undeuterated

264 samples by a combination of data independent acquisition (MS<sup>E</sup>) and exact mass measurement  
265 (below 5.0 ppm mass error) using the same chromatographic conditions than for the deuterated  
266 samples.

267

268 **Data processing.** The initial peptide map of HcA1 was generated by database searching in  
269 ProteinLynX Global server 3.0 (Waters corporation, Milford, MA) using the following  
270 processing and workflow parameters: low and elevated intensity thresholds set to 100.0 and  
271 50.0 counts; intensity threshold sets to 750.0 counts; automatic peptide and fragment tolerance;  
272 non-specific primary digest reagent; false discovery rate sets to 4%. Each fragmentation  
273 spectrum was manually inspected for assignment confirmation. The peptide map was refined  
274 in DynamX 3.0 (Waters corporation, Milford, MA) using the following Import PLGS results  
275 filter: minimum intensity = 1500; minimum products per amino acid = 0.4; minimum score =  
276 6.8; maximum MH<sup>+</sup> error (ppm) = 5; file threshold = 2.

277 DynamX 3.0 was used to extract the centroid masses of all peptides selected for HDX-MS  
278 analyses. Only one unique charge state was considered per peptide and no back-exchange  
279 correction was performed. HDX results are reported as relative deuterium exchange level  
280 expressed in either mass unit or fractional exchange. Fractional exchange data were calculated  
281 by dividing the experimental uptake value by the theoretically maximum number of  
282 exchangeable backbone amide hydrogens that could be replaced into each peptide in 90%  
283 excess deuterium. MEMHDX (36) was used to visualize and statistically validate the HDX data  
284 (Wald test, false discovery rate of 5%).

285 The presence of bimodal *m/z* envelopes (i.e., EX1 behavior) was determined by visually  
286 inspecting the shape of the isotopic distribution for all peptides. HX-Express2 software was  
287 used to extract the uptake value of bimodal *m/z* envelopes using a Double-Gaussian fitting (37).

288

## 289 RESULTS

### 290 TA12 efficiently neutralizes BoNT/A1

291 BoNT/A is divided into 8 distinct subtypes (BoNT/A1 to BoNT/A8) according to their  
292 amino acid sequence variations ranging from 2.9 % to 15.6 % (1). Although the BoNT/A  
293 toxinotype is defined by its neutralization with polyclonal anti-BoNT/A antibodies, each  
294 subtype can be distinctly neutralized by individual mAbs (1). We recorded that TA12 efficiently  
295 neutralized BoNT/A1 and displayed an inhibitory spectrum on BoNT/A subtypes A2, A3, and  
296 A7 (**Table 1**) (23). However, it showed a weak neutralizing activity against BoNT/A5 (**Table**  
297 **1**). Taken together, these results confirm that TA12 is a highly neutralizing mAb of the most

298 potent subtype BoNT/A1 with medium neutralizing activity against BoNT/A2 and A3 subtypes  
299 and low neutralizing activity against BoNT/A5 (38, 39).

300

### 301 **TA12 binding affinity to BoNT/A subtypes**

302 TA12 binding affinity to the H<sub>C</sub> domain of different BoNT/A subtypes was determined by  
303 Bio-Layer Interferometry. The binding affinity of TA12 was very high for H<sub>C</sub>A1, H<sub>C</sub>A2, and  
304 H<sub>C</sub>A3 with K<sub>D</sub> values in the low pM range (i.e., < 100 pM; **Table 2**) in good agreement with  
305 previous data obtained for H<sub>C</sub>A1 using Biacore instrument (24). The TA12 affinity was slightly  
306 lower with H<sub>C</sub>A2 and significantly reduced with H<sub>C</sub>A4, H<sub>C</sub>A5, and H<sub>C</sub>A7 with K<sub>D</sub> values in the  
307 low nM range. These differences in K<sub>D</sub> mainly result from faster dissociation rate values for  
308 H<sub>C</sub>A2, H<sub>C</sub>A4, H<sub>C</sub>A5, and H<sub>C</sub>A7 as compared to H<sub>C</sub>A1. Indeed, TA12 interacts with the H<sub>C</sub>  
309 domain of the different BoNT/A subtypes with similar on-rates values (**Table 2**). Consistent  
310 with the protection of TA12 on BoNT/A subtypes, TA12 binds BoNT/A Hc domains with  
311 maximal affinities for BoNT/A1, A2, and A3. These results reveal that low picomolar affinities  
312 for the H<sub>C</sub> domain are required to achieve maximal TA12 neutralizing power in the mouse  
313 lethality assay.

314

### 315 **TA12 recognizes a conformational epitope at the junction between the H<sub>CC</sub> and the H<sub>CN</sub>** 316 **subdomains of H<sub>C</sub>A1**

317 To better understand the mechanism underlying BoNT/A1 neutralization by TA12, the free  
318 and TA12-bound state of H<sub>C</sub>A1 were subjected to HDX-MS analysis. The quench and pepsin  
319 digestions were first optimized to generate a peptide map with high sequence coverage and  
320 peptide redundancy. Urea was included in the quench buffer to favor the dissociation of the  
321 TA12: H<sub>C</sub>A1 complex and increase the accessibility of both proteins to pepsin during the  
322 digestion step. A total of 98 unique peptides covering 85.2% of the H<sub>C</sub>A1 sequence were  
323 confidently identified, of which 62 were selected for HDX-MS (**Extended Figure 2**).  
324 Overlapping peptides were only used to increase the spatial resolution when their back-  
325 exchange values were similar (i.e., < 10%; **Extended file HDX.xls**).

326 The binding of TA12 reduces the solvent accessibility of 12 peptides covering six distinct  
327 regions within H<sub>C</sub>A1 (**Figure 1A, Extended Figure 3**). The main decreases in solvent  
328 accessibility occur in region 2 (peptide 1005-1016; loop β31-β32, β32), in region 4 (peptides  
329 1114-1134, 1115-1134, 1116-1134 and 1117-1134; loop-β38-loop), and in region 5 (peptides  
330 1135-1144, 1135-1147 and 1148-1154; β39-loop-β40-loop-β41). Inspection of the different

331 overlapping peptides in regions 2 and 4 reveals that the reduction of solvent accessibility is  
332 restricted to segments 1005-1009 and 1116-1124. In addition, a slight but statistically  
333 significant reduction in solvent accessibility was observed in region 1 (peptides 946-955, 946-  
334 958 and 946-961; loop  $\beta$ 27- $\beta$ 28) and in region 6 (peptide 1250-1261;  $\beta$ 49-loop) (**Figure 1A**  
335 **and Extended Figure 4A**).

336 As shown in **Figure 1B**, TA12 binds H<sub>C</sub>A1 at the junction between the N-terminal lectin  
337 subdomain (H<sub>CN</sub>) and the C-terminal trefoil subdomain (H<sub>CC</sub>). The TA12 epitope appears  
338 conformational and mainly formed by the  $\beta$ 27- $\beta$ 28 and  $\beta$ 31- $\beta$ 32 loops of H<sub>CN</sub> and by the  
339 structural elements  $\beta$ 38 to  $\beta$ 41,  $\beta$ 49 and the  $\beta$ 37- $\beta$ 38,  $\beta$ 39- $\beta$ 40,  $\beta$ 40- $\beta$ 41 and  $\beta$ 49- $\beta$ 50 loops of  
340 H<sub>CC</sub>. Interestingly, the HDX-MS defined TA12 epitope contains elements identified as  
341 important for the binding of both the SV2C luminal domain (SV2C-LD) and the GT1b  
342 ganglioside (**Figure 1B, regions 4, 5 and 6**). Indeed, the SV2C-LD binding site has been  
343 characterized in the H<sub>CN</sub>-H<sub>CC</sub> interface and the ganglioside binding pocket in the C-terminal  
344 part of H<sub>CC</sub> (Glu1203-Gly1279 with Tyr1117 and Phe1252 playing a critical role) (6, 9, 40).  
345 Finally, TA12 also increases the deuterium uptake in region 3 (peptide 1017-1035;  $\beta$ 32-loop-  
346  $\beta$ 33) in the lectin subdomain (**Figures 1A, 1B**). Upon complex formation, the isotopic profile  
347 of peptide 1017-1035 adopts a bimodal distribution characterized by a low and high  $m/z$  protein  
348 population (**Figure 1C**). Bimodal isotopic patterns result from cooperative exchanges (also  
349 known as EX1 regime) and are very rare in proteins under physiological conditions. Several  
350 experimental factors, such as carry-over or abnormal gas-phase back-exchange are known to  
351 generate false EX1 signatures. To rule out these possibilities, a blank was acquired between  
352 each injection and the pepsin column was carefully washed after each run. In addition, specific  
353 settings were applied on the mass spectrometer (source and ion transfer) to prevent gas-phase  
354 deuterium loss (41). We are therefore confident that the bimodal pattern observed with peptide  
355 1017-1035 is not an experimental artefact but a true change in dynamics imposed by the binding  
356 of TA12. To extract the deuterium uptake level of the low and high  $m/z$  protein populations, the  
357 bimodal isotopic envelop was fitted with two Gaussians. The low  $m/z$  population showed no  
358 difference in deuterium uptake compared to free H<sub>C</sub>A1 (**Figure 1C**). The high  $m/z$  population  
359 however displayed an evident and constant increase in deuterium uptake over time, with the  
360 incorporation of four additional deuteriums. This data confirms that segment 1017-1035 is  
361 destabilized in the complex.

362 Taken together, these data reveal that TA12 targets a conformational epitope located at the  
363 junction between the H<sub>CN</sub> and H<sub>CC</sub> subdomains that overlaps with the the SV2C-LD and the

364 GT1b binding site. The formation of the complex triggers a conformational change in the H<sub>CN</sub>  
365 domain that was not observed with the two previously described neutralizing antibodies ciA-  
366 C2 and CR1 (see discussion) (42-45).

367

### 368 **TA12 prevents BoNT/A1 interaction with SV2C-LD**

369 The HDX-defined TA12 epitope overlaps with the SV2C-LD and the GT1b binding sites  
370 identified by X-ray crystallography (6, 40). SV2C-LD and GT1b both correspond to the  
371 neuronal cell surface receptors used by BoNT/A to efficiently and specifically enter neurons  
372 via a synergetic dual-binding process (8, 46-48). Based on this observation, we hypothesized that  
373 TA12 binding might interfere with the interaction of both SV2C-LD and GT1b.

374 We first focused our attention on the H<sub>CA1</sub>:SV2C-LD interaction. The deuterium uptake  
375 profil of H<sub>CA1</sub> was monitored in the SV2C-LD:H<sub>CA1</sub> complex and compared to the one  
376 obtained in the presence of TA12. The binding of SV2C-LD induced several statistically  
377 significant changes (Wald test,  $p < 0.05$ ) in deuterium uptake in H<sub>CA1</sub> (**Figure 2A; Extended**  
378 **Figures 4B, 5**). As observed in the H<sub>CA1</sub>:TA12 complex, major reductions were observed in  
379 region 2 of the H<sub>CC</sub> domain (peptides 1145-1154 and 1148-1154, region 5 in **Figure 1**) covering  
380 loops  $\beta$ 39- $\beta$ 40 and  $\beta$ 40- $\beta$ 41. Region 2 corresponds to the main SV2C-LD interaction site  
381 identified by X-ray crystallography and contains critical residues for binding such as T1145  
382 and T1146 (**Figures 2B**) (6, 10). Therefore, these data confirm that TA12 and SV2C both target  
383 a common binding region on H<sub>CA1</sub> (Compare **Figures 1A, 1B and 2A, 2B**). Two additional  
384 regions surrounding residues 1145-1154, namely region 1 (peptides 946-955, 946-958 and 946-  
385 961) in the H<sub>CN</sub> domain and region 3 (peptides 1282-1296, 1283-1296, 1284-1296 and 1285-  
386 1296) in the C-terminal part of H<sub>CC</sub>, also display minor but statistically significant reductions  
387 in uptake upon complex formation. Region 3 contains several overlapping peptides with similar  
388 back-exchange values (**Extended file HDX.xls**) allowing to confine the effects of SV2C-LD  
389 binding to segment 1285-1296 *only*. This segment contains residue R1294 involved in the  
390 interaction and the binding specificity with SV2C-LD. Finally, although region 1 is very close  
391 to the main SV2C-LD interaction site (i.e., region 2), this latter was not identified as part of the  
392 SV2C-LD binding interface by X-ray crystallography (**Figure 2B**). Based on this observation,  
393 the slight decrease in solvent accessibility observed in 946-961 might be attributed to a change  
394 in dynamics rather than a masking effect.

395 We next performed *in vitro* experiments to analyze the interference of TA12 on the binding  
396 of recombinant H<sub>CA1</sub> to SV2C-LD immobilized on glutathione-Sepharose 4B beads. As shown  
397 in **Figure 2C**, H<sub>CA1</sub> binding to immobilized SV2C-LD was prevented in a TA12 dose-

398 dependent manner. More than 90% inhibition of binding was obtained at an equimolar ratio  
399 between TA12 and H<sub>C</sub>A1. These results confirm that TA12 prevents the formation of the  
400 toxin/receptor complex via direct interaction with H<sub>C</sub>A1. Thus, as observed with the  
401 neutralizing ciA-C2 VHH, TA12 blocks the entry of BoNT/A1 into neuronal cells by targeting  
402 one critical area of the toxin-cell receptor interface.

403

#### 404 **TA12 affects H<sub>C</sub>A1 binding to GT1b**

405 We next focused our attention on the H<sub>C</sub>A1:GT1b interaction. Our HDX-MS data shows  
406 that TA12 affects the solvent accessibility of elements involved in the GT1b-binding site  
407 (**Figure 3A**). Region 1116-1124 contains residue Y1117 which directly coordinates GT1b  
408 through hydrogen bondings with sialic acid 5 (Sia5); residue F1252 in region 1250-1252 also  
409 hydrogen bonds to galactose 4 (Gal4) of the GT1b ganglioside (40). Compared to SV2C-LD,  
410 GT1b only shares a limited overlapping binding area with TA12 on H<sub>C</sub>A1 (**Figure 3A**).  
411 Therefore, TA12 is not expected to directly compete with GT1b binding on H<sub>C</sub>A1 as observed  
412 with SV2C-LD but rather to affect the H<sub>C</sub>A1:GT1b interaction mainly through steric clashes.

413 To determine the effects of TA12 on H<sub>C</sub>A1 binding to GT1b, an inhibition binding assay  
414 was performed using GT1b coated on plastic plate and incubated with H<sub>C</sub>A1 either alone or in  
415 the presence of TA12. As shown in Figure 3B, increased concentrations of TA12 result in  
416 inhibition of H<sub>C</sub>A1 binding to GT1b. Complete inhibition of H<sub>C</sub>A1 binding to GT1b was  
417 achieved at a much higher TA12/H<sub>C</sub>A1 molar ratio compared to SV2C-LD (10:1 for GT1b; 1:1  
418 for SV2C-LD), hence suggesting that TA12 mainly causes indirect interferences with GT1b.  
419 Altogether, these data confirm that TA12 is also able to interfere with H<sub>C</sub>A1 binding to GT1b  
420 albeit with a lower efficiency compared to the binding inhibition of H<sub>C</sub>A1 to SV2C-LD.

421

#### 422 **Residue N1006 is important for TA12 binding.**

423 Although TA12 recognizes the H<sub>C</sub> domain of BoNT/A subtypes with high affinity (i.e., in  
424 the low pM-nM range; **Table 2**), the efficiency of neutralization appears subtype dependent.  
425 The quantity of TA12 required to neutralize 50% of the activity of both BoNT/A5 and A7  
426 appears 400- and 40-fold higher than for BoNT/A1, respectively (**Table 1**). Interestingly, the  
427 H<sub>C</sub> domain of BoNT/A1 shares respectively 94.1 and 91.7% sequence identity with H<sub>C</sub>A5 and  
428 H<sub>C</sub>A7, suggesting that small variations in the primary sequence are sufficient to explain the  
429 difference of neutralizing potency by TA12. To test this hypothesis, specific residues mainly  
430 positioned within the HDX-MS defined TA12 epitope where major changes in deuterium  
431 uptake occur upon binding were selected for substitution (**Extended Figure 6**). Four distinct

432 residues of H<sub>C</sub>A1 including N1006 (region 2, **Figure 1**), and V1143, M1144 and R1156 (region  
433 5, **Figure 1**) were replaced by the corresponding residues found in H<sub>C</sub>A5 or H<sub>C</sub>A7. The  
434 modified residues correspond to natural H<sub>C</sub> variants and their mutation is therefore not expected  
435 to cause any local secondary structural changes on H<sub>C</sub>A1. The substitution of V1143 alone or  
436 in combination with M1144 and R1156 showed no significant effect on TA12 binding (**Table**  
437 **2**). By contrast, the substitution of N1006 with alanine strongly affects the binding of TA12 to  
438 H<sub>C</sub>A1 with a 280-fold reduction in affinity compared to wild type (**Table 2**) confirming the  
439 importance of N1006 in complex formation.

440

## 441 **DISCUSSION**

442 Neutralizing antibodies against BoNT represent the only available antidote against  
443 botulism considering that vaccination is not an appropriate strategy due to the expanding  
444 therapeutic applications of BoNTs. Although equine anti-BoNT polyclonal sera are able to  
445 efficiently neutralize all seven known BoNT toxinotypes, their use can cause dramatic side  
446 effects (13, 49-53). Alternative therapeutic antitoxin approaches, such as mAbs or peptide  
447 inhibitors (6), are currently explored to reduce the risks of serum sickness and cardiac arrest.  
448 Human anti-BoNT immune globulins obtained from vaccinated donors have been developed  
449 for the treatment of infant botulism, but their production is not easily scalable (14).  
450 Recombinant mAbs and more specially recombinant humanized mAbs represent a promising  
451 and safer alternative strategy for botulism treatment. Their production is highly reproducible  
452 compared to animal hyperimmune sera thereby preventing batch to batch variations in antibody  
453 affinity (50). Over the last decade, several neutralizing mAbs against the distinct BoNT types  
454 have been generated (17, 18, 54). However, mAbs are specific of single epitopes *only* and  
455 extended protective coverage most often requires the combination of several mAbs. For  
456 example, the association of three distinct mAbs targeting non-overlapping epitopes of BoNT/A  
457 was found to be highly protective, whereas each single mAb showed no significant protection.  
458 Moreover, the three mAb in combination induced a 90-fold greater neutralizing potency of  
459 BoNT/A than human hyperimmune globulins (21). Although effective to treat botulism, the  
460 association of several mAbs introduces unique development and manufacturing challenges for  
461 clinical application. Recently, a single tri-epitopic IgG1-based mAb (TeAb) containing the  
462 binding sites of three individual anti-BoNT/A1 mAbs was engineered (22). TeAb was found to  
463 be much more potent in mouse-neutralization assay than any of the three single mAbs and  
464 almost as potent as the combination of the three individual mAbs against BoNT/A1. Though

465 the neutralization potency of TeAb against the other BoNT/A subtypes was not reported, this  
466 work highlights the potential of using single mAbs as alternative antitoxin solution (22).

467

468 In this study, we showed that the single TA12 mAb raised against H<sub>C</sub>A1 is a potent  
469 inhibitor of the two most frequent subtypes involved in human botulism, namely A1 and A2  
470 (55-57), but also A3. TA12 recognizes a conformational epitope mainly composed of solvent  
471 accessible loops located at the interface between the H<sub>CN</sub> and the H<sub>CC</sub> subdomain. The TA12  
472 binding site overlaps with both the SV2C and the GT1b binding interfaces on H<sub>C</sub>A1, causing  
473 interferences with the interaction of the two neuronal cell surface receptors used by BoNT/A to  
474 enter neurons. The strong neutralization potency of TA12 is therefore achieved by the  
475 synergetic and simultaneous inhibition of BoNT/A binding to GT1b and the peptide moiety of  
476 the SV2C luminal domain.

477 Two additional BoNT/A neutralizing antibodies have been reported to block the  
478 recognition of SV2C: the camelid single domain ciA-C2 antibody (42, 43) and the human  
479 monoclonal antibody CR1 currently in clinical trials (44, 45). The identification of the TA12  
480 binding site provides a means to compare the different epitopes on H<sub>C</sub>A1 and the neutralization  
481 mechanism of each antibody. All three antibodies target the same H<sub>CN</sub>/H<sub>CC</sub> junction, which was  
482 shown to elicit neutralizing antibodies against BoNT/A (28), but they adopt distinct orientation  
483 on H<sub>C</sub>A1 (**Figure 4**). CR1 mainly occupies the SV2C glycan binding site on the H<sub>CN</sub>  
484 subdomain. The CR1 epitope locates on the same H<sub>C</sub>A1's face than the glycosylated SV2C  
485 (gSV2C) without sharing any overlapping peptide regions with gSV2C. The large size of CR1  
486 and its close proximity with gSV2C are sufficient to generate local steric clashes between  
487 portions of CR1 and gSV2C, causing indirect interferences with gSV2C binding on H<sub>C</sub>A1 (10,  
488 45). The strong neutralizing potency of CR1 is therefore driven by two simultaneous blocking  
489 effects: an indirect effect with the SV2C peptides moieties and a direct effect through the  
490 occupation of the SV2C glycan binding site. As observed with CR1, ciA-C2 largely occupies  
491 the binding site for the SV2C glycan but it also interacts with a key region in the H<sub>CN</sub> subdomain  
492 containing critical residues for gSV2C binding (**Figure 4**). Thus, ciA-C2 simultaneously  
493 inhibits both the protein- and the glycan- interactions between BoNT/A1 and gSV2C via direct  
494 blocking effects (43). The TA12 epitope shares common structural features with ciA-C2 as they  
495 both occupy the same gSV2C peptide binding site on H<sub>C</sub>A1 and directly block gSV2C binding  
496 to BoNT/A (**Figure 4**). However, the TA12 binding interface lies on the face opposite  
497 recognized by both ciA-C2 and CR1, confirming that the H<sub>CN</sub>/H<sub>CC</sub> junction contains distinct  
498 antigenic regions able to elicit highly potent and unique BoNT/A neutralizing antibodies (28).



499 In contrast to both ciA-C2 and CR1, TA12 does not compete with the SV2C glycan but  
500 interferes with H<sub>C</sub>A1 binding to GT1b.

501 The identification of the TA12 epitope on BoNT/A also provides a means to understand  
502 the variations in potency observed with the different H<sub>C</sub>A subtypes (**Tables 1, 2**). The three  
503 main regions defining the TA12 epitope (i.e., I1005-D1009, Y1117-V1125, and L1136-L1154;  
504 **Extended Figure 6**) are relatively well conserved hence explaining the capabilities of TA12 to  
505 recognize all H<sub>C</sub>A subtypes with high affinity. As previously reported, the antitoxin efficacy of  
506 antibodies largely depends on their binding affinity (42). Thereby, the highest TA12 binding  
507 affinities and neutralization potencies were obtained with subtypes A1, A3 and A2 whereas the  
508 lowest were observed with A5 and A7. TA12 binds A5 and A7 with affinity values comparable  
509 to both ciA-C2 and CR1 (i.e., in the low nanomolar range), and yet only poorly neutralizes  
510 BoNT/A5 in the mouse lethality assay. Low nanomolar  $K_D$  are therefore not sufficient because  
511 TA12 interferes simultaneously with the two BoNT/A cell binding partners. The unique  
512 neutralization mechanism of TA12 requires therefore low picomolar range affinity to prevent  
513 the displacement of the bound antibody from the toxin by either gSV2C, GT1b or both. We  
514 believe therefore that the differences in potency observed between H<sub>C</sub>A subtypes are due to  
515 subtle amino-acid changes within the TA12 binding interface, resulting in lower  $K_D$  values, as  
516 we showed with residue 1006 in H<sub>C</sub>A5. In addition, TA12 interferes on H<sub>C</sub>A1 interaction with  
517 gangliosides. Albeit TA12 prevented H<sub>C</sub>A1 binding to GT1b with lower efficiency compared  
518 to SV2C-LD, the cumulative effect of TA12 on binding to both types of H<sub>C</sub>A1 cell receptors  
519 likely contributes to the highly efficient *in vivo* neutralization observed in mouse protection  
520 assay. To our knowledge, TA12 is the first antibody able to neutralize BoNT/A by  
521 simultaneously interfering with the binding to the two cell surface receptor types on  
522 motoneurons.

523

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527

#### 528 **Author contributions**

529 M.R.P. designed the project. S.B., M.R.P, CRE, AW, SS, MM, designed and performed the  
530 experiments. S.B., M.R.P and CRE analyzed the data. S.B. and M.R.P. wrote the manuscript.  
531 All authors discussed and commented on the manuscript.

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747



748 **FIGURE LEGENDS**

749

750 **Figure 1 | Effects of TA12 binding on the deuterium uptake behavior of H<sub>C</sub>A1.** **A,** The  
 751 fractional uptake difference plot was generated for each peptide and at each time point by  
 752 subtracting the deuterium uptake values in the TA12-unbound from those in the bound state.  
 753 Positive numbers indicate TA12-induced protection (regions 1, 2, 4, 5 and 6) while negative  
 754 values indicate an increase in solvent accessibility (region 3). The six regions displaying  
 755 statistically significant changes of deuterium uptake upon TA12 binding (Wald test,  $p < 0.05$ )  
 756 are highlighted in gray. Each dot corresponds to an average of three independent replicates. **B,**  
 757 Mapping of the HDX-MS results obtained after 60 min labeling onto the cartoon and surface  
 758 representations of H<sub>C</sub>A1 (pdb # 2NZ9). The largest reductions of deuterium uptake mainly  
 759 occur within loops (regions 2, 4 and 5) located at the interface between the N-terminal lectin  
 760 subdomain (H<sub>CN</sub>) and the C-terminal trefoil subdomain (H<sub>CC</sub>) whereas changes in dynamics are  
 761 observed in H<sub>CN</sub> *only*. “No change” refers to regions of H<sub>C</sub>A1 that show no statistically  
 762 significant uptake difference between states during the time scale of the experiment. **C,** Mass  
 763 spectra and deuterium uptake plot of peptide 1017-1035 that displays a bimodal isotopic pattern  
 764 (EX1) in the presence of TA12. The EX1 behavior is characterized by an increase in the peak  
 765 width due to the overlap of two distinct isotopic distributions corresponding to the low (red)  
 766 and high (green)  $m/z$  populations. The center of these two distributions (blue, mix  $m/z$ ) was  
 767 used to generate the fractional uptake difference values for peptide 1017-1035 (panel A).

768

769 **Figure 2 | TA12 blocks BoNT/A1 binding to SV2C-LD by targeting a common area on**  
 770 **H<sub>C</sub>A1.** **A,** Effects of SV2C-LD binding on the deuterium uptake behavior of H<sub>C</sub>A1. The  
 771 Fractional uptake difference plot was generated for each peptide and at each time point by  
 772 subtracting the uptake values measured in the SV2C-LD unbound-state from those in the bound  
 773 state. Positive numbers indicate SV2C-LD induced protection (regions 1, 2, and 3). Regions  
 774 displaying statistically significant changes of deuterium uptake upon SV2C-LD binding (Wald  
 775 test,  $p < 0.05$ ) are highlighted in gray. Each dot corresponds to an average of three independent  
 776 replicates. **B,** Mapping of the HDX-MS results obtained after 60 min labeling onto the surface  
 777 representation of H<sub>C</sub>A1 (pdb # 2NZ9). As observed with TA12, the largest reduction in  
 778 deuterium uptake occurs within region 2 (i.e., 1145-1154 corresponding to region 5 in **Figure**  
 779 **1A, 1B**). The position of the SV2C-LD binding site identified by X-ray crystallography is also  
 780 reported (magenta). The H<sub>C</sub>A1 residues in direct interaction with SV2C-LD are indicated. “No  
 781 change” refers to regions of BoNT/A1 that show no statistically significant uptake difference

782 between states during the time scale of the experiment. **C**, Inhibition of H<sub>C</sub>A1 binding to SV2C-  
783 LD by mAb TA12. H<sub>C</sub>A1 (200 pmol) was preincubated with or without TA12 for 15 min at  
784 room temperature (Input) and then incubated with immobilized SV2C-LD on glutathione-  
785 Sepharose 4B beads for 90 min at 4°C. Bound H<sub>C</sub>A1 was detected by 10% SDS-PAGE analysis  
786 after Coomassie blue staining. Load input volumes on gel were 1:10 of the total input volumes.  
787 TA12 was dissociated in heavy and light chains. A representative figure of 4 is shown.

788

789 **Figure 3 | Effects of TA12 on the binding of the GT1b ganglioside.** **A**, Structure of the  
790 H<sub>C</sub>A1:GT1b complex (pdb # 2VU9) showing the position of the GT1b-binding site relative to  
791 the HDX-MS defined TA12 epitope. GT1b is shown as green sticks. A close-up view of the  
792 GT1b-binding interface is shown on the right side, where the position of residues Y1117 and  
793 F1252 involved in the coordination of GT1b are reported. The GT1b sialic acid 5 (Sia5) and  
794 galactose 4 (Gal4) hydrogen bonded respectively to Y1117 and F1252 are also indicated. The  
795 variations in deuterium uptake imposed by TA12 binding on H<sub>C</sub>A1 are colored as in Figure 1.  
796 **B**, TA12 inhibition of H<sub>C</sub>A1 binding to GT1b. H<sub>C</sub>A1 (10 nM) was preincubated with or without  
797 increasing concentration of TA12 for 15 min at room temperature and then exposed to GT1b  
798 coated on 96-well plate (1 µg/well). Bound H<sub>C</sub>A1 to GT1b was detected with rabbit anti- H<sub>C</sub>A1  
799 antibodies and HRP-goat anti-rabbit IgG. Representative experiment in triplicate is shown.  
800 TA12 128 nM and above completely prevent the binding of H<sub>C</sub>A1 (10 nM) to GT1b.

801

802 **Figure 4 | TA12 binds H<sub>C</sub>A1 on the face opposite occupied by both ciA-C2 and the**  
803 **BoNT/A1-neutralizing therapeutic human monoclonal antibody CR1.** Superposition of the  
804 structures of the H<sub>C</sub>A1:ciA-C2 (pdb # 5L21), H<sub>C</sub>A1:CR1 (pdb # 2NYY), H<sub>C</sub>A1:GT1b (pdb #  
805 2VU9) and H<sub>C</sub>A1:gSV2C (pdb # 5JLV) complexes. GT1b and the N559-glycan of gSV2C are  
806 shown as green and cyan sticks respectively. CiA-C2 (dark red) partially occupies the binding  
807 site on H<sub>C</sub>A1 of both the N-glycan (N559) and the peptide moieties of gSV2C, thereby  
808 interfering directly with gSV2C (cyan) binding to H<sub>C</sub>A1. The region of H<sub>C</sub>A1 containing  
809 critical residues for gSV2C binding (i.e., T1145 and T1146) and targeted by ciA-C2 is indicated  
810 by a black box. The BoNT/A1-neutralizing CR1 antibody (ribbon representation, gold) directly  
811 targets the gSV2C N599-glycan binding site on H<sub>C</sub>A1 without competing with the gSV2C  
812 peptide binding site (black box). The variable region in the CR1 light chain overlaps almost  
813 completely with the ciA-C2 binding areas containing the gSV2C N599-glycan interaction site.  
814 The HDX-defined TA12 epitope (see color code in Figure 1) occupies the gSV2C peptide  
815 binding site targeted by ciA-C2 (black box), causing direct interferences with the peptide

816 moieties of gSV2C. In contrast to both ciA-C2 and CR1, TA12 does not affect the gSV2C  
817 glycan binding region but recognize a region of the GT1b-binding site. Thus, TA12 partially  
818 impaired H<sub>c</sub>A1 binding to GT1b (Fig. 3B). The position of the TA12 epitope relative to the ciA-  
819 C2, CR1 and gSV2C binding sites is better visualized using the H<sub>c</sub>A1 surface representation  
820 shown on the right side. TA12 shares a common binding interface with ciA-C2 but mainly  
821 occupies the face opposite on H<sub>c</sub>A1 to both ciA-C2 and CR1 (black circle). The position of the  
822 N1006 residue identified as important for TA12 binding is indicated.

823

824

825

826 **Table 1.** Neutralization activity of TA12 mAb with BoNT/A subtypes827  
828

<b>TA12 mAb quantity (ng/mouse)</b>	<b>0</b>	<b>10,000</b>	<b>2,500</b>	<b>1,000</b>	<b>250</b>	<b>25</b>	<b>2.5</b>	<b>mAb quantity (ng/mouse) yielding 50% neutralization</b>
BoNT/A1	6/6	0/2	/	0/4	1/7	4/10	6/6	2.5<25
BoNT/A2*	9/9	/	0/9	/	6/9	9/9	/	250
BoNT/A3*	9/9	/	0/9	/	0/9	6/9	/	25<250
BoNT/A5	6/6	5/8	4/4	8/8	5/5	2/2	/	1,000<10,000
BoNT/A7	6/6	1/6	5/6	5/8	8/8	2/2	/	250<1,000

829

830

831 mAb neutralization activity was determined using the mouse protection assay with estimated

832 5 MLD<sub>50</sub>/mL of BoNT/A subtype and serial dilutions of mAb. 5 estimated MLD<sub>50</sub>/mouse

833 were incubated with 2.5 to 10,000 ng of TA12 mAb for 30 min at room temperature and the

834 mixture (0.5 mL) was injected intraperitoneally into each mouse.

835 Results are expressed as the number of dead mice versus the total number of mice.

836 \*Data from (23).

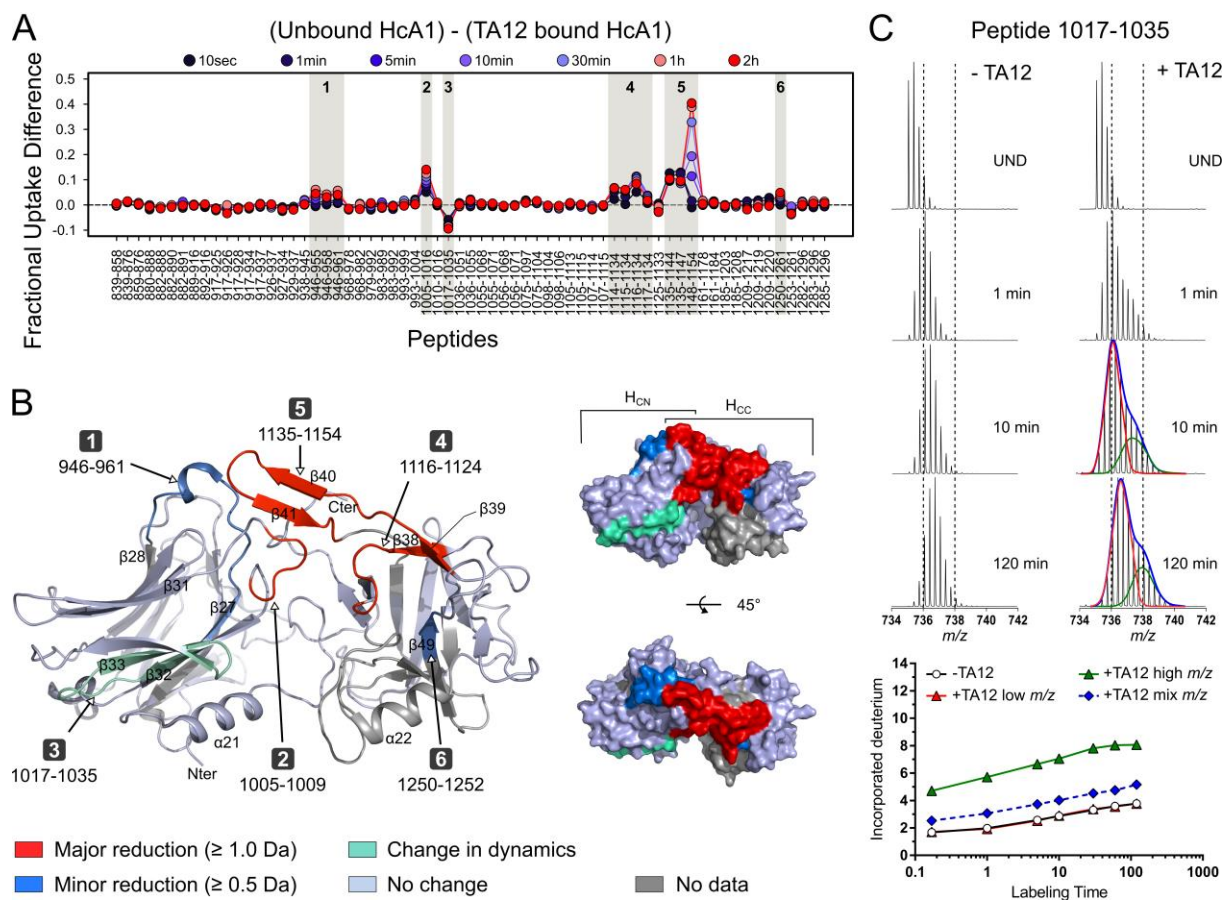
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838  
839**Table 2.** Binding affinities of mAb TA12 with BoNT/A subtypes H<sub>C</sub>

BoNT/A H <sub>C</sub>	Amino acid identity <sup>a</sup> (%)	k <sub>on</sub> (1/Ms)	k <sub>off</sub> (1/s)	K <sub>D</sub> (pM)
H <sub>C</sub> A1	-	3.01 10 <sup>5</sup>	7.1 10 <sup>-6</sup>	23.6
H <sub>C</sub> A2	87.0	6.96 10 <sup>5</sup>	7.74 10 <sup>-5</sup>	111
H <sub>C</sub> A3	86.58	1.13 10 <sup>6</sup>	6.58 10 <sup>-5</sup>	58.1
H <sub>C</sub> A4	91.76	2.13 10 <sup>5</sup>	5.91 10 <sup>-4</sup>	2780
H <sub>C</sub> A5	94.11	7.95 10 <sup>5</sup>	7.55 10 <sup>-4</sup>	949
H <sub>C</sub> A7	91.76	2.83 10 <sup>5</sup>	5.94 10 <sup>-4</sup>	2100
H <sub>C</sub> A1 R1156M		4.79 10 <sup>5</sup>	1.47 10 <sup>-5</sup>	30.7
H <sub>C</sub> A1 V1143I/M1144V		6.42 10 <sup>5</sup>	5.63 10 <sup>-6</sup>	8.77
H <sub>C</sub> A1 V1143I/M1144V/R1156M		4.09 10 <sup>5</sup>	4.61 10 <sup>-5</sup>	113
H <sub>C</sub> A1 N1006A		1.88 10 <sup>5</sup>	1.27 10 <sup>-3</sup>	6755

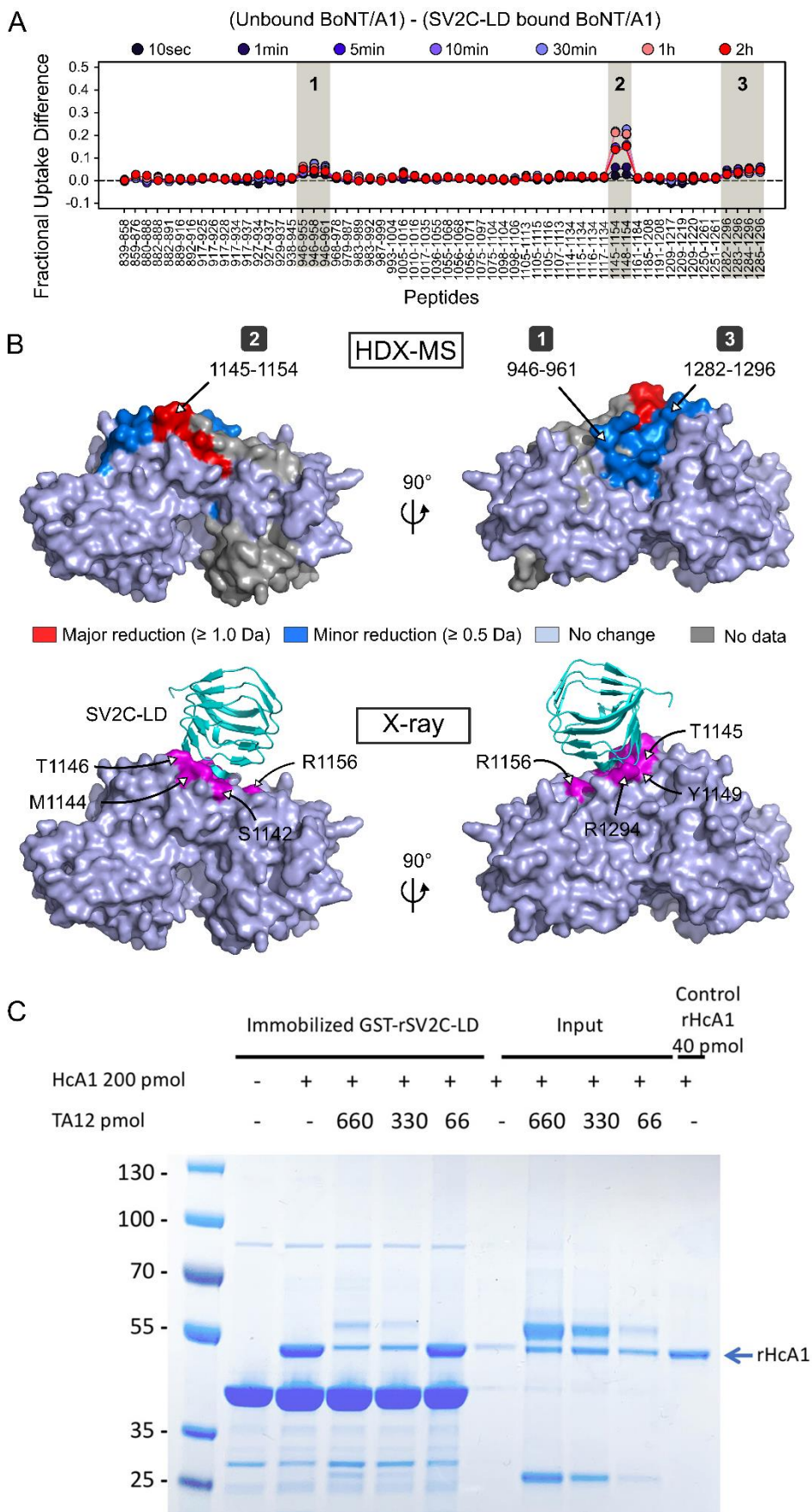
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844<sup>a</sup> Amino acid identity between H<sub>C</sub>A1 and the other H<sub>C</sub>A subtypes

845 **Figure 1**  
846



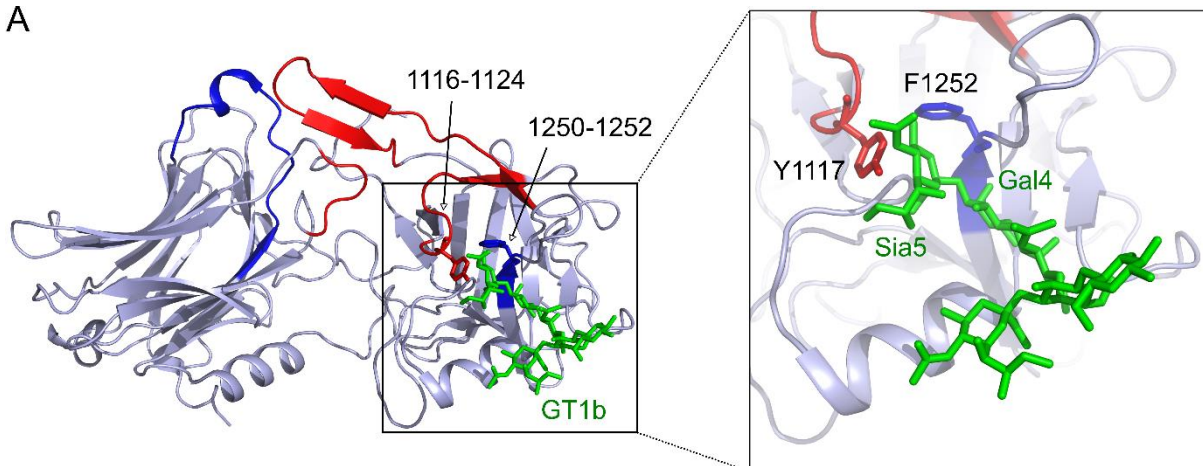
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849 **Figure 2**  
850

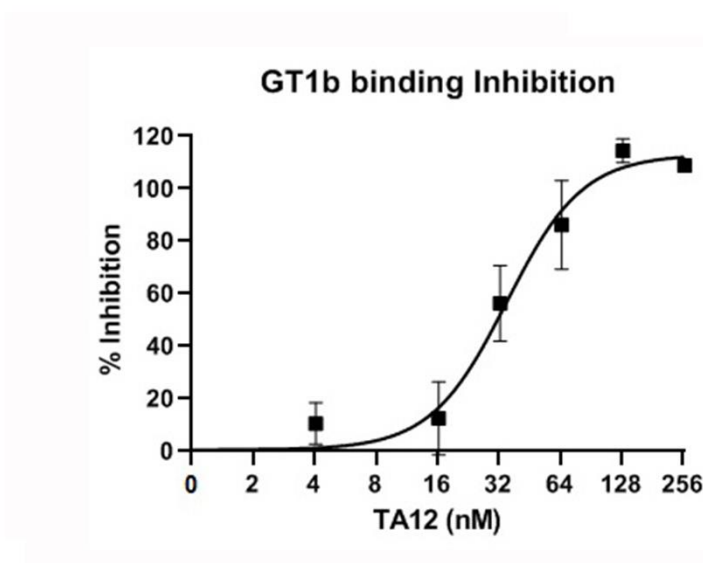


852 **Figure 3**  
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A



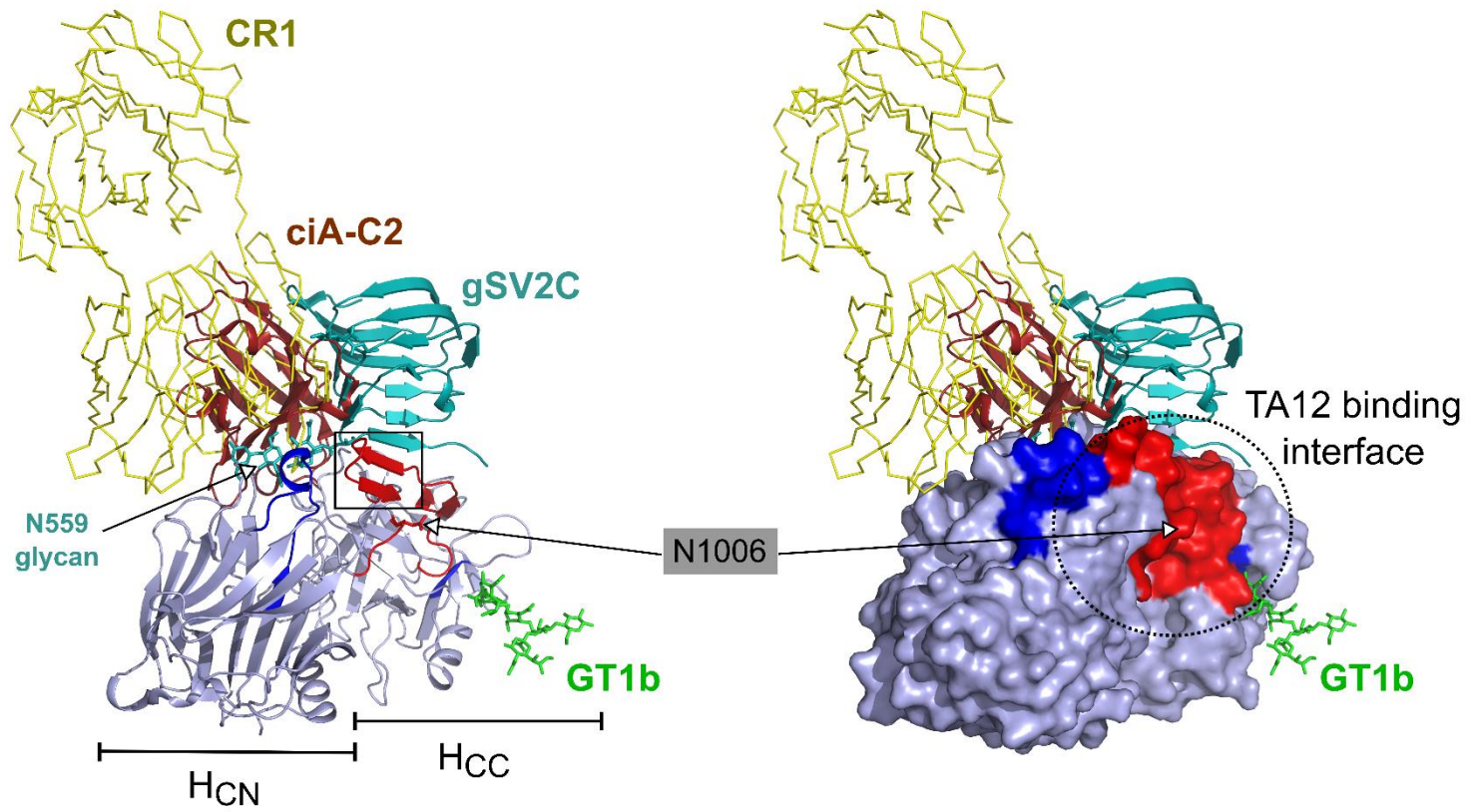
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855 **Figure 4**  
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857