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Human synaptotagmin-II is not a high affinity receptor for botulinum neurotoxin B and G: Increased therapeutic dosage and immunogenicity

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ABSTRACT

Botulinum neurotoxins (BoNTs) inhibit neurotransmitter release by hydrolysing SNARE proteins essential for exocytosis. The synaptic vesicle protein synaptotagmin-II of rat and mouse acts as neuronal high affinity receptor for BoNT/B and BoNT/G. Here, we show that human synaptotagmin-II is not a high affinity receptor for BoNT/B and G due to a phenylalanine to leucine mutation in its luminal domain present only in humans and chimpanzees. It eliminates one of three major interactions between synaptotagmin-II and BoNT/B and hereby explains the disparity in potency of BoNT/B in humans and mice as well as the 40-fold higher dosage of rimabotulinumtoxinB versus onabotulinumtoxinA.

Structured summary of protein interactions:
rSyt-II binds to **BoNT/G** by pull down (View Interaction: 1, 2)
rSyt-II binds to **BoNT/B** by pull down (View Interaction: 1, 2)

1. Introduction

Botulinum neurotoxins (BoNTs) are extremely poisonous bacterial protein toxins causing the disease botulism due to blockade of the acetylcholine release at the neuromuscular junction. On the other hand they are extensively employed as highly effective medicines to treat diseases like cervical dystonia, blepharospasm, hyperhidrosis and overactive bladder syndrome. The seven BoNT serotypes A–G are composed of four domains which play individual roles in their intoxication mechanism. The C-terminal H_{CC} domain harbours the two receptor binding sites for gangliosides and/or a synaptic vesicle protein [1]. The function of the adjacent H_{CN} domain is the least understood. It can interact with phosphatidylinositol phosphates [2] and might trigger the translocation step [3]. Together, they constitute the 50 kDa H_C-fragment which forms together with the H_N domain the 100 kDa heavy chain (HC). The H_N domain spans a pore into the synaptic vesicle membrane thereby translocating the partially unfolded light chain (LC), a Zn^{2+} dependent endopeptidase, into the neuronal cytosol to specifically hydrolyse certain soluble *N*-ethylmaleimide sensitive attachment protein receptors of the vesicular fusion machinery [4]. This results in disruption of the Ca^{2+} triggered fusion of synaptic vesicles with the presynaptic membrane thereby blocking release of acetylcholine.

Local injections of BoNT are widely recognised as the most effective treatment for cervical dystonia. BoNT/B (rimabotulinumtoxinB) and BoNT/A (onabotulinumtoxinA) have been shown to be effective for cervical dystonia in multiple open-label and controlled trials [5]. Both drugs were compared within the indication cervical dystonia (CD) using a randomized, double-blind, parallel-arm study design [6]. Both serotypes had equivalent benefit in subjects with CD at 4 weeks. However, the mean dosage was 205 ± 50 units (U) onabotulinumtoxinA and 8520 ± 1892 U rimabotulinumtoxinB (one U is defined as one mouse LD_{50}) which corresponds to ~40-fold higher dosage of rimabotulinumtoxinB [6]. Such high protein dosage causes a high frequency of antibody induced therapy failure [7,8]. On the other hand, the specific activities in mice were determined to be 20–35 U/ng for onabotulinumtoxinA [9,10] and to be 90–107 U/ng for rimabotulinumtoxinB [11] which differs only by factor 3–5. Hence, this approximately 10-fold lower potency of rimabotulinumtoxinB in human versus mouse has to be attributable to species differences on the molecular level. Analysis of the amino acid sequences 53–92 and 51–90 of VAMP-1 and VAMP-2, respectively, the neuronal substrates of BoNT/B, revealed 100% identity between human and mouse VAMP-1 and -2, respectively, excluding impaired substrate hydrolysis in human [12]. Therefore a difference in receptor recognition might cause the disparity in potency.

Abbreviations: BoNT, botulinum neurotoxin; HC, heavy chain; H_C, carboxyl-terminal half of HC; H_{CC}, carboxyl-terminal half of H_C; H_{CN}, amino-terminal half of H_C; H_N, amino-terminal half of HC; LC, light chain; SV2, synaptic vesicle glycoprotein 2; hSyt, mSyt, rSyt, human, mouse and rat synaptotagmin, respectively; CD, cervical dystonia

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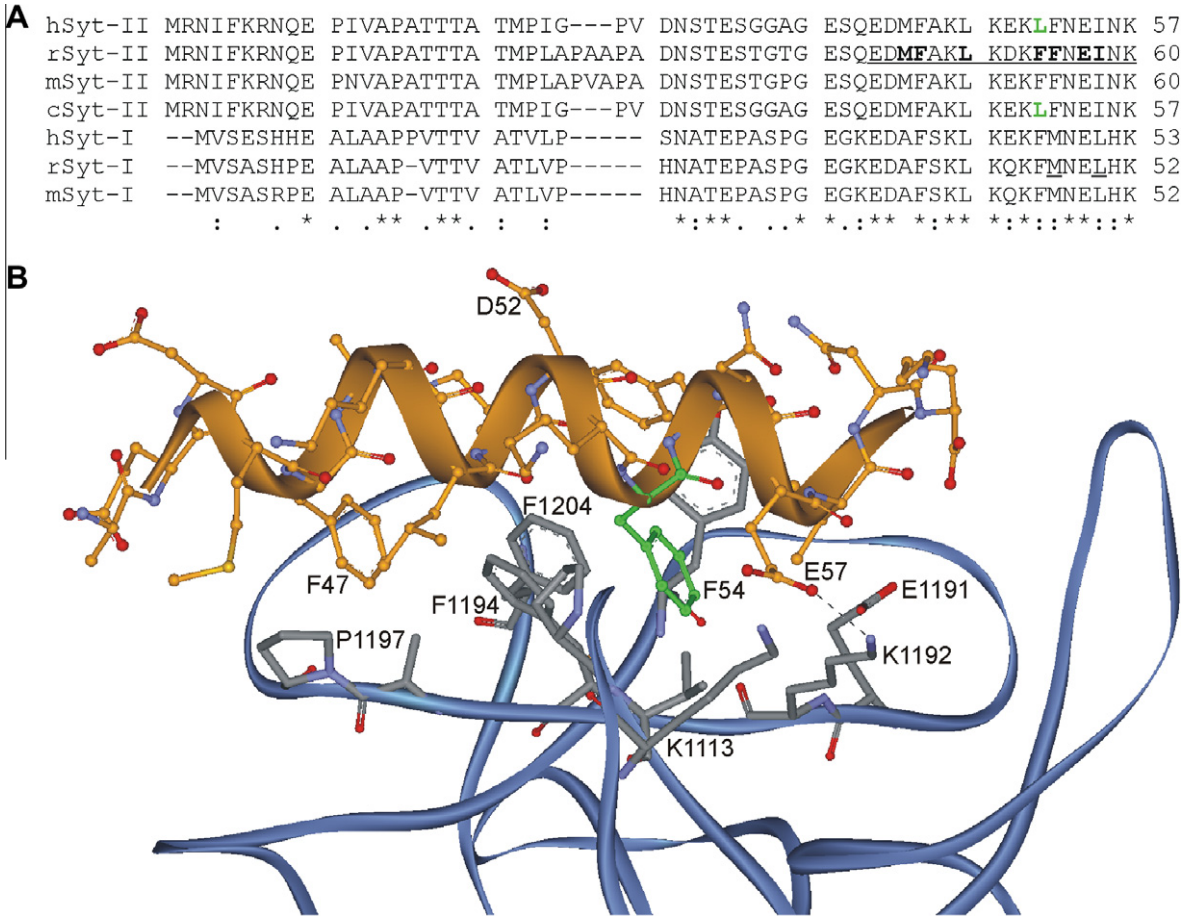

Fig. 1. F54 is not conserved in human Syt-II. (A) Sequence alignment of the intraluminal domain of human (h), rat (r), mouse (m) and chimpanzee (c) Syt-II and Syt-I, respectively. The 17 residues of rSyt-II forming a α-helix upon binding to BoNT/B are underlined. The seven residues mediating the interaction of rSyt-II with BoNT/B are highlighted in bold. The two residues turning rSyt-I into a high affinity receptor are underlined. The substitution L51 in hSyt-II and cSyt-II corresponding to F54 in rat and mouse Syt-II is highlighted in green. (B) Binding of the transmembrane juxtaposed 17mer peptide of the intraluminal domain of rSyt-II in an α-helical conformation (amino acids in orange ball and stick presentation) to the saddle like site in the distal tip of the H_{CC}-domain of BoNT/B (residues in grey stick presentation) (2nm1.pdb). K1192 forms an important salt bridge to E57 of rSyt-II (dashed line). F54 corresponding to L51 in hSyt-II is highlighted in green ball and stick presentation.

BoNT are taken up by a double receptor mechanism [13,14]. First, abundant polysialo gangliosides trap and accumulate BoNTs in the plane of the plasma membrane thus increasing the probability to contact their sparsely occurring synaptic vesicle protein receptors [1]. The intravesicular parts of these proteins are only temporarily exposed upon fusion of synaptic vesicles at the synaptic cleft. BoNT/B and BoNT/G use as their receptors the membrane juxtaposed 20 amino acids of the intraluminal domain of Synaptotagmin (Syt)-I and Syt-II (residues 32–52 and 40–60, respectively) [15–17], two homologous type I membrane proteins of synaptic vesicles that link membrane fusion to Ca^{2+} entry [18–21]. The mode of interaction of BoNT/B with its protein receptor Syt-II was elucidated in parallel by co-crystallization of full-length BoNT/B with a 21mer peptide encoding mouse (m) Syt-II 40-60 [22] and by crystallizing the BoNT/B H_C-fragment fused to the peptide encoding rat (r) Syt-II 8-61 [23]. Interestingly, the in solution unstructured luminal domain of mSyt-II and rSyt-II Syt-II formed a 17 residue long α-helix upon binding to a saddle like crevice at the distal tip of the BoNT/B H_C-fragment in the direct neighbourhood of the single ganglioside binding pocket (Fig. 1B). Only six hydrophobic residues, especially F47 and F54, of Syt-II interact via two adjacent pockets in H_CB (Fig. 1A). In addition, E57 forms an important salt bridge with K1192 of H_CB. Syt-I has one to two orders of magnitude lower affinity for BoNT/B compared to Syt-II [23,24] due to the substitutions Syt-I M47 instead of Syt-II F55 and Syt-I L50 instead of Syt-II I58 (Fig. 1A). Double mutation M47F/L50I converted Syt-I to a Syt-II-like high affinity receptor thereby substantiating a conserved binding mode for both Syt isoforms [23]. Another study evidenced that Syt-I and Syt-II bind to the equivalent place in BoNT/G [14].

In the present study, we investigated whether amino acid changes in the intraluminal domains of hSyt-I and hSyt-II could be responsible for the observed potency difference of BoNT/B in humans compared to mice. We identified in the intraluminal domain of the BoNT/B high-affinity receptor rSyt-II that F54, which forms an important interaction with BoNT/B, is mutated to leucine in hSyt-II and chimpanzee (c) Syt-II. Introduction of the corresponding mutation F54L into rSyt-II abolished its binding to the H_C-fragments of BoNT/B and G in GST-pull-down assays. Hence, a drastically impaired receptor recognition by BoNT/B provides an explanation for its decreased potency in humans. Targeted modification of the BoNT/B Syt-binding site might improve affinity to hSyt-II and thereby increase the potency in humans.

2. Materials and methods

Fig. 1B was prepared using the programme Discovery Studio Visualizer 2.5 (Accelrys Software Inc.). Sequence alignment was made using ClustalW.

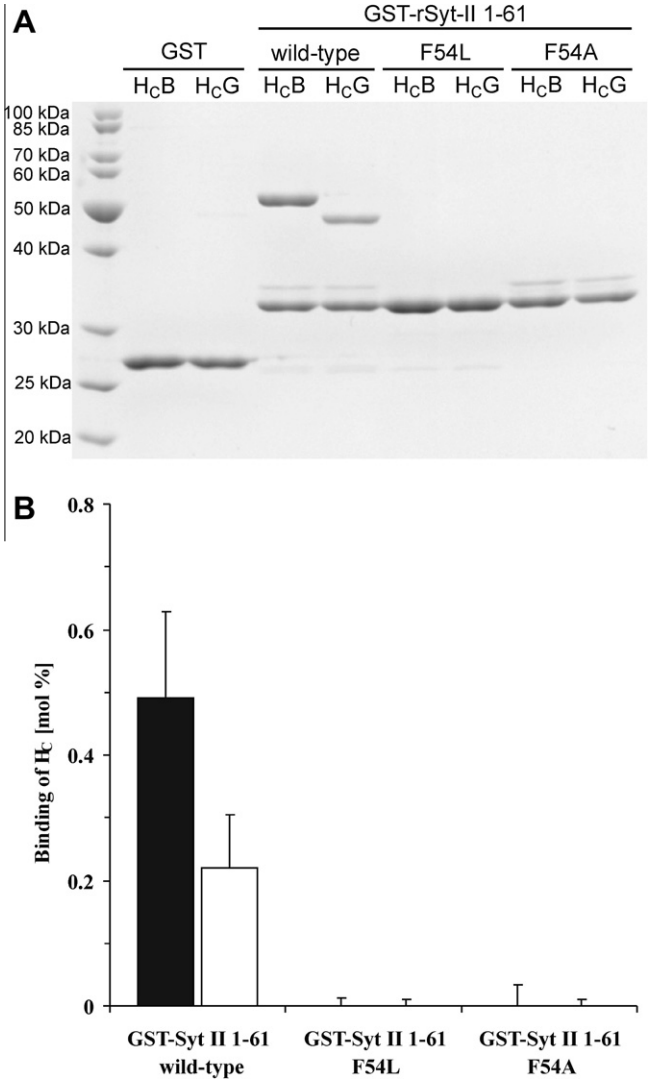

Fig. 2. BoNT/B and G H_C-fragments do not bind to GST-rSyt-II 1-61 F54L. (A) GST (25 kDa), GST-rSyt-II 1-61 wild-type (33 kDa) and its mutants F54A and F54L were immobilised on glutathione-sepharose beads and incubated with H_CB and H_CG (50 kDa) for 2 h at 4 °C. Proteins bound to the solid phase were visualised by Coomassie blue staining. (B) Amounts of bound H_CB (filled) and H_CG (empty column) are quantified ($n = 4$, ±S.D.).

2.1. Plasmid constructions and production of proteins

Production and purification of the H_C-fragments of BoNT/B and G carrying a carboxyl-terminal StrepTag as well as GST-rSyt-II 1-61 were previously described [17,25]. Mutations of GST-rSyt-II were generated by the Genetailor method (Invitrogen) using suitable primers and pGEXSytII-61C as template DNA [17]. Nucleotide sequences of all mutants were verified by DNA sequencing.

2.2. GST-pull-down assays

GST-fusion-proteins (150 pmol each) immobilised to 10 µl of glutathione-sepharose-4B matrix were incubated with recombinant H_C-fragments (100 pmol each) in a total volume of 100 µl 20 mM Tris–HCl, 150 mM NaCl, pH 7.2 supplemented with 0.5% Triton X-100 (Tris/NaCl/Triton) buffer for 2 h at 4 °C. Beads were collected by centrifugation and washed three times each with 160 µl Tris/NaCl/Triton buffer. Washed pellet fractions were boiled in SDS sample buffer, analysed by 12.5% SDS-polyacrylamide gel electrophoresis, detected by Coomassie blue staining and subsequently quantified.

3. Results and discussion

3.1. Human and chimpanzee Syt-II display a unique single mutation at position 51

The amino acid sequences of the intraluminal domains of human, rat, and mouse Syt-I and Syt-II were aligned (Fig. 1A). According to biochemical data and crystallographic studies, only the membrane juxtaposed 17 amino acids of Syt-I and Syt-II interact with the H_C-fragment of BoNT/B (Fig. 1B) [15,17,22–24]. Here, F47 and F54 as well as E57 of rSyt-II and mSyt-II form the main interactions with BoNT/B [22,23]. Identical residues are found within the homologous segment of human, mouse and rat Syt-I (F39, F46 and E49). In contrast, residue 51 of human and chimpanzee Syt-II corresponding to F54 in mouse and rat Syt-II is a leucine (Fig. 1A). According to the receptor-toxin structure H_CB-rSyt-II, F54 constitutes one of two major hydrophobic interactions with BoNT/B, and its mutation to alanine completely abolished binding of H_CB in a pull-down assay [23]. Thus, L51 of hSyt-II is a likely candidate for impaired affinity to BoNT/B and G.

3.2. BoNT/B and G H_C-fragments do not bind GST-rSyt-II 1-61 F54L

To test the effect of the Phe to Leu replacement at position 51 in hSyt-II we generated the GST-fusion protein GST-rSyt-II 1-61 F54L to allow a better comparison with previous binding experiments of H_CB employing rSyt-II. The substitution D52 in rSyt-II versus E52 and E49 in mSyt-II and hSyt-II, respectively, is irrelevant since residue 52 does not interact with H_CB and the mutant mSyt-II E52A displays wild-type binding characteristics [22]. The GST-rSyt-II 1-61 F54L mutant was recombinantly expressed in *Escherichia coli* and could be isolated in yields comparable to the wild-type construct. Subsequently, we performed GST-pull-down assays and tested the binding of H_CB and H_CG to the mutant F54L in comparison to wild-type and mutant F54A [23]. H_CB and H_CG bound to GST-rSyt-II 1-61 wild-type with 49 ± 14 and 22 ± 9 mol%, respectively (Fig. 2B). The lower affinity of H_CG to Syt-II versus H_CB is in accordance with previous data [14,17]. Removing the phenyl ring of residue 54 (F54A) abolished binding of H_CB as well as of H_CG to GST-rSyt-II 1-61 as shown previously [22,23,26]. Finally, mutant F54L corresponding to hSyt-II similarly deletes binding of H_CB and H_CG to GST-rSyt-II 1-61. As F54L is the only replacement of hSyt-II versus mSyt-II among interacting amino acids, human Syt-II will bind BoNT/B and G with much lower affinity than mouse or rat Syt-II. This result underlines the importance of the phenyl side chain at residue 54 for mediating the hydrophobic interaction with both BoNT serotypes and is further supported by the mutation of F54 to methionine which also deleted binding with BoNT/B [26].

In conclusion, this finding explains the minimum 10-fold lower potency of BoNT/B in humans compared to mice. Furthermore, although BoNT/B binds Syt-I with a 10- to 100-fold higher dissociation constant [23,24], it seems that in humans presumably Syt-I mediates the neuronal uptake of BoNT/B since the three key residues (F40, F47 and E50) are conserved in human Syt-I. In addition, hereby we provide one explanation for the rareness of human botulism cases caused by BoNT/G [27]. Moreover, this study also sheds light onto the problematic issue of extrapolating results of animal experiments or non-human cell line based assays for pharmaceutical applications in humans. In this case, only experiments in chimpanzees and no other mammals would have yielded results valid for humans. In the future, employing the detailed knowledge of

BoNT-Syt receptor interaction, one could increase the low binding affinity to human Syt-II by rationale site directed mutagenesis in the Syt binding site thereby increasing the potency of BoNT/B in humans to provide a highly effective alternative treatment for BoNT/A-non-responding patients with movement disorders.

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