**Engineered botulinum neurotoxin B with improved binding to human receptors has enhanced efficacy in preclinical models**

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Although botulinum neurotoxin serotype A (BoNT/A) products are common treatments for various disorders, there is only one commercial BoNT/B product, whose low potency, likely stemming from low affinity toward its human receptor synaptotagmin 2 (hSyt2), has limited its therapeutic usefulness. We express and characterize two full-length recombinant BoNT/B1 proteins containing designed mutations E1191M/S1199Y (rBoNT/B1MY) and E1191Q/S1199W (rBoNT/B1QW) that enhance binding to hSyt2. In preclinical models including human-induced pluripotent stem cell neurons and a humanized transgenic mouse, this increased hSyt2 affinity results in high potency, comparable to that of BoNT/A. Last, we solve the cocrystal structure of rBoNT/B1MY in complex with peptides of hSyt2 and its homolog hSyt1. We demonstrate that neuronal surface receptor binding limits the clinical efficacy of unmodified BoNT/B and that modified BoNT/B proteins have promising clinical potential.

**INTRODUCTION**

Botulinum neurotoxins are a family of potent bacterial toxins, including seven major serotypes (BoNT/A to BoNT/G) and many subtypes (designated with numerical number, e.g., BoNT/A1). Because of their highly specific action on blocking neurotransmission, BoNTs are used for the treatment of muscle hypertonicity in various medical indications (1, 2). Treatment consists of injecting the BoNT-containing product directly into affected areas and is typically well tolerated (2, 3). However, generation of neutralizing antibodies that renders future treatments ineffective can occur (4). Adverse effects that are sometimes associated with BoNT treatment seem linked to unwanted spread and diffusion of the toxin away from the site of injection (5, 6). In the clinical setting, multiple factors influence this unwanted spread and diffusion, with the administered dose being a recognized factor affecting toxin spread (7).

Most available BoNT therapeutics are derived from BoNT/A1, with just one commercial BoNT/B1 product (2). In clinical practice, the latter displays a lower potency than the available BoNT/A1 products, commonly necessitating the administration of higher doses of the BoNT/B1 product (8) and leading to a higher frequency of clinically undesirable effects (2, 9). A BoNT/B1 protein engineered to require doses as low as the available BoNT/A1 products thus has the potential to match the efficacy and safety profile of the BoNT/A1 products.

The prospect of developing an engineered BoNT/B1 protein with these properties has come into view after recent insights into the way that BoNT/B1 interacts with the membrane proteins of the targeted neuron. Unlike BoNT/A, which uses synaptic vesicle protein 2 isoforms A to C, BoNT/B recognizes synaptotagmins 1 and 2 (Syt1 and Syt2, respectively) (10, 11). Of these two isoforms, Syt2 appears to be the major isoform at the mature neuromuscular junction (NMJ) (12). BoNT/B1 displays a higher affinity to the rodent homolog of Syt2 as compared to the human homolog (13, 14). The molecular basis of this interaction has been determined by x-ray crystallography (15). Using a bacterial adenylate cyclase two-hybrid saturation mutagenesis screen, we previously identified a series of point mutations that can be introduced into the receptor-binding domain of BoNT/B (Hc/B) to enhance its binding to human receptor Syt2 (hSyt2) (16). Furthermore, introducing an E1191M/S1199Y double mutation to the full-length molecule [recombinant BoNT (rBoNT) (B1MY)] was shown to have increased functional efficacy in cultured rodent neurons that expressed hSyt2 via lentiviral transduction (16). Notably, the toxin used in this earlier study was a histidine-tagged version.

Here, we produce the full-length rBoNT/B1MY without this histidine tag, as well as a second untagged modified toxin containing a different set of mutations (E1191Q/S1199W; rBoNT/B1QW). We characterize the activity of both toxins in several preclinical models including human neurons derived from induced pluripotent stem cells (iPSCs) and a humanized transgenic mouse model that expresses the hSyt2 toxin binding region. Our studies demonstrate that the increased affinity to the human receptor results in increased activity of both modified toxins in these models with a potency similar to that of rBoNT/A1. Our data demonstrate that neuronal surface receptor binding is likely to be the major factor limiting the clinical efficacy of BoNT/B1. Furthermore, we also solve the crystal structures of rBoNT/B1MY in complex with both human Syt isoforms, as well as a molecular basis for their interactions.
In addition, we report the cocrystal structure of BoNT/B1 in complex with Syt1. Our data suggest that rBoNT/B1\textsubscript{MY} and rBoNT/B1\textsubscript{QW} are interesting candidates to be developed into new clinical treatment options for patients suffering from a spectrum of disorders.

### RESULTS

**rBoNT/B1\textsubscript{MY} and rBoNT/B1\textsubscript{QW} have higher activity than rBoNT/B1 in cell-based assays**

To characterize the biological activity of the enzymatically active toxins, we first tested the efficacy of blocking \(^{[1]}\text{H}\)-glycine release from primary rat spinal cord neurons (SCNs). The toxin concentration required for 50% maximal inhibition (IC\(_{50}\)) of \(^{[1]}\text{H}\)-glycine release was 1.1 pM for rBoNT/B1\textsubscript{MY} and rBoNT/B1\textsubscript{QW} \((n = 3\) for both toxins), demonstrating functionality of both modified toxins (Fig. 1A). For comparison, the IC\(_{50}\) for rBoNT/B1 in this assay was 3.5 pM \((n = 4)\).

We next characterized the activity of rBoNT/B1\textsubscript{MY} and rBoNT/B1\textsubscript{QW} in various human-derived or humanized models, henceforth including rBoNT/A1 and rBoNT/B1 for comparison. We first tested the toxins on neurons derived from human iPSCs (hiPSCs), iCell GABANeurons, which have been shown to be highly sensitive to BoNTs (17). As hypothesized from the increased affinity of both mutants to hSyt1 and hSyt2 (table S1), the IC\(_{50}\) of \(^{[1]}\text{H}\)-\(\gamma\)-aminobutyric acid (GABA) release was significantly lower for the two rBoNT/B1 mutants as compared to unmodified rBoNT/B1 \([0.3\) pM for rBoNT/B1\textsubscript{MY} and rBoNT/B1\textsubscript{QW} \((n = 3\) for each toxin) versus 10.7 pM for rBoNT/B1 \((n = 4);\) Fig. 1B]. Likewise, the IC\(_{50}\) for the two rBoNT/B1 mutants was also significantly lower as compared to rBoNT/A1, which had an IC\(_{50}\) of 2.3 pM \((n = 5)\). Analysis of the expression levels of Syt1 and Syt2 confirmed the presence of both isoforms at the time when GABA release assays were performed (16 to 18 days in vitro; fig. S1A). The presence of other proteins involved in soluble NSF (N-ethylmaleimide–sensitive factor) attachment protein receptor (SNARE)–mediated release in these cells was also confirmed (fig. S1B). Note that the values reported in table S1 reflect on the binding of the BoNT/B mutants to the Syt isoforms only, without the ganglioside co-receptor. In the functional studies, the BoNT/B toxins will have a much higher affinity to the neuronal surfaces since they can interact with the Syt isoforms and the ganglioside co-receptor.

**A transgenic mouse model with a humanized luminal domain of Syt2**

To further assess the activity of modified rBoNT/B1\textsubscript{MY} and rBoNT/B1\textsubscript{QW} in models that express the hSyt2 toxin binding site (Fig. 2A), we generated transgenic mice in which the luminal domain of the murine Syt2 was replaced by the human sequence (hereafter referred to as “hSyt2 mice”). These transgenic mice were viable, but their fertility rate was reduced. Husbandry data were consistent with the idea that this was due to partial male infertility (Fig. 2B). Phenotypic characterization of the mice did not indicate other abnormalities; for example, hSyt2 mice displayed normal body weights (BWs) (Fig. 2C) with no signs of ataxia when compared to wild-type (WT) mice. Likewise, histopathological analysis of several tissues did not detect any differences between the hSyt2 mice and their WT littermates. Distribution of Syt1, Syt2, and other proteins involved with central nervous system synaptic function was not different between hSyt2 and WT mice (Fig. 2, D and E).

**Syt2 has a major role in mediating toxin efficacy in the hemidiaphragm muscle but not in the detrusor smooth muscle**

The activity of the toxins (10 pM) was assessed in phrenic nerve hemidiaphragm striated muscle prepared from hSyt2 mice and compared to those from WT littermates. In hSyt2 tissue, the time taken to achieve half-maximal paralysis \((t_{50})\) for electrically evoked hemidiaphragm muscle contractions was 53.7 ± 3.3 min \((n = 5)\) and 121.2 ± 4.8 min \((n = 6)\) for rBoNT/A1 and rBoNT/B1, respectively. This compared to 53.9 ± 3.5 min \((n = 5)\) and 60.3 ± 6.3 min \((n = 4)\) for rBoNT/B1\textsubscript{MY} and rBoNT/B1\textsubscript{QW}, respectively.
in WT tissue, respectively. The difference between hSyt2 and WT mice values was significant for rBoNT/B1 (P < 0.0001, unpaired t test; Fig. 3, A and D), suggesting that the activity of BoNT/B1 in the hemidiaphragm model is, to a large degree, mediated through interaction with Syt2. The t50 in hSyt2 tissue for rBoNT/B1MY (Fig. 3B) and rBoNT/B1QW (Fig. 3C) was 62.5 ± 4.7 min (n = 7) and 71.8 ± 3.0 min (n = 6), respectively. These values were not significantly different to those obtained in tissue from WT littermates with these two toxins (t50 = 54.3 ± 3.9 min (n = 5) and 69.2 ± 3.5 min (n = 5), respectively; Fig. 3D), consistent with the idea that the mutations, while improving affinity to hSyt2, only have minor effects on the affinity to murine Syt2, as compared to rBoNT/B1 (table S1). The potency of the mutated toxins was significantly higher in hSyt2 mice as compared to rBoNT/B1 (P < 0.0001, one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison), but there was no significant difference between mutated and WT rBoNT/B1 toxins in WT mice (Fig. 3D).

We next tested the toxins in a mouse detrusor smooth muscle preparation. As reported earlier (18), low nanomolar concentrations of toxins are needed to induce paralysis in this tissue. In our studies, contraction strength was gradually reduced in both the WT and the hSyt2 mice after bath application of 1 nM toxins (Fig. 4, A and B). In contrast to the hemidiaphragm striated muscle, rBoNT/B1 had similar activity in detrusor smooth muscle tissue from WT and hSyt2 mice with 55.2 ± 3.8 min (n = 5) and 57.3 ± 3.6 min (n = 5), respectively. Likewise, both mutants had similar high activity in WT and hSyt2 preparations. The t50 values were 38.7 ± 4.4 min (n = 3) and 39.4 ± 3.8 min (n = 3) in WT tissue and 40.2 ± 3.2 min (n = 4) and 37.1 ± 0.9 min (n = 3) in hSyt2 tissue for rBoNT/B1MY and rBoNT/B1QW, respectively (Fig. 4C). Although the values for the...
_mutants were, in WT and hSyt2 tissue, lower than those for the unmodified rBoNT/B1, this was not statistically significant. These data suggest that Syt1, rather than Syt2, plays a major role in mediating toxin efficacy in bladder smooth muscle. This was corroborated by immunohistochemical (IHC) analysis, which consistently detected immunoreactivity for Syt1 in the bladder tissue from WT and hSyt2 mice but failed to detect staining for Syt2 in either preparation, despite positive signals in spinal cord control preparations (Fig. 4D).

As expected from previous work with natural toxins (19), rBoNT/A1 had significantly lower rates of blockade than the rBoNT/B1 toxins in the smooth muscle bladder preparation (hSyt2 mice, \( t_{50} = 106.6 \pm 7.3 \) min \((n = 4); \) WT mice, \( t_{50} = 112.4 \pm 8.2 \) min \((n = 5); \) Fig. 4C).

The in vivo activity of rBoNT/B1_{MY} and rBoNT/B1_{QW} matches that of rBoNT/A1

We next used the digit abduction scoring (DAS) assay in mice (20) to test the activity of the toxins in vivo. In WT mice, rBoNT/A1 and rBoNT/B1 had similar high activity with half-maximal effective dose (ED_{50}) values close to 1 pg per animal (table S2). As expected, rBoNT/A1 potency was unchanged in the hSyt2 mice, but rBoNT/B1 had reduced DAS scores, and the ED_{50} value was approximately fourfold higher than that in WT animals (Fig. 5A and table S2).

In contrast, rBoNT/B1_{MY} and rBoNT/B1_{QW} induced muscle weakness in the hSyt2 mice with ED_{50} values close to 1 pg per animal. ED_{50} values with the modified toxins in the WT mice were close to 1.5 pg per animal (Fig. 5A and Table S2).

Transient BoNT dose-dependent BW loss is considered evidence of a generalized toxin effect in mice (21). Thus, BW was assessed in parallel to the daily DAS measurement. The 0% BW dose was higher than the respective ED_{50} values for all toxins in WT and hSyt2 mice (table S2). The tolerability index of the toxins, defined as the ratio between the calculated dose inducing ∆ 0% BW and DAS ED_{50}, was similar for all toxins in both WT and transgenic mice (Table S2).

Besides humans, five other species were found to present a leucine at position 54 (murine sequence numbering) in Syt2, including gorilla, chimpanzee, mole rat, kangaroo rat, and lesser hedgehog (fig. S2A). Using a Syt2 peptide representing the sequence of the kangaroo rat, we confirmed that the presence of the leucine residue at this position reduced the interaction with BoNT/B (fig. S2, B and C).

Considering that results in the WT mice for rBoNT/B1_{MY} and rBoNT/B1_{QW} did not overestimate the activity of the toxins in the hSyt2 mice (table S2), we next tested one of the two modified toxins, rBoNT/B1_{MY}, in a second common laboratory species. Because rat VAMP1 (vesicle associated membrane protein 1), the predominant isoform in motor neurons, has a mutation that renders skeletal
muscles in rats insensitive to BoNT/B (22, 23), we tested rBoNT/B1 MY in a muscle force study in rabbits in which we evaluated potency, as well as the duration of action of the toxin. We compared the results to those obtained with rBoNT/A1 in the same model and at the same doses (Fig. 5B). Injection of rBoNT/B1 MY induced a long-lasting, dose-dependent decrease in the muscle force of the treated hind limb. This decrease appeared similar in duration and strength to the decrease of muscle force induced by injections of rBoNT/A1 at
all tested doses. Effects on the muscle force of the contralateral, non-injected, hind limb were seen for both toxins at 100 pg/kg but not at lower doses. This likely indicates systemic spread of the toxins at the 100 pg/kg dose, which therefore was the highest dose tested.

The key electrostatic interactions between BoNT/B1 and Syt are conserved in rBoNT/B1MY

To obtain a mechanistic insight into the interaction of rBoNT/B1MY with hSyt2, we crystallized this toxin in complex with hSyt2 peptide (Fig. 6 and table S3). We also determined the x-ray crystal structure of Hc/B in complex with hSyt1 peptide to allow for a direct comparison. In all cases, the bound Syt peptides take on an α-helical structure and occupy the crevice at the tip of the binding domain, similar to what was previously reported for the binding of rat Syt2 to Hc/B (Fig. 6B) (15, 24). The key electrostatic interactions between the toxin and all Syt peptide species are conserved between rBoNT/B1 and rBoNT/B1MY; in particular, Syt E50 (hSyt1)/E54 (hSyt2) forms salt bridges with K1192, and F1994 and offers an important anchoring point for the binding of the N-terminal part of hSyt1 (Fig. 6C).

The second pocket is formed by residues V1118, Y1183, E1191, F1194, and F1994 and offers an important anchoring point for the binding of the N-terminal part of hSyt1 to rBoNT/B1MY (Fig. 6C). The E1191M mutation in rBoNT/B1MY provides greater hydrophobicity to this site (fig. S4). Replacement of this residue by methionine caused a significant repositioning of the charged residue side chain of E1191 from an outward-facing direction (in Hc/B) to a bulky side group (M1191) buried inside the pocket (Fig. 6C). The new interface in rBoNT/B1MY thus allows for stronger interaction with the hydrophobic side chains of L51 (hSyt1)/I55 (hSyt2). This seems particularly important for hSyt2 recognition by rBoNT/B1MY where I55 is attracted deeper within the binding pocket, likely causing a slight transitional shift (1 Å) of the whole peptide compared to hSyt1 (Fig. 6D).

**DISCUSSION**

The present study investigates the effects of two modified rBoNT/B1 toxins, rBoNT/B1MY and rBoNT/B1QW, both with higher affinity to hSyt1 and hSyt2 as compared to rBoNT/B1, in preclinical models.
Fig. 6. Syt recognition by BoNT/B1 variants. (A) Crystal structure of rBoNT/BMY (blue) in complex with hSyt1 (yellow). The three functional domains of BoNT/B1 are labeled, with the catalytic light chain (LC), the translocation domain (HN), and the binding domain (HC). (B) Superposition of the crystal structures of Hc/B (gray) with hSyt1 (yellow) and rSyt2 [red; Protein Data Bank (PDB) code: 2nm1]. Structures were aligned using the binding domain only. Residues E1191 and S1199 are highlighted in blue; the N and C termini of Syt are indicated. (C) Superposition of the crystal structures of the Hc/B (gray)–hSyt1 (green) complex with rBoNT/BMY (blue)–hSyt1 (yellow). Movement of loop 1197-1201 and the difference in the position of the peptides’ N termini are indicated with blue and black arrows, respectively. (D) Superposition of the crystal structures of rBoNT/BMY (blue) with hSyt1 (yellow) and hSyt2 (red). The shift between the two peptide positions is indicated with a black arrow.
Our main finding is that this improved affinity to the human receptors translates into increased activity in all models that express human Syt isoforms, including transgenic mice that express the hSyt2 toxin binding region. This finding shows that the neuronal binding step does limit the potency of BoNT/B in humans. This is also consistent with our recent finding that increasing the catalytic activity of rBoNT/B1 does not increase the activity in complex physiological systems and thus is not limiting the potency of BoNT/B (25).

We found a rather modest fourfold difference in the mouse DAS ED₅₀ between rBoNT/A1 and rBoNT/B1 in the hSyt2 mice, as well as for rBoNT/B1 between the WT and hSyt2 mice. This contrasts with the roughly 40-fold conversion factor that is being used in clinical practice between BoNT/A1 and BoNT/B1 products for skeletal muscle indications (3). Although this may suggest that the low affinity of BoNT/B1 to hSyt2 is not the only reason for the low clinical efficacy of BoNT/B1, the hemidiaphragm data from the hSyt2 mice, the human-derived cell-based data, and the strong increase in Syt2 affinity do agree with the idea that the large difference between BoNT/A1 and BoNT/B1 in the clinic would mostly be due to the low affinity of BoNT/B1 to hSyt2. In this context, it is important to remember that the mouse DAS assay is performed on healthy mice. This model may be inadequate to predict clinical efficacy in spasticity at the quantitative precision required. There is clear evidence that spastic muscles show not only histopathological changes (26) but also changes in their transcriptional profile (27).

Another point of observation is that, in our previous study (16), we found that rBoNT/B₁MY had ~11-fold higher activity than rBoNT/B1 using patch-clamp recording of miniature inhibitory postsynaptic currents in rat cortical neurons that express hSyt2 via lentivirus transduction. In the present study, we found an ~40-fold difference in activity between rBoNT/B₁MY and rBoNT/B1 in hiPSCs using GABA release as a readout. This larger difference in the present study may simply be due to the different technologies used to measure activity. However, there are also other differences between the two studies. For example, the humanized model used in the earlier study expressed only hSyt2 but not Syt1 (16). In contrast, hSyt1 and hSyt2 are both present in iCell GABANeurons. Another difference between the studies is the presence of a C-terminal His-tag on the toxin used in our earlier study. While these tags are commonly used tools in the purification of recombinant proteins, they may alter the biological activity of purified proteins (28). We found that C-terminally histidine-tagged rBoNT/B1 and rBoNT/B₁MY showed reduced potency than the untagged version in mouse hemidiaphragm preparations and in vivo in mice (fig. S5).

Our ex vivo data suggest that Syt2 is the dominant isoform mediating the paralyzing effects of BoNT/B in the hemidiaphragm. This is supported by studies showing expression of Syt2 at NMJs of striated muscle (12), including the hemidiaphragm (29). The presence of Syt1, also described in the latter tissue, is developmentally regulated with a strong down-regulation within the first 2 weeks after birth (29). Our studies, however, were performed on mice aged 5 to 6 weeks. In contrast, Syt1 seems to mediate the effect of BoNT/B in the urinary bladder tissue, and there is evidence that Syt1 is the predominant isoform in autonomic and sensory neurons (30). Our functional data are thus consistent with these earlier expression studies.

With regard to the ex vivo data, it is also interesting to note that the increase in affinity of the mutated toxins to hSyt2 as compared to the unmodified BoNT/B was not reflected on a statistical level by the functional data in the bladder strips preparation. This may be because the nerve endings in the bladder urothelium are embedded within the muscle structure, possibly leading to a dilution effect. This is likely to also account, at least in parts, for the higher toxin concentrations needed in this preparation as compared to the hemidiaphragm, where the NMJ of the phrenic nerve is easily accessible at the muscle surface.

By solving the crystal structures of Hc/B and rBoNT/B₁MY bound to fragments of the human Syt receptors, we find that they bind to two hydrophobic pockets, which is consistent with previous structural studies with the homologous rat Syt2 receptor (15, 24). Notably, the E1191M/S1997Y mutations do not perturb the network of electrostatic interactions seen between the native toxin and its receptor. Each mutation flanks the side of the binding crevice, providing additional hydrophobicity in key positions. For E1191M, the methionine improves hSyt2 binding by better accommodating L51 and I55 within the adjacent pocket and avoiding any conflict with the charged side chain of the glutamate. This in turn does not perturb the binding to hSyt1 that also presents hydrophobic residues in these positions (F47 and L51). On the other side of the crevice, the S1199Y mutation offers a bulky side group that widens the binding pocket and provides improved hydrophobic binding to F40(hSyt1)/F44(hSyt2) of Syt. This position is highly conserved across Syt species; thus, it is unlikely that S1199Y plays a significant role in the observed variation in Syt specificity. Overall our crystal structures validate previous results in which rBoNT/B₁MY showed improved affinity for hSyt2 and hSyt1 as compared to rBoNT/B1 (16). These detailed atomic structures of rBoNT/B₁MY also offer a template to explore further mutations that could provide the toxin with enhanced receptor binding properties by, for example, targeting the plasticity of the loops surrounding the main site.

One unexpected consequence of generating hSyt2 mice was the observation of a partial infertility phenotype. While a previous study with a transgenic Syt2 mice also reported a decreased fertility (31), there was evidence that the specific mutation tested in this earlier study induced protein instability. Consequently, these transgenic mice not only showed fertility deficits but also had other gross phenotypic changes, such as small BW and ataxia, as well as reduced Syt2 protein expression (31). In contrast, besides a fertility phenotype, we did not observe other phenotypic changes with hSyt2 mice in the present study. Notably, the husbandry data can be explained by a simple dominant partial male infertility.

Our finding that rBoNT/B₁MY and rBoNT/B₁QW display high activity in all models that express human Syt isoforms suggests that these toxins could also have superior activity in the clinic, as compared to the currently available BoNT/B1 product. Our data allude to the possibility that the modified toxins may be as active as the BoNT/A1 products. Furthermore, the tolerability of these modified toxins in the preclinical studies was comparable to rBoNT/A1. Thus, modified rBoNT/B1 toxins with improved affinity to hSyt1 and hSyt2 may be viable alternatives to BoNT/A1 treatments, especially for patients who have developed neutralizing antibodies against BoNT/A1.

**MATERIAL AND METHODS**

**Experimental design**

The main objective of this study was to characterize the activity of two modified recombinant BoNT/B in several preclinical models,
including hiPSCs and a transgenic mouse with humanized Syt2 (hSyt2 mice), the major receptor for BoNT/B in skeletal muscle. The activity of the modified toxins was compared to unmodified BoNT/B1 and BoNT/A1. Last, we crystallized one of the recombinant modified BoNT/Bs to obtain mechanistic insight into how the modified BoNT/B interacts with its human receptor.

Cloning and expression of recombinant neurotoxins in *Escherichia coli* and their purification

The production of rBoNT/A1 (32) and rBoNT/B1 (25) has been described previously. In summary, constructs were cloned in the pET32a expression vector and mutations introduced by site-directed mutagenesis. Recombinant molecules were expressed in *E. coli* in selective media using 1 mM isopropyl-β-D-thiogalactopyranoside at 16°C for 20 hours. Cells were harvested and lysed, and the lysate was clarified before purification of target protein by fast protein liquid chromatography with BuHP Sepharose (GE Healthcare Life Sciences) and Q Sepharose High Performance (GE Healthcare Life Sciences). The partially purified molecule was then cleaved with Lys-C to yield the active di-chain, which was polished down a phenyl Sepharose High Performance (GE Healthcare Life Sciences) column. All recombinant toxins were purified and activated to more than 85%, as determined by densitometry, and molecule identity was confirmed by SDS–polyacrylamide gel electrophoresis (PAGE) and Western blot analysis (fig. S6, A and B).

The production of Hc/B was performed, as described previously (15). Briefly, the codon-optimized gene was cloned into the pET28a expression vector and expressed as above. Hc/B was purified by immobilized metal affinity chromatography with HisTrap HP (GE Healthcare Life Sciences), followed by gel filtration with Superdex 200 (GE Healthcare Life Sciences). The sample was kept in 20 mM Hepes (pH 7.5), 300 mM NaCl, 10% glycerol, and 0.5 mM TCEP [tris(2-carboxyethyl)phosphine].

Measurement of light-chain activity

The light-chain activities were assessed using the BoTest cell-free assay (BioSentinel, WI, USA), as previously described (25).

Glutathione S-transferase pull-down and biolayer interferometry assays

Glutathione S-transferase (GST) pull-down and biolayer interferometry assays were performed, as previously described (16).

X-ray crystallography

Samples for crystallization were prepared by preincubation of Hc/B (15 mg/ml) or rBoNT/B1 MY (10 mg/ml) with 2 mM hSyt1 or hSyt2 peptides (GenScript, USA). Crystals were obtained from a sitting-drop vapor diffusion setup against Morpheus screen H5 (Molecular Dimensions, UK) for the Hc/B:hSyt1 complex; against 1.1 M sodium malonate dibasic monohydrate, Hepes (pH 7.0), and 0.5% (v/v) Jefameine ED-2003 (JCSG screen, F10, Molecular Dimensions, UK) for the rBoNT/B1 MY:hSyt1 complex; and against 0.2 M magnesium chloride, tris (pH 7.0), and 10% (w/v) PEG 8000 (JCSG screen, B8, Molecular Dimensions, UK) for the rBoNT/B1 MY:hSyt2 complex. A drop of 200 nl of sample was mixed with an equal amount of reservoir and incubated at 16°C, and the crystal grew within 1 week. Crystals were transferred briefly into a cryoprotectant solution, consisting of their respective growth condition supplemented with 25% glycerol, before freezing in liquid nitrogen.

Diffraction data were collected at stations I02, I03, and I04–1 of the Diamond Light Source (Oxon, UK), equipped with a PILATUS-6M detector (Dectris, Switzerland). Complete datasets to 2.5 and 2.0 Å were collected from single crystals at 100 K for each complex. Raw data images were processed and scaled with XDS (33) or Mosflm (34), and Aimless (35) using the CCP4 suite 7.0 (36). The resolution cutoff chosen was based on the CC1/2 (37).

Molecular replacement was performed with the coordinates of BoNT/B (PDB code: 1EPW) (38) to determine initial phases for structure solution in Phaser (39). The working models were refined using Refmac5 (40) and manually adjusted with Coot (41). Validation was performed with MolProbity (42). Crystallographic data statistics are summarized in table S3. Figures were drawn with PyMOL (Schrodinger LLC, NY, USA).

**Rat SCN culture and [3H]-glycine release assay**

All studies using rat SCN cultures were performed in the laboratories in the United Kingdom on material obtained from animals euthanized under Schedule 1 to the Animals (Scientific Procedures) Act 1986. Rat SCNs were prepared from E15 Sprague-Dawley rat embryos (Charles River, UK) and maintained in culture, as described previously (25).

Glycine release was assessed in SCNs at 20 to 23 days in vitro (DIV). Cells were treated with rBoNT/B1, rBoNT/B1 MY, or rBoNT/B1 QW for 24 hours at 37°C. [3H]-glycine release was determined, as described previously (25).

**Release studies in iCell GABANeurons**

iCell GABANeurons (Cellular Dynamics International, USA) were plated at a density of 60,000 cells per well in 96-well plates coated with poly-l-ornithine and laminin (Sigma-Aldrich, UK). iCell GABANeurons were maintained according to the manufacturer’s instructions. iCell GABANeurons were fed every 3 to 4 days by replacement of half media.

GABA release was assessed in iCell GABANeurons at DIV 16 to 18. Cells were treated with neurotoxins for 24 hours at 37°C. Following removal of neurotoxin, cells were washed three times in Neurobasal medium containing 1% B27 and 0.5 mM GlutaMAX (assay medium). Cells were loaded with [3H]-GABA (2 μCi/ml; PerkinElmer, UK) in assay medium for 120 min at 35°C. Following removal of [3H]-GABA, cells were washed three times with assay medium. Basal and stimulated [3H]-GABA release was established by incubation at 35°C for 5 min with assay medium (50 μl per well) containing low potassium (5.3 mM KCl) or high potassium (60 mM KCl), respectively. To determine retained [3H]-GABA, cells were lysed by adding radiolucemoprecipitation assay buffer (50 μl per well; Sigma-Aldrich, UK). Supernatants and cell lysates were transferred into 96-well IsoPlates (PerkinElmer, UK), and OptiPhase Supermix scintillation fluid (200 μl per well) was added. Radioactivity was quantified using a MicroBeta2 plate reader (PerkinElmer, UK).

**Generation of hSyt2 mice**

The Syt2 gene-targeting vector was constructed from genomic C57BL/6N mouse strain DNA (genOway, Lyon, France). Given that the intracellular and transmembrane regions of murine and hSyt2 are identical except for a single conservative amino acid substitution in the transmembrane region (see Fig. 2A), only the luminal domain of the protein was humanized. Humanization was achieved...
by replacing the region coding for the luminal domain of the murine Syt2 protein (exon 4) with a complementary DNA (cDNA) encoding the human luminal domain, thus preserving the overall intron–exon structure of the murine gene. An FRT (flippase recognition target)–flanked neomycin cassette was inserted in intron 4. Access to the Syt2 knock-out was given by insertingloxP sites on each side of the exon 4 containing the ATG codon. Upon Cre-mediated excision, the humanized exon 4, containing the ATG codon and the neurotoxin interaction site, was deleted.

A linearized targeting vector was transfected into C57BL/6N embryonic stem (ES) cells (genOway, Lyon, France) per genOway’s electroporation procedures. Recombined ES cell clones were microinjected into C57BL/6N blastocysts and gave rise to male chimeras with a significant ES cell contribution. Breeding was established with C57BL/6N mice expressing the Flp-recombinase to produce the Syt2 humanized heterozygous line devoid of the neomycin cassette. Heterozygous humanized animals were interbred to produce the Syt2 humanized homozygous line.

Handling of animals
Except for the muscle force studies on rabbits, all ex vivo and in vivo experiments were done within the Ipsen laboratory premises in France and were conducted in compliance with the relevant animal health regulation in place (Council Directive No. 2010/63/UE of 22 September 2010 on the protection of animals used for scientific purposes and Institutional Animal Care and Use Committee (IACUC) guidelines). These studies were also approved by an internal ethics committee at Ipsen. Muscle force studies on rabbits were performed at Charles River Laboratories in Lyon, France. The study design was reviewed and approved by the ethical committee of the test facility of Charles River, was submitted to French authorities, and followed the IACUC guidelines.

For ex vivo or in vivo mouse studies, female C57BL/6N mice weighing 18 to 25 g were purchased from Charles River (Lyon, France) or Janvier (Le Genest-Saint-Isle, France) and allowed to acclimatize for at least 5 days before the experiments. Animals were housed with six mice per cage and acclimatized for 7 days before the beginning of the experiments. The mice had free access to water and were fed with pelleted complete diet ad libitum.

For rabbits muscle force test, male New Zealand white rabbits [Crl: KBL(NZW), 3.1 to 4 kg] were purchased from Charles River (Chatillon sur Chalaronne, France) and acclimatized for at least 7 days before the experiment. Animals were housed in individual cages, had free access to filtered (0.2 μm) mains drinking water, and were fed with pelleted complete rabbit diet, sterilized by irradiation, ad libitum. In addition, irradiated hay (Special Diet Services) was provided daily.

Phenotypic characterization of hSyt2 mice
Mice were euthanized by exsanguination under deep anesthesia. WT male and female mice were compared to hSyt2 mice. There were three mice per sex per group, and 51 tissue sections per animal were examined. Tissues were fixed in formalin or in Davidson’s solution (eyes, lacrimal and Harderian glands, testes, and epididymides) for 24 hours and then embedded in paraffin. Tissue sections were hematoxylin and eosin–stained, and additional testicular sections were stained using periodic acid–Schiff for a better examination. Tissue sections were examined by a board-certified veterinary pathologist.

Immunochemistry
Tissue sections were deparaffinized and submitted to antigen retrieval treatments, as previously described (43). After two blocking steps with 3% H2 O2 (Sigma-Aldrich) for 10 min, and 10% horse serum (Vector) for 30 min, slides were incubated for 60 min at room temperature with the primary antibody (table S4). A secondary biotinylated antibody (Vector Labs) was applied for 30 min, followed by detection (30 min) with an avidin/biotin system (VECTASTAIN ABC Elite, PK-7100, Vector). The signal was revealed with Dako Envision system using DAB substrate (3,3’-diaminobenzidine; K5007, Dako). Nuclei were stained with hematoxylin (K8018, Dako) for 3 min. Isotype controls immunoglobulins (Vector) were included in all experiments to attest of the specificity of the staining. Sections were dehydrated in the Leica AutoStainer XL and mounted with DPX Mountant (VWR International).

Mouse phrenic nerve hemidiaphragm and detrusor strip ex vivo organ bath assays
Preparation of mouse hemidiaphragm and mouse detrusor bladder strips and recordings from these tissues were done, as previously described (19, 25).

Mouse DAS assay
The DAS assay (20, 44) was used to test efficacy in mice in vivo, as described previously (25). Mice presenting an abnormal paw, or a control DAS score different from 0, were excluded. Mice were identified by a mark on the tail or via implanted ID microchips. In each study, experiments were performed on four to six mice per dose. Toxins were evaluated in hSyt2 mice and WT littermates in parallel. Mice were assigned to doses in a randomized manner. Two to three independent studies were performed for each toxin.

In parallel to the DAS measurements, mice were weighed daily. The variation of the BWs after treatments was calculated for each day from the baseline BW (before injections) for each mouse and then averaged in groups. The 0% BW dose was calculated for the day of the largest average percentage variation during the entire study by linear regression of the part of the curve covering 0% average percentage variation. The tolerability index of the toxins was subsequently calculated as the ratio of 0% BW and ED50.

Rabbit muscle force generation test
A method to measure the muscle force developed by the triceps surae group of the left and right hind limb was adapted to rabbits from a previously described rat model (45). Briefly, animals were anaesthetized with isoflurane (0.5 to 5%) in oxygen. The hair on each hind limb was clipped before the first experiment and as necessary during the study. Stimulation sites were identified by a spotted tattoo. Rabbits were placed onto a specially designed apparatus securing each hind limb such that only the tibiotarsal joint was mobile. An isometric force transducer (model K1000, HSE, Harvard Apparatus) was secured to the forefoot such that the tibiotarsal angle was at 90°. A pair of needle electrodes was then placed near the tibial nerve. The tibial nerve was electrically stimulated [square shocks, 40 V (50 μs at 0.5 Hz); Stimulator C type 224, HSE, Harvard Apparatus], and the muscle force was recorded using the force transducer connected to a computerized data acquisition system (Notocord-hem software, NOTOCORD Systems S.A., Croissy-Sur-Seine, France). The signal was continuously sampled at 500 Hz and recorded from each animal twice for pretest measurement and then at days 3, 8, 15,
22, and 31 after injection. Animals were injected with the vehicle (Gelatin Phosphate Buffer) at a dose volume of 0.1 ml/kg in the left gastrocnemius muscle and BoNTs at a dose volume of 0.1 ml/kg in the right gastrocnemius muscle at day 1, under anesthesia. Absolute muscle force values were recorded for the vehicle- and toxin-injected hind limb. Data were normalized to the arithmetic mean of the pretest measurements.

**Expression analysis by Western blot in iCell GABANeurons and WT and hSyt2 mice**

Proteins from the cortex, cerebellum, and hippocampus of six WT and eight hSyt2 mice were extracted from approximately 20 mg of each brain region in 500 μl of ice-cold T-PER (Tissue Protein Extraction Reagent; Thermo Fisher Scientific) supplemented with 10% of Complete Protease Inhibitor Cocktail (Roche) using Precells (Ozyme).

iCell GABANeurons (Cellular Dynamics International) from three wells of six-well plates were harvested at 1, 7, 14, 21, and 28 days after plating in M-PER (Mammalian Protein Extraction Reagent; Thermo Fisher Scientific) supplemented with 10% of Halt Protease Inhibitor Cocktail (Thermo Fisher Scientific).

Proteins (20 μg) from each sample were resolved by SDS-PAGE (Invitrogen) and transferred onto nitrocellulose membranes. Membranes were blocked with 5% nonfat milk in 0.1% PBS-T (phosphate-buffered saline with Tween20) and probed with an anti-Syt1 antibody (1/1000; ab126253, Abcam) or anti-Syt2 antibody [syy105123, SYNaptic SYstems (mice samples), both at 1/1000]. Immunoreactive bands were detected using horseradish peroxidase–conjugated secondary anti-mouse (1/1000; A4416, Sigma-Aldrich) or anti-rabbit (1/1000; A6154, Sigma-Aldrich) antibodies and SuperSignal (Thermo Fisher Scientific).

Proteins were visualized on a PXi imager (Ozyme), and intensities were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1/1000; MAB374, Millipore) or SNAP-25 (1/1000; S9684, Sigma-Aldrich).

**SUPPLEMENTARY MATERIAL**

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/5/1/eaau7196/DC1

**Fig. S1. Longitudinal study of SNARE protein expression in iCell GABANeurons.**

**Fig. S2. Evaluation of Syt2 sequences with L54 in BoNT/B binding site.**

**Fig. S3. Molecular mechanisms of Syt recognition by BoNT/B1 variants.**

**Fig. S4. Electron density maps of the bound peptides and surface electrostatic potential of toxins.**

**Fig. S5. Effect of C-terminal histidine tag on rBoNT/B1 activity.**

**Fig. S6. Production of recombinant BoNTs.**

**Table S1. Dissociation constants (in micromolar) of rBoNT/B1 binding domains for human (h) and rodent (r) GST-tagged Syt1 and Syt2, measured by biolayer interferometry.**

**Table S2. Efficacy and safety of toxins in murine in vivo studies.**

**Table S3. X-ray crystallography: Data collection and refinement statistics.**

**Table S4. Antibodies used in immunohistochemistry studies.**

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Engineered botulinum neurotoxin B with improved binding to human receptors has enhanced efficacy in preclinical models

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