

Research Article

Construction of Recombinant Protein of Botulinum Neurotoxin Light Chain and Analysis of Cleavage Effect on VAMP1 Protein in Brown Rat

Li Shengqing^{1,3,*}, Han Shengyi^{1,3}, Li Shuping^{1,3}, Hu Guoyuan^{1,3}, Liu Huaixing^{1,3},
Li Lingxia^{2,3}, Shi Tian^{1,2,3}, Zhang Shinan^{1,2,3}

¹Qinghai Academy of Animal Science and Veterinary Medicine, Qinghai University, Xining, China

²College of Agriculture and Animal Husbandry, Qinghai University, Xining, China

³Qinghai Provincial Key Laboratory of Pathogen Diagnosis for Animal Diseases and Green Technical Research for Prevention and Control, Xining, China

Abstract

In order to explore the feasibility of the new rodenticide of type D Botulinum toxin to prevent and control the population density of pests in farmland from a molecular perspective. This study aimed to analyze the feasibility of D-type botulinum toxin for the control of urban rodents from a molecular perspective, and to detect the cleavage effect of D-type botulinum neurotoxin light chain recombinant protein on the synaptic vesicle membrane protein VAMP-1 of brown house mouse. The genomic DNA of Clostridium botulinum D 8901 strain was used as a template. Based on the botulinum toxin D gene sequence reported in GenBank, specific primers were designed. Plasmids were constructed by gene synthesis and subcloned into the pET28a expression vector. The recombinant plasmid was transformed into BL21 (DE3) Rosetta competent cells, and induced expression by IPTG. The expression products were purified by Ni-NTA affinity chromatography, identified by SDS-PAGE and Western blotting, and the recombinant proteins were analyzed by SDS-PAGE and Western blotting. The cleavage of VAMP1 protein in SD brown house mouse. The pET-28a- BDLc expression plasmid was successfully constructed and transformed into E. coli BL21 (DE3) Rosetta. Western blot confirmed that the recombinant protein pET-28a- BDLc (residues Met 1-Met 94) obtained soluble expression, and obtained 5 mg, Recombinant protein with purity > 90%. It has good biological activity as determined by animal method. The recombinant protein can decompose VAMP1 protein into two fragments. Type D botulinum toxin protein can specifically cleave synaptic vesicle membrane protein (VAMP1) of brown house mouse, and type D botulinum toxin is feasible for urban rodent control.

Keywords

Botulinum Neurotoxin Type D, Expression of Recombinant Protein, Detection of Biological Activity

*Corresponding author: Lsq.8008@163.com (Li Shengqing)

Received: 7 January 2021; Accepted: 16 January 2021; Published: 31 December 2024



1. Introduction

Since the isolation of Botox in 1897, seven toxin types A, B, C, D, E, F, and G have been discovered, a large amount of literature has been reported on *Clostridium botulinum* at home and abroad, but most of them focus on the structural characteristics of neurotoxins [1], mechanisms of toxin poisoning [2], detection methods [3, 4], and treatment of toxin poisoning [5, 6] and so on.

This laboratory uses a strain of *Clostridium botulinum* D isolated from animals for the first time in China in 1989, and developed a toxin produced by it into a new biological rodenticide. And rodent control in Qinghai Voles, etc., achieved remarkable results [7]. However, by measuring the sensitivity of D-type botulinum to different pests, it was found that the sensitivity of the toxin protein to rats is significantly different. Take plateau pika and plateau voles as an example. The oral lethal doses differ by 10 times (5010 MLD/mL and 51,900 MLD/mL, respectively), while the brown mice have a higher half lethality of 1 million MLD/mL (intravenous injection in mice), which is more sensitive. Poor, this phenomenon severely limits the rat species to which the rodenticide is applicable, and the cause of this phenomenon is currently unclear. It has been reported that due to changes in the 48-residue site of the VAMP1 protein in some rats, the D-botulinum toxin cannot cleave the substrate protein VAMP normally, and thus appears as non-susceptible animals [8].

Therefore, in this paper, the recombinant protein was expressed by botulinum neurotoxin light chain protein through molecular biology method, and the degradation effect of recombinant protein DBLc on the synaptic vesicle membrane protein VAMP1 protein of brown rat was examined. The feasibility of D-type botulinum toxin for urban rodent prevention and control will lay a foundation for expanding the D-type botulinum rodenticide and expanding the applicable rat species and scope.

2. Materials and Methods

2.1. Materials

Strains and plasmid: D-type botulinum D8901 strain was isolated from botulinum-poisoned sheep in Qinghai. The strain produced good toxic properties, had hemagglutinin, grew well on VF medium, and produced toxin. It is 2 million MLD (intramuscular injection) / ml. The dialysis culture has a virulence of 20-40 million MLD / ml; BL21 (DE3) chemically competent cells, and the pET28a expression vector is a product of Shanghai Shenggong Biological Company. Recombinant VAMP1 protein from brown house mouse was constructed and stored in our laboratory.

Tool enzymes and main reagents: Restriction endonucleases BamHI and XhoI were purchased from New England Biolabs; Western Blot primary antibody, Western Blot secondary antibody, protein concentration quantification kit,

plasmid mini extraction kit, gel recovery kit, IPTG, LB medium, primers, and gene sequencing were completed by Biotech Bioengineering (Shanghai) Co., Ltd.

Experimental animals: Kunming mice, weighing 16-18g, were purchased from the Small Animal Breeding Base of Qinghai Institute of Endemic Diseases.

2.2. Experimental Methods

Design and Synthesis of Primers: Primers were designed based on the full sequence of the D-type botulinum neurotoxin gene registered in GenBank. The primer sequences are as follows: 5'-atgtagtattatgacatggccag-3' (introduced XhoI digestion site); 5'-ttgtacattagtctcatctgtaa-3' (introduced BamHI site). The primers were synthesized by Shanghai Shenggong Biological Company.

Pure culture of *Clostridium botulinum* strain: Aseptically inoculate *Clostridium botulinum* D8901 strain into the home-made VF broth liver and stomach enzyme digestion broth culture medium, incubate at 37 °C to the logarithmic growth phase, wipe the microscope inspection and observation of strain morphology.

Genomic DNA extraction: Take 1.5 mL of the above pure culture into a dorf tube, centrifuge, and discard the supernatant. Add TE suspension pellet, use bacterial DNA extraction and kit to extract genomic DNA.

PCR amplification of LC fragment of botulinum neurotoxin light chain: Using the extracted *Clostridium botulinum* genomic DNA as a template, specific primers were used to amplify the LC fragment of botulinum neurotoxin type D. The expected amplification product was 1450 bp.

PCR reaction system: 2 µl of template genomic DNA, 0.5 µl of upstream and downstream primers, 1 µl of 25mM dNTP, 5 µl of 10×PCR Buffer, 1 µl of Taq DNA polymerase, and water supply to 50 µl. Reaction conditions: preheating at 95 °C for 3 min, denaturing at 95 °C for 22 sec, annealing at 53 °C for 20 sec, extending at 72 °C for 40 sec, 22 cycles, and finally extending at 72 °C for 5 min. The amplified product was subjected to 1% agarose electrophoresis, and the size of the product was determined using DL2000 as the standard molecular weight.

Construction of recombinant expression plasmid: The recovered and purified target DNA fragments were ligated with the vector, and a 20 ul ligation system was used (8 ul. Digestion target fragment, pET28a 4 ul. Digestion vector 10X T4 DNALigase Buffer 2ul, T4 DNALigase 1ul (5u/ul), ddH2O supplemented to 20ul), the ligation solution was transferred into TOP10 competent state, and positive clones were detected and screened for sequencing verification.

Induced expression of recombinant proteins: Transfer the pET28a recombinant plasmid into BL21 (DE3) E. coli competent cells, heat-shock at 42 °C and spread on a plate containing 30 µg / ml kanamycin. Pick a single clone to a card containing 30 µg / ml kanamycin. When the bacterial solution

was cultured in the medium of natamycin, when the OD value reached 0.6, 0.5 mM inducer IPTG was added, and the cells were cultured overnight at 20 °C to express a large amount, and the cells were collected by centrifugation. Buffer A was added to the collected bacterial cells and suspended using an ultrasonic crusher. Centrifuge. The precipitate after centrifugation was dissolved with buffer B. The supernatant and the precipitate were treated separately. Samples were prepared for SDS-PAGE detection.

Purification of recombinant protein, dialysis and concentration: The cells were dissolved with buffer C, sonicated, and the crude protein was collected by centrifugation. 5 ml of Ni-NTA was taken. The equilibrium column was washed with Binding buffer at 5 times the bed volume. The flow rate was 5 ml / min. After the equilibrated column packing was incubated for 1 h, the product after incubation was loaded onto the column, collected and flowed out, the equilibrium column was washed with Binding buffer, and the column was washed with Washing buffer, collected and flowed out, eluted with Elution buffer, collected and flowed out. Washed out and eluted outflow were processed separately, sample preparation, SDS-PAGE detection.

The better-purified fraction 5 was dialyzed into 500 mM L-Arginine, 1 mM DTT, 0.1% SKL, pH 8.5 buffer. After the end of dialysis, dialysis was performed to 50 mM Tris, 300 mM NaCl, 1 mM DTT, 0.1% SKL, pH 8.5. After the end of dialysis, it was concentrated with PEG20000, filtered through a 0.45 µm filter membrane, and aliquoted in 1 ml / tube, -80 °C. save.

Western blot identification of expression products: After the expression products were subjected to SDS-PAGE, they were transferred to a nitrocellulose membrane, and after blocking, they were reacted with horse anti-BoNT/D homotoxin polyclonal antibody (1: 500 dilution) and HRP-rabbit anti-horse IgG (1: 1,000 dilution). DAB develops color.

Detection of Recombinant Protein Activity and Determination of Kinetic Parameters: The constructed D. brown domestic rat VAMP1 recombinant protein and the recombinant protein pET-28a- DBLc were treated in a reaction solution (50 mmol/L HEPES, 2.5 mmol/L DTT, 10 µmol / L ZnCl₂, pH 7.4) for 40 minutes at 37 °C. A set of negative controls without BoNT/D light chain was also set. After 20% SDS-PAGE electrophoresis of VAMP-1 activity, Coomassie brilliant blue was used to detect the cleavage.

3. Results

3.1. Gene Cloning of DBLc and Construction of Expression Plasmid pET-22b-DBLc

The DBLc gene fragment obtained by PCR amplification was about 1450 bp in size (Figure 1 A), which was consistent with the predicted size. The obtained product was sequenced with 99% identity to the gene sequence reported in GenBank.

The PCR product was cloned into a cloning vector under the action of T4 DNA ligase, and positive clones were selected after transformation of DH5α. The sequenced DNA fragment was digested with BamHI and XhoI and ligated into pET-28a prokaryotic expression vector. The resulting recombinant plasmid was subjected to BamHI Identified with XhoI and obtained a target band of about 1450 bp in size, indicating that the target gene has been inserted into the vector (Figure 1 B).

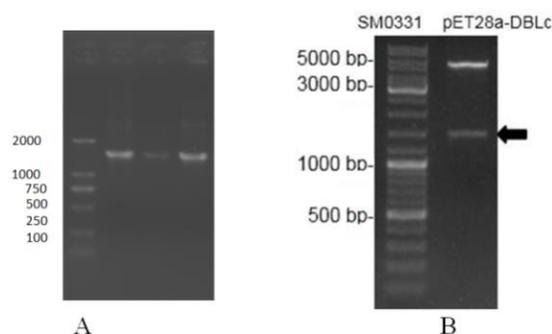


Figure 1. Construction of recombinant plasmid pET-28a-DBLc.

A. DBLc gene PCR product. The arrow indicates the DBLc gene fragment obtained by PCR amplification.

B. Agarose gel electrophoresis of the double digested product. The pET-28a-DBLc expression vector was identified by double digestion with BamH I and Xho I.

3.2. Identification of Recombinant Protein Expression Products

The correctly identified pET-28a-DBLc plasmid was transformed into the expression strain BL21 Rosetta competent cells and expressed under the induction of IPTG. SDS-PAGE analysis showed that the induced bacteria had obvious expression bands, and the relative molecular mass of the expression product was about 50 KDa, which was consistent with the expected size of the recombinant protein (Figure 2). After purification by HisTrap FF column, the target band is relatively single and the purity is about 98%, and high-purity recombinant DBLc protein is obtained (Figure 3).

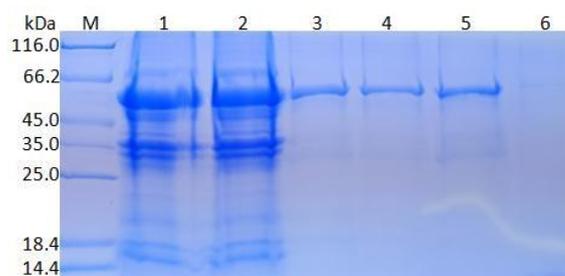
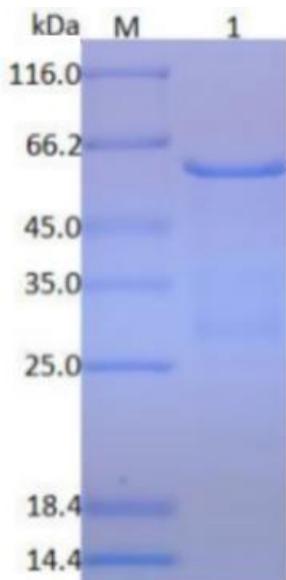


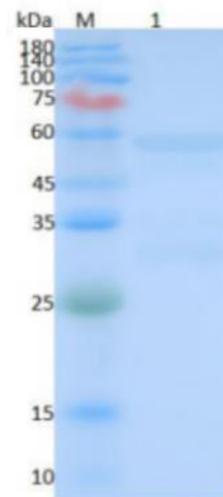
Figure 2. SDS-PAGE analysis of fusion protein nickel agarose affinity chromatography purification.

M: Protein marker (Cat. No.: C600525); 1: loading; 2: elution; 3: 20 mM Imidazole elution fraction; 4: 50 mM Imidazole elution fraction; 5-6: 500 mM Imidazole elution fraction



M: Protein marker (Cat. No.: C600525); 1: fusion target protein

Figure 3. SDS-PAGE analysis of the final purified protein.



M: Protein marker (Cat. No.: C610013); 1: fusion target protein

Figure 4. Western Blot analysis of the final purified protein.

3.3. Western Blot Identification and Concentration Determination of 6 Expression Products

Western blot identification: The expression of recombinant protein pET-28a-DBLc in E.coli was detected by Western blot using HRP-labeled histidine antibody. (Figure 4).

Table 1. Recombinant expression protein concentration determination table.

Test1	Test2	Average	BSA(μg)
0.997	1	0.9985	0
0.968	0.982	0.975	8
0.909	0.922	0.9155	16
0.848	0.849	0.8485	24
0.718	0.715	0.7165	40
0.647	0.656	0.6515	50
0.817	0.808	0.8125	Target protein

Expression product concentration determination: The non-interfering protein quantification kit Cat. No.: C503071 was used to determine the protein concentration of 2.81 mg/ml and the volume of the sample to be tested was 10 μl (Table 1). After purification, 2 ml of the target protein was obtained at a concentration of 2.81 mg / ml, for a total of 5.62 mg.

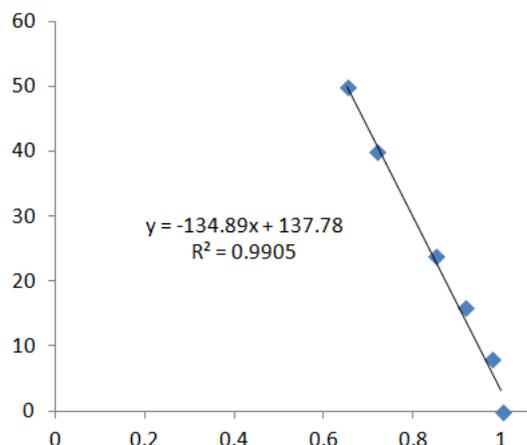


Figure 5. Analysis of protein concentration.

3.4. Activity Detection and Kinetic Parameter Determination of Recombinant Protein VAMP1

The BoNT/D light chain can specifically recognize Q60-K61 [9], which cleaves its substrate VAMP1 protein, and cleaves VAMP-1 into VAMP-1-60 and the tail small peptide VAMP-61-96. Recombinant protein VAMP-1 and BoNT/D light chain were incubated in a reaction solution (50 mmol/L HEPES, 2.5 mmol/L DTT, 10 μmol / L ZnCl₂, pH 7.5) at 37°C

for 30 minutes, and the reaction was analyzed by SDS-PAGE result. The results showed that the VAMP-1 protein with a relative molecular mass of 13×10^3 was digested by BoNT/D to produce 11×10^3 VAMP-1-60 and 2×10^3 tail peptides VAMP-61-96 (Figure 6), indicating that the recombinant protein VAMP1 can be specifically cleaved by BoNT/D.

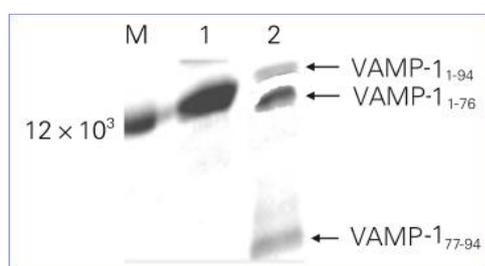


Figure 6. Degradation effect of recombinant DBLc protein on VAMP1 protein of brown house mouse.

4. Discussion

Botulinum toxin is secreted by *Clostridium botulinum* and can cause special neurotoxicity symptoms. It has a high lethality to humans and animals and is one of the most toxic proteins [10]. But according to the different serotypes, it can be divided into 7 types: A, B, C (C α and C β), D, E, F, G. Each type of botulinum toxin molecule has many similar characteristics in structure. H chain, relative molecular mass (100 000) and light chain (L, 50 000 relative molecular mass), both of which are connected by a disulfide bond [11]. The carboxy terminus of the heavy chain is bound to the target cell, and the amino terminus is involved in neutralization and blocking the release of neurotransmitters [12]. In view of this, this study cloned and expressed *Clostridium botulinum* neurotoxin D8901 for the first time in our laboratory in China, and constructed a recombinant D-type light chain botulinum toxin. It is prospective to study the cleavage effect of light chain neurotoxin proteins on specific substrate proteins in target animals, analyze the sensitivity of different mouse species to botulinum toxin type D, and to apply mouse species to the control of toxin rodenticides in the next step.

According to data, neurotoxins specifically cleave SNARE protein, preventing the release of transmitters in the transport vesicles, causing muscle paralysis. The substrates and peptide bond cleavage sites of various serotypes of BoNT are different: Types A and E mainly act on the SNAP-25 protein, while types B, D, F, and G mainly act on the VAMP protein; there are 2 types of C toxins. Substrates are SNAP-25 and syntaxin proteins [1, 13, 14]. The latest results show that VAMP2 is not detected in rat motor nerve endings. It was replaced by another VAMP1 protein [15], and it has been reported that VAMP1 and VAMP2 proteins are co-expressed at nerve junctions [16]. However, there have been no reports of recombinant expression of intact VAMP1 protein or partial

VAMP1 protein after dehydrophobic region for in vitro analysis of endopeptidase activity. In order to analyze molecularly whether *Clostridium botulinum* toxin is suitable for the prevention and control of urban rodents (Brown domestic rat), this study used genetic engineering to recombinantly prepare the recombinant protein DBLc to degrade VAMP1 in brown domestic rat. The results show that D-type botulinum toxin can effectively degrade VAMP1 protein and can be used for urban rodent control.

The latest research report indicates that some rats have VAMP1 protein mutations at 48 positions, which can be divided into two genotypes I48 and M48. The sensitivity of the two genotypes to type D botulinum toxin is extremely high. The difference is large, and the article concludes that the gene sequence of the VAMP1 protein in humans may be insensitive to type I48, so that the human body is very insensitive to type D botulinum toxin [8]. The MAMP type of VAMP1 protein at 48 sites in the brown rat was preserved in this experiment. It can also be determined that the brown rat is a sensitive animal to botulinum toxin type D, which is consistent with the results of the degradation test. However, its sensitivity is weaker than that of plateau zokor and plateau pika. Combined with the previous work in the laboratory, the results of the analysis of the damage intensity of different intestinal contents of different rodents against type D botulinum toxin, we speculate whether it is intestinal against the brown rat's intestine. The destruction of toxin proteins is related by a certain degree.

5. Conclusion

The pET-28a-BDLc recombinant protein was constructed through this study. And it has good biological activity as determined by animal method. The recombinant protein can decompose VAMP1 protein into two fragments. Type D botulinum toxin protein can specifically cleave synaptic vesicle membrane protein (VAMP1) of brown house mouse, and type D botulinum toxin is feasible for urban rodent control.

Author Contributions

S. L. designed and performed experiments, analyzed results, and wrote the manuscript. S. H., S. L., G. H. and H. L. Methodology: T. S., S. Z. provided advice. L.L.

Funding

Qinghai Provincial Science and Technology Plan (2021-ZJ-923).

Conflicts of Interest

The authors declare that they have no conflict of interest.

References

- [1] Turton K, Chaddock JA, Acharya KR: Botulinum and tetanus neurotoxins: structure, function and therapeutic utility. *Trends Biochem Sci*, 27(11): 552-558, 2002. [https://doi.org/10.1016/s0968-0004\(02\)02177-1](https://doi.org/10.1016/s0968-0004(02)02177-1)
- [2] Mas MF, Li S, Francisco GE: Centrally mediated late motor recovery after botulinum toxin injection: Case reports and a review of current evidence. *J Rehabil Med*, 49(8): 609-619, 2017. <https://doi.org/10.2340/16501977-2257>
- [3] Woudstra C, Skarin H, Anniballi F, Auricchio B, De Medici D, Bano L, Drigo I, Hansen T, Lofstrom C, Hamidjaja R, van Rotterdam BJ, Koene M, Bayon-Auboyer MH, Buffereau JP, Fach P: Validation of a real-time PCR based method for detection of Clostridium botulinum types C, D and their mosaic variants C-D and D-C in a multicenter collaborative trial. *Anaerobe*, 22: 31-37, 2013. <https://doi.org/10.1016/j.anaerobe.2013.05.002>
- [4] von Berg L, Stern D, Pauly D, Mahrhold S, Weisemann J, Jentsch L, Hansbauer EM, Muller C, Avondet MA, Rummel A, Dorner MB, Dorner BG: Functional detection of botulinum neurotoxin serotypes A to F by monoclonal neopeptide-specific antibodies and suspension array technology. *Sci Rep*, 9(1): 5531, 2019. <https://doi.org/10.1038/s41598-019-41722-z>
- [5] Thant ZS, Tan EK: Emerging therapeutic applications of botulinum toxin. *Med Sci Monit*, 9(2): RA40-48, 2003.
- [6] Shi Y, Lin H, Cao J, Cui C: Botulinum toxin type A induces protective autophagy in human dermal microvascular endothelial cells exposed to an in vitro model of ischemia/reperfusion injury. *Exp Ther Med*, 16(6): 4379-4386, 2018. <https://doi.org/10.3892/etm.2018.6741>
- [7] Han Z, Song W, Deng X, Xu X: Grassland ecosystem responses to climate change and human activities within the Three-River Headwaters region of China. *Sci Rep*, 8(1): 9079, 2018. <https://doi.org/10.1038/s41598-018-27150-5>
- [8] Peng L, Adler M, Demogines A, Borrell A, Liu H, Tao L, Tepp WH, Zhang SC, Johnson EA, Sawyer SL, Dong M: Widespread sequence variations in VAMP1 across vertebrates suggest a potential selective pressure from botulinum neurotoxins. *PLoS Pathog*, 10(7): e1004177, 2014. <https://doi.org/10.1371/journal.ppat.1004177>
- [9] Benagiano V, Lorusso L, Flace P, Girolamo F, Rizzi A, Bosco L, Cagiano R, Nico B, Ribatti D, Ambrosi G: VAMP-2, SNAP-25A/B and syntaxin-1 in glutamatergic and GABAergic synapses of the rat cerebellar cortex. *BMC Neurosci*, 12: 118, 2011. <https://doi.org/10.1186/1471-2202-12-118>
- [10] Schiavo G, Rossetto O, Montecucco C: Clostridial neurotoxins as tools to investigate the molecular events of neurotransmitter release. *Semin Cell Biol*, 5(4): 221-229, 1994. <https://doi.org/10.1006/scel.1994.1028>
- [11] Seo MS, Kim YI, Kang KK, Oh SK, Sung SE, Jung YS, Cho JY, Song H, Hwang DY, Park SJ, Kim KS: Comparison study of the response with botulinum toxin muscle injection in the ICR mice from three different sources. *Lab Anim Res*, 35: 11, 2019. <https://doi.org/10.1186/s42826-019-0010-4>
- [12] Schiavo G, Matteoli M, Montecucco C: Neurotoxins affecting neuroexocytosis. *Physiol Rev*, 80(2): 717-766, 2000. <https://doi.org/10.1152/physrev.2000.80.2.717>
- [13] Meunier FA, Lisk G, Sesardic D, Dolly JO: Dynamics of motor nerve terminal remodeling unveiled using SNARE-cleaving botulinum toxins: the extent and duration are dictated by the sites of SNAP-25 truncation. *Mol Cell Neurosci*, 22(4): 454-466, 2003. [https://doi.org/10.1016/s1044-7431\(02\)00016-7](https://doi.org/10.1016/s1044-7431(02)00016-7)
- [14] Rozas JL, Gomez-Sanchez L, Mircheski J, Linares-Clemente P, Nieto-Gonzalez JL, Vazquez ME, Lujan R, Fernandez-Chacon R: Motoneurons require cysteine string protein-alpha to maintain the readily releasable vesicular pool and synaptic vesicle recycling. *Neuron*, 74(1): 151-165, 2012. <https://doi.org/10.1016/j.neuron.2012.02.019>
- [15] Li JY, Edelmann L, Jahn R, Dahlstrom A: Axonal transport and distribution of synaptobrevin I and II in the rat peripheral nervous system. *J Neurosci*, 16(1): 137-147, 1996.
- [16] Liu Y, Sugiura Y, Lin W: The role of synaptobrevin1/VAMP1 in Ca²⁺-triggered neurotransmitter release at the mouse neuromuscular junction. *J Physiol*, 589 (Pt 7): 1603-1618, 2011. <https://doi.org/10.1113/jphysiol.2010.201939>