

# Comparison of Structure and Mechanism in BoNT/A1 and BoNT/A2: Implications for Therapeutic Applications

Brookfield Academy Upper School SMART Team

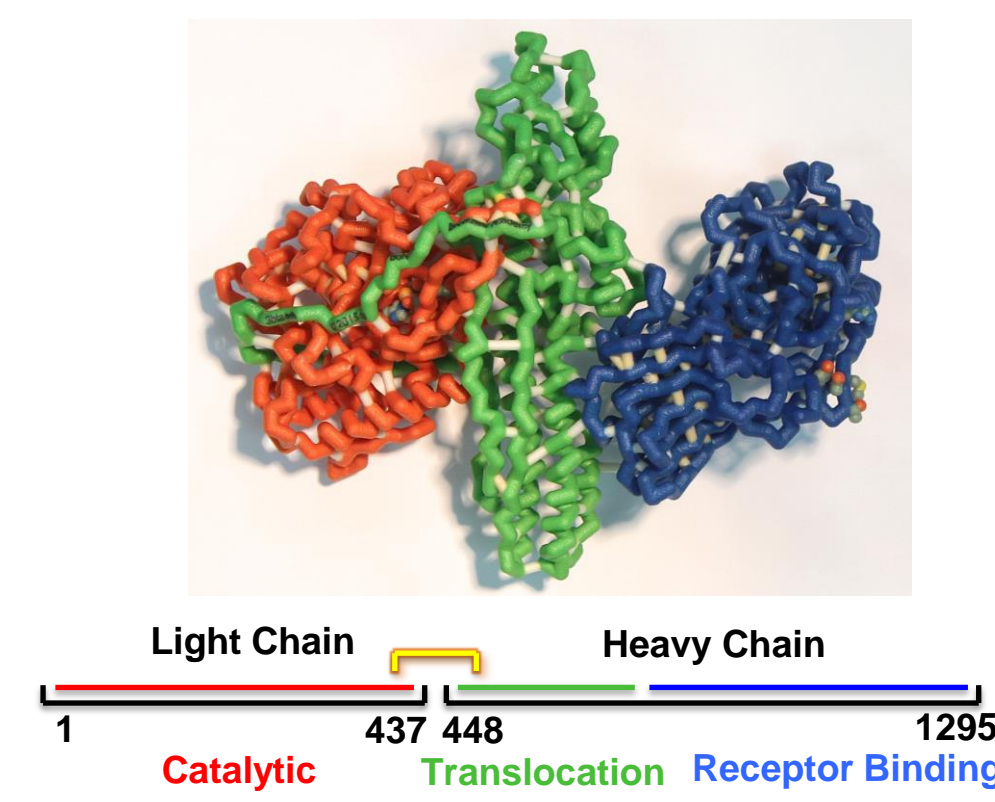
Elizabeth Cinquegrani, Shalini Gundamraj, Harry Kalsi, Leah Wang, Lena Ding, Alex Dortzbach, Suneri Kothari, Srimayi Mylavarapu, Juliana Vollmer

Advisor: Robbyn Tuinstra, Ph.D., Mentors: Joseph T. Barbieri, Ph.D. and Madison Zuverink, Medical College of Wisconsin, Department of Microbiology and Molecular Genetics



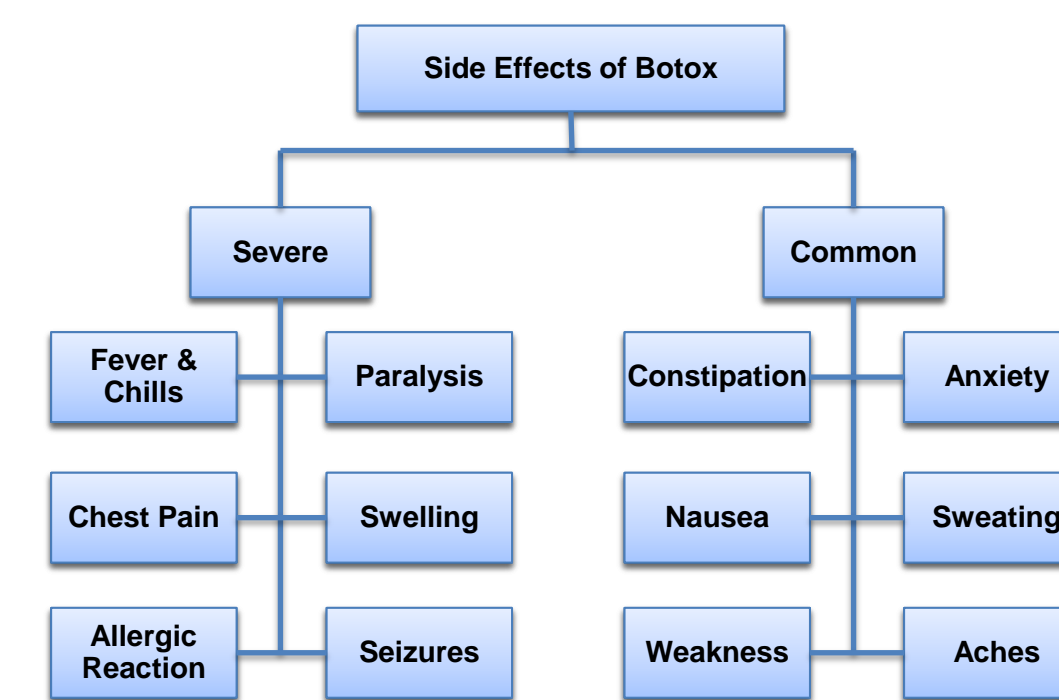
## 1. INTRODUCTION

Botulinum neurotoxin type A (BoNT/A) is a potent neurotoxin, causing muscle paralysis in the host by blocking the release of the neurotransmitter, acetylcholine, from motor neurons associated with skeletal muscle. Despite this toxicity, BoNT/A is used pharmaceutically as a treatment for numerous neurological diseases, such as migraines and dystonias, and as an anti-wrinkle agent in cosmetic surgery. BoNT/A is one of seven serotypes of botulinum (A-G), which, along with tetanus toxin, are produced by several species of Clostridium. While the use of BoNT as a therapeutic agent is promising for treating various conditions, adverse side effects have prompted researchers to investigate the use of alternative serotypes, such as BoNT/A2, for clinical treatment. The Brookfield Academy SMART (Students Modeling A Research Topic) Team students modeled BoNT/A by using 3D printing technology, highlighting amino acid residues associated with protease activity in the catalytic domain, and observing GT1b and SV2 interactions within the receptor binding domain. This model is meant to help understand and communicate structure/function relationships of BoNT/A and promote potentially therapeutic uses of the toxin.



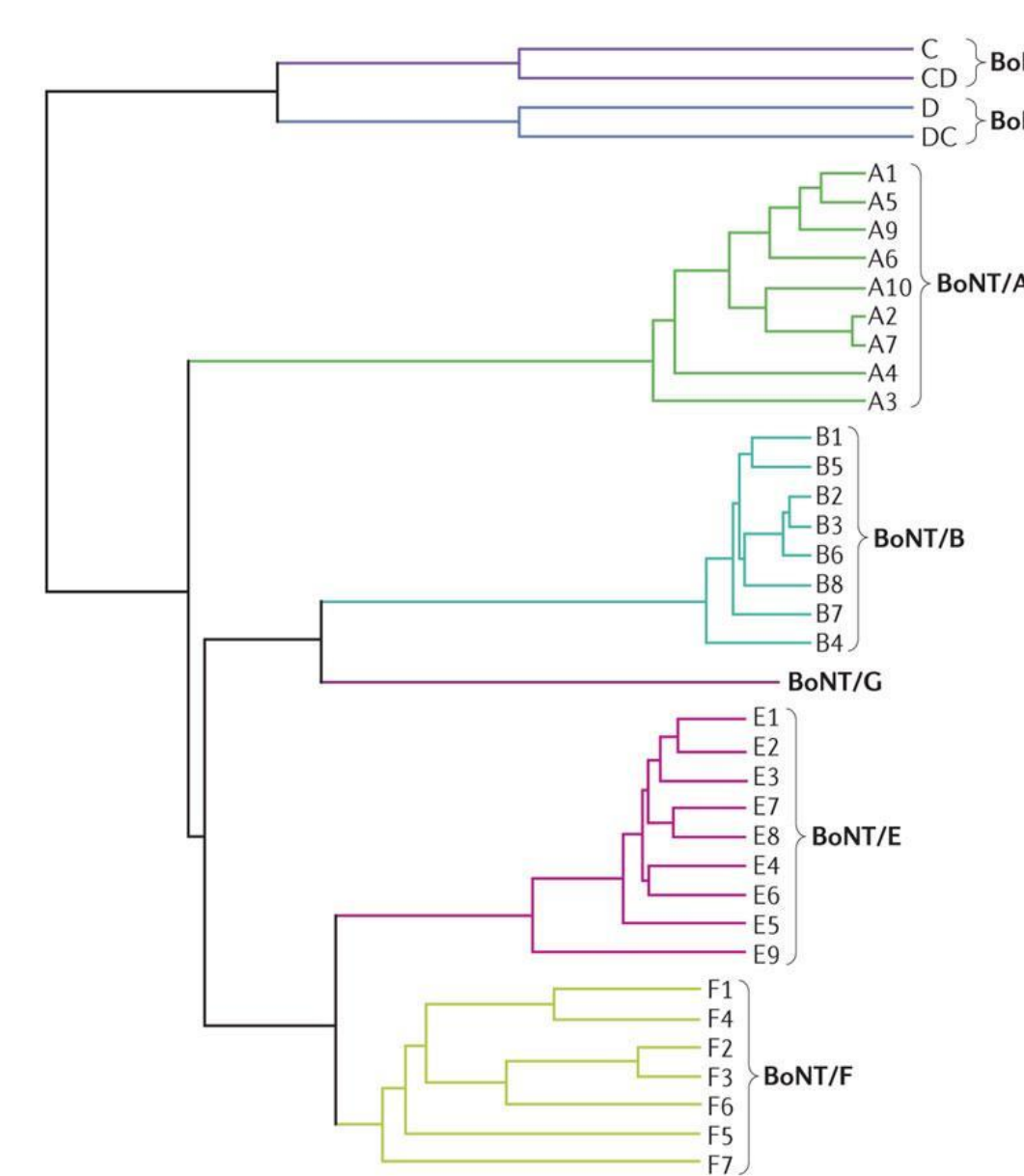
**Figure 1. Structure of the BoNT holotoxin.** All clostridial neurotoxins, such as BoNT/A, consist of two protein chains, the heavy chain (100,000 Da) and the light chain (50,000 Da), connected by a disulfide bond. The heavy chain contains the translocation domain (green) and the receptor binding domain (blue), while the light chain is the catalytic domain (red). Figure generated using Jmol and PDB #3BTA.

## 3. CLINICAL APPLICATIONS OF BoNT AND ADVERSE EFFECTS OF BoNT THERAPIES

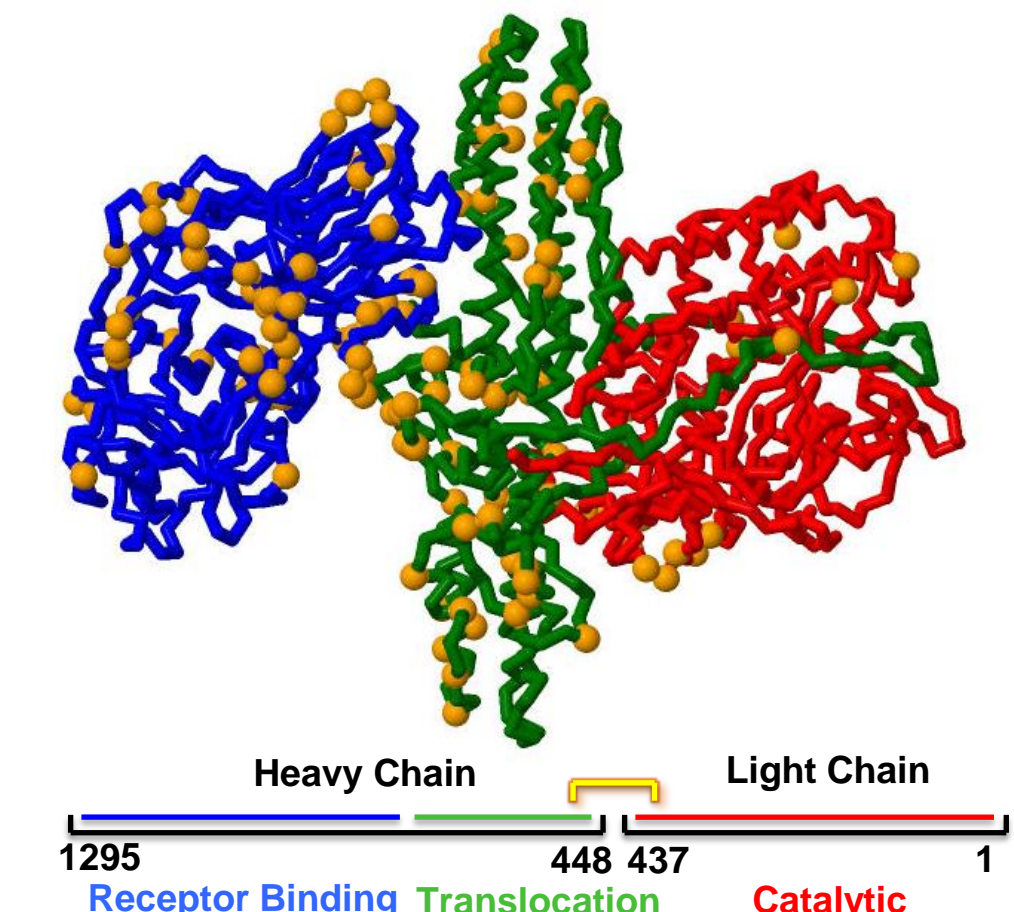
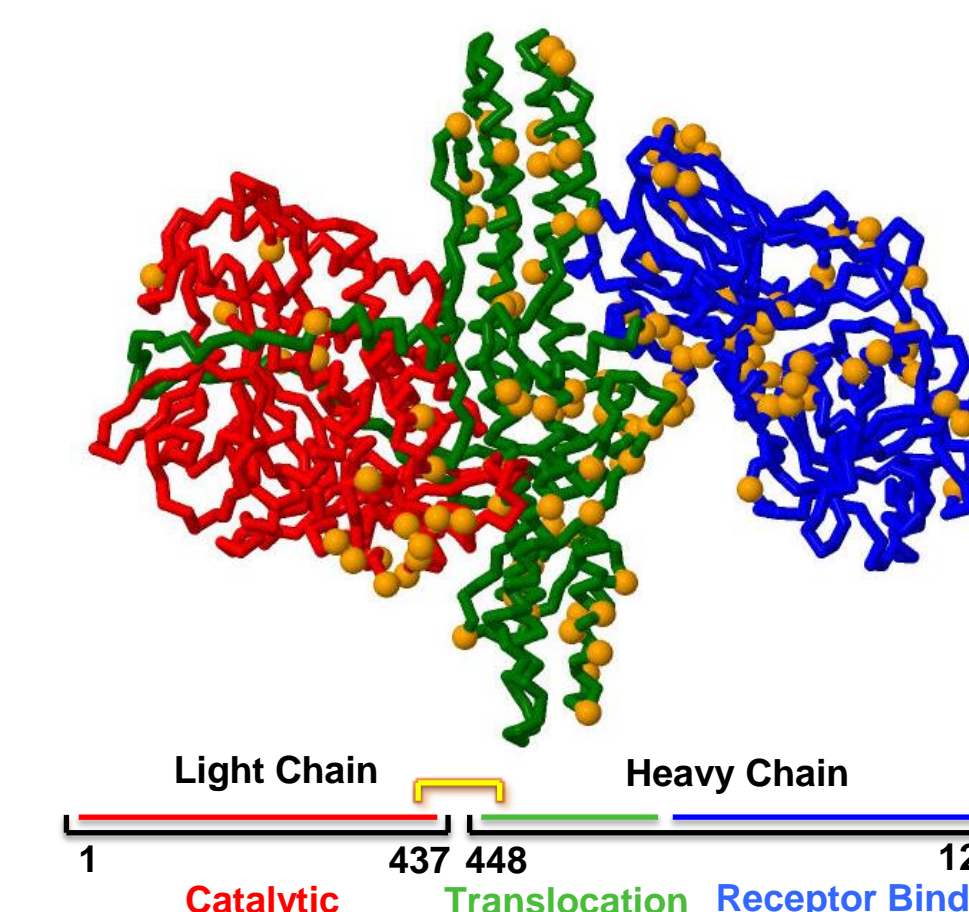


The two main commercial types of BoNT used are serotype A and serotype B. BoNT/A can also be used to treat a range of conditions including upper motor neuron syndrome, profuse sweating, involuntary closing of the eyelid, poor eye muscle control and involuntary grinding of the teeth. For cosmetic applications, BoNT/A can be injected to paralyze facial muscles in order to prevent wrinkles. Some patients have experienced temporary muscle weakness in the areas treated, and some developed flu-like symptoms. In April 2009, the FDA announced that they were releasing a warning on products involving the use of the BoNT/A. The warning stated that there is a risk of BoNT/A spreading away from injection site, causing adverse side effects such as: difficulty with speaking, swallowing, and breathing, muscle weakness, loss of bladder control, drooping eyelids, and blurred vision.

## 4. BoNT/A2 AS A THERAPEUTIC ALTERNATIVE TO BoNT/A1



**Figure 6. There are seven serotypes of BoNT and various subtypes; BoNT/A has 7 subtypes.** Currently, BoNT/A1 is the only subtype approved for clinical use. BoNT/A2 is being evaluated in clinical trials and preliminary studies suggest that BoNT/A2 displays a greater efficacy with fewer adverse side effects seen with BoNT/A1. In addition, BoNT/A2 may be more effective for treatment of Parkinson's disease, epilepsy, and post stroke spasticity. Figure from Rossetto *et al.*

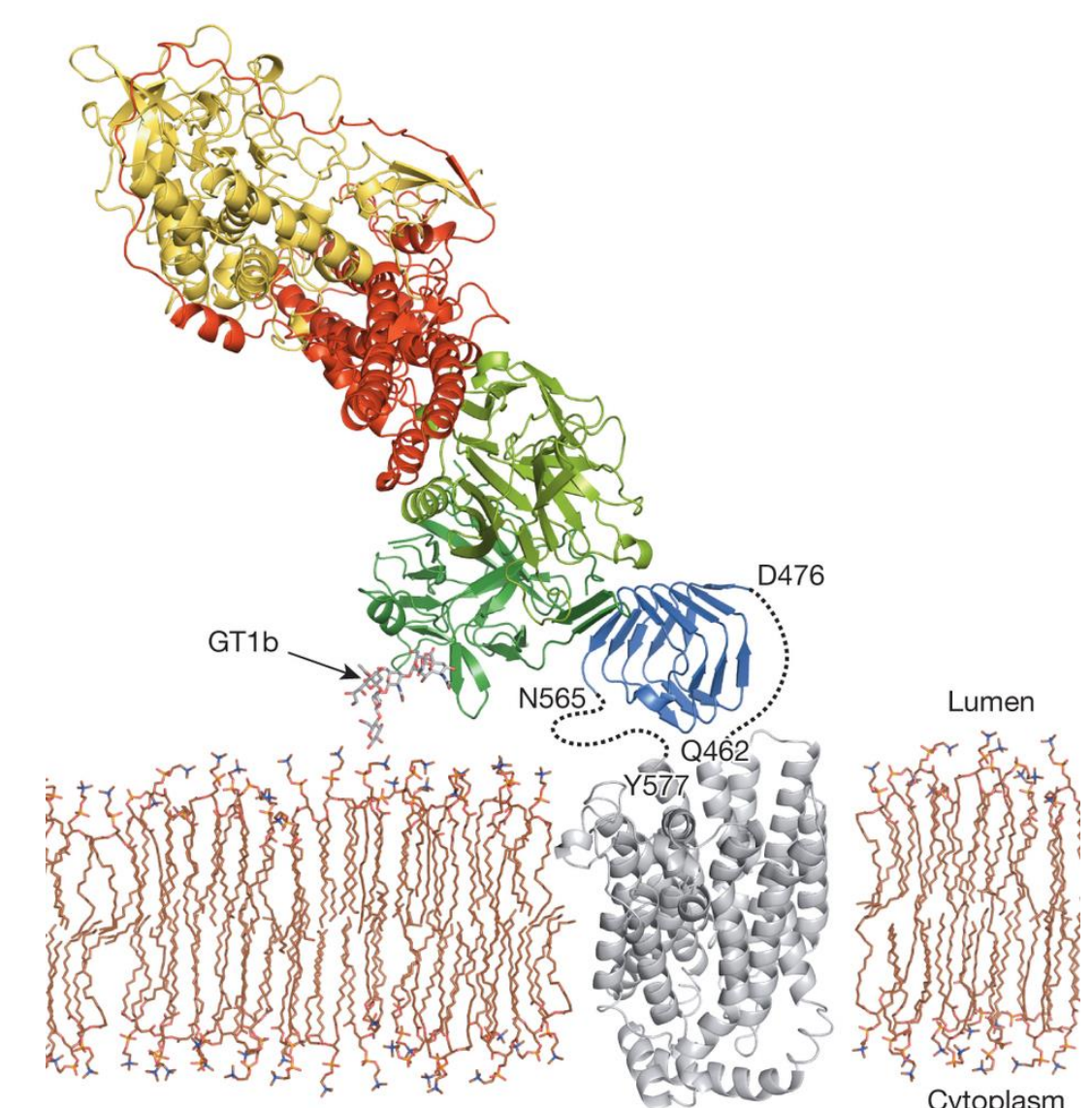
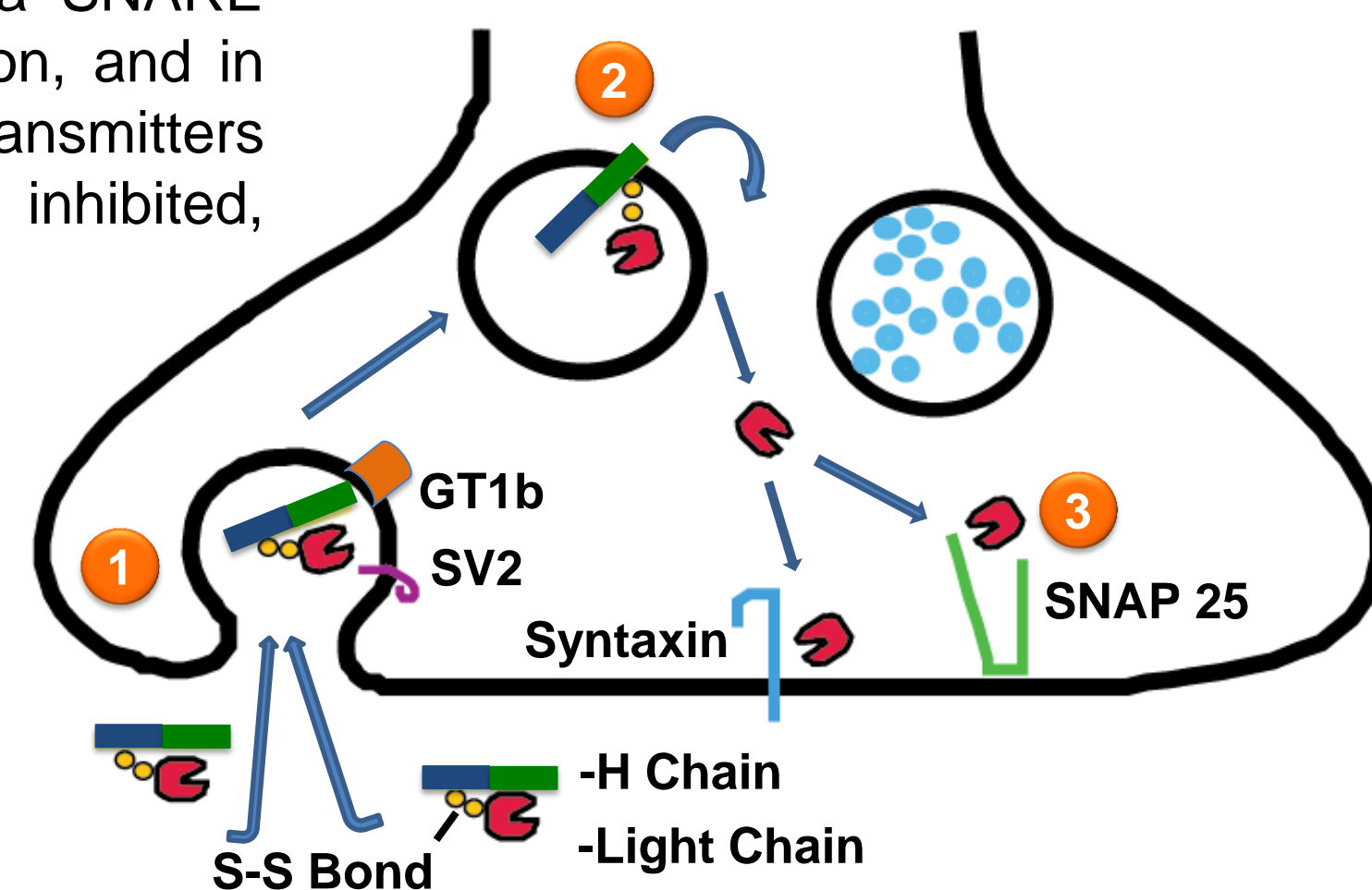


**Figure 7. While BoNT/A2 differs from BoNT/A1 in only 10% of its amino acid sequence, efficacy of BoNT/A2 is approximately 1.5x that of BoNT/A1, with reduced diffusion from the site of injection.** alpha-Carbon of residues that differ between A1 and A2 are indicated in orange spacefill. There is little difference in function of the catalytic and translocation domains, suggesting the difference in efficacy between the two serotypes may be attributed to difference in the receptor binding domain. Figure generated using Jmol and PDB #3BTA.

## 2. MECHANISM OF BoNT TOXICITY IS A MULTI-STEP PROCESS

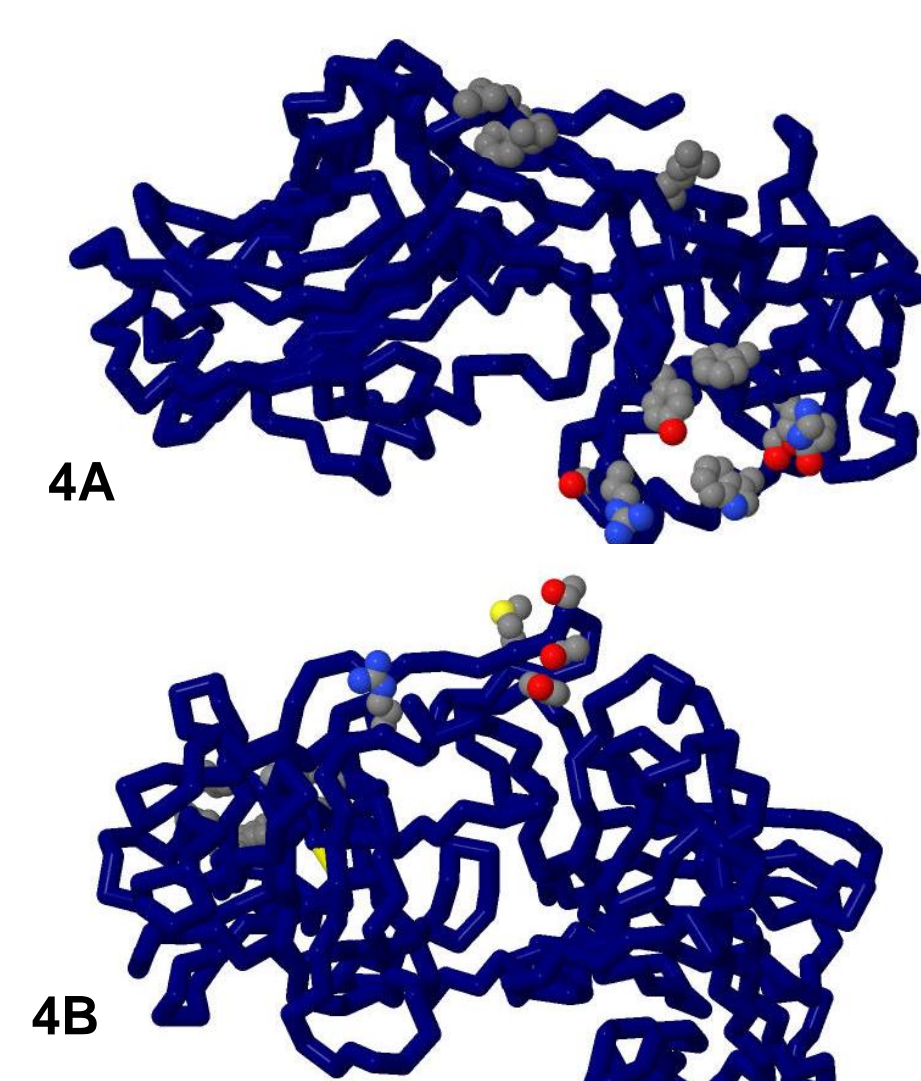
BoNT lethality is due to the toxin's ability to cleave and disable a SNARE protein. This SNARE protein is responsible for synaptic vesicle fusion, and in processes such as muscle contraction, fusion allows essential neurotransmitters such as acetylcholine (ACh) to be released. Since ACh release is inhibited, resulting in flaccid muscle paralysis.

**1 Binding:** First, BoNT/A binds to presynaptic nerve endings through interactions of the receptor-binding domain with the ganglioside, GT1b, and the protein co-receptor, SV2. SV2 is a synaptic vesicle protein that becomes exposed to the neuron cell surface as vesicles fuse with plasma membrane, releasing neurotransmitter into the synapse. BoNT/A then enters the neuron by receptor-mediated endocytosis.

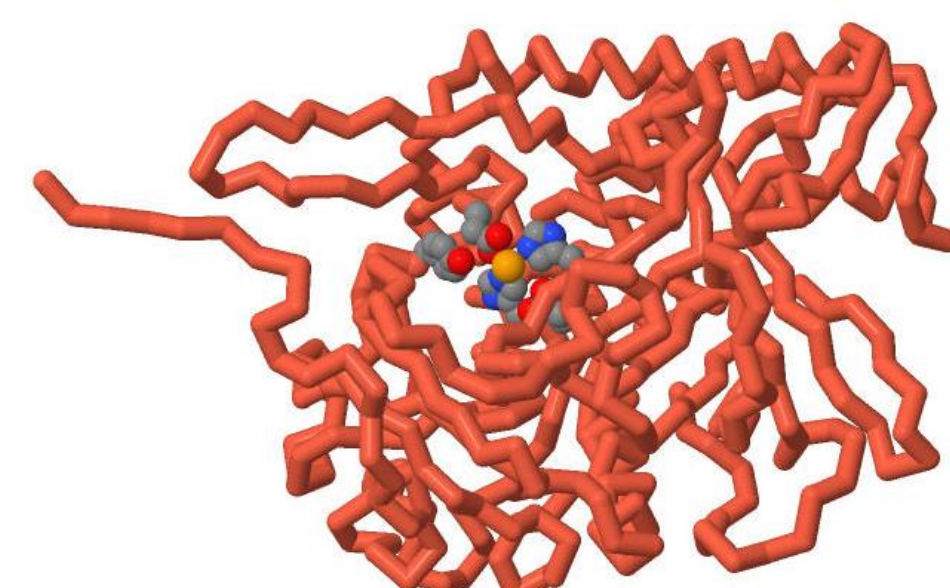


**Figure 3 (left). The receptor binding domain of BoNTs is responsible for entry of BoNT into the motor neuron.** BoNTs bind to two co-receptors on the surface of the motor neuron, GT1b and SV2, through interactions with distinct binding regions of the RBD. Figure from Benoit *et al.*

**Figure 4 (right). The receptor binding domain of BoNT/A contains two binding pockets for interacting with GT1b (panel A), and SV2 (panel B).** Residues involved in interactions with co-receptors are displayed in CPK coloring. Figures generated using Jmol and PDB #3BTA.



**2 Translocation:** Following endocytosis, the synaptic vesicle acidifies, allowing for neurotransmitter uptake, which triggers the translocation domain to insert and transport the catalytic domain into the cytosol. Following reduction of the disulfide bridge connecting the two chains, the translocation domain is retained in the vesicle, while the catalytic domain travels to SNARE complex.



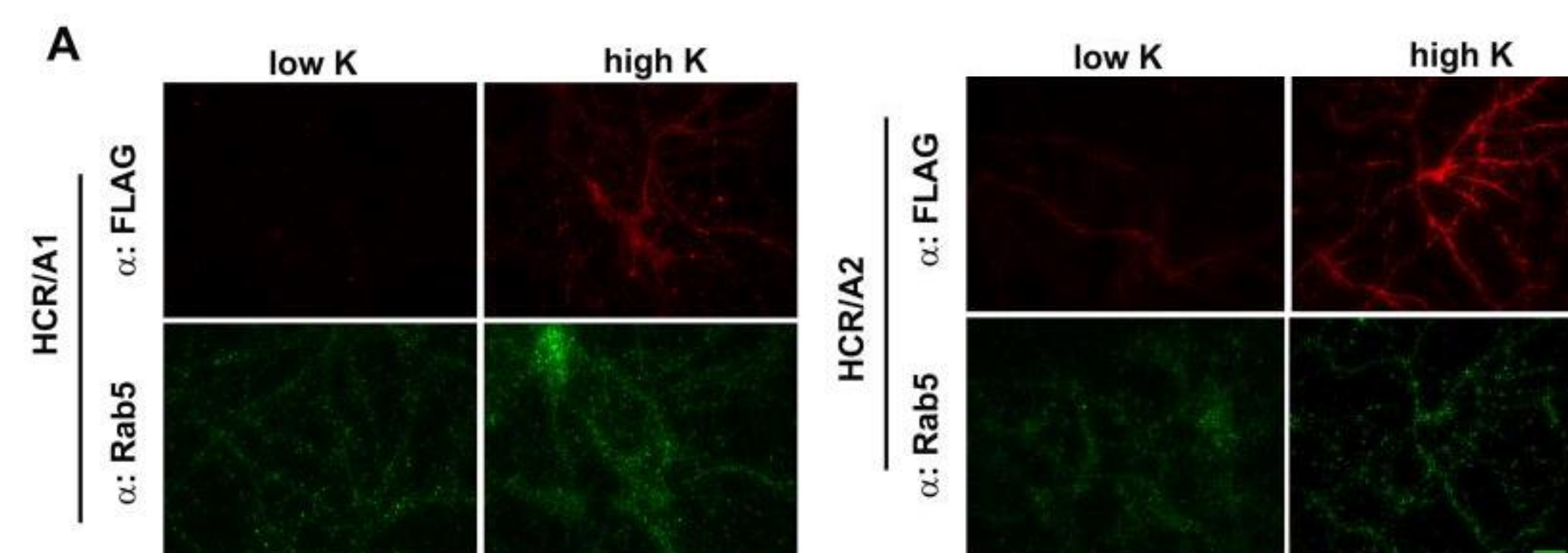
**Figure 5. Catalytic domain of BoNT/A.** The Zn<sup>2+</sup> cofactor (orange) and conserved active site residues Glu-261, His-226, Glu-223, Tyr-365 and His-222 (cpk coloring) are highlighted. Figure generated using Jmol and PDB #3BTA.

**3 Cleavage of SNARE proteins:** The catalytic domain is a Zn-dependent protease that cleaves SNAP-25, which is one of three major proteins present in the SNARE complex. The SNARE protein complex is required for the fusion of synaptic vesicles with the neuronal membrane. Cleavage of SNAP-25 inhibits fusion of synaptic vesicles to the plasma membrane to inhibit acetylcholine release and muscle contraction, leading to flaccid paralysis.

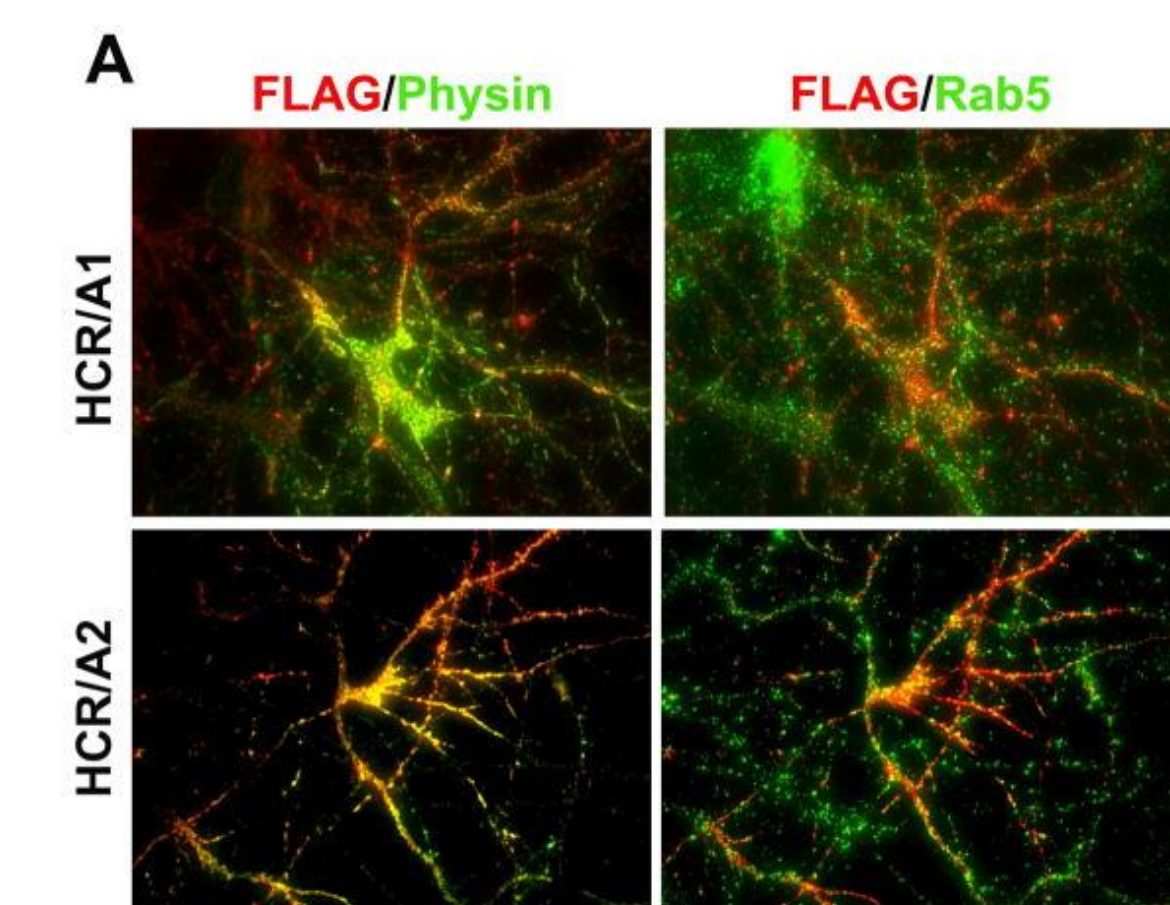
## 5. BoNT/A2 BINDS AND ENTERS MOTOR NEURONS MORE EFFECTIVELY THAN BoNT/A1

Heavy chain receptor binding domains (HCR) of BoNT/A1 and BoNT/A2, containing a FLAG tag, were recombinantly expressed in bacterial cells and incubated with rat cortical neurons. Entry was monitored by fluorescent microscopy using fluorescent-antibodies directed against the FLAG marker. Fluorescence intensity was normalized to cell markers for endosomes (Rab5) or synaptic vesicles (synaptophysin).

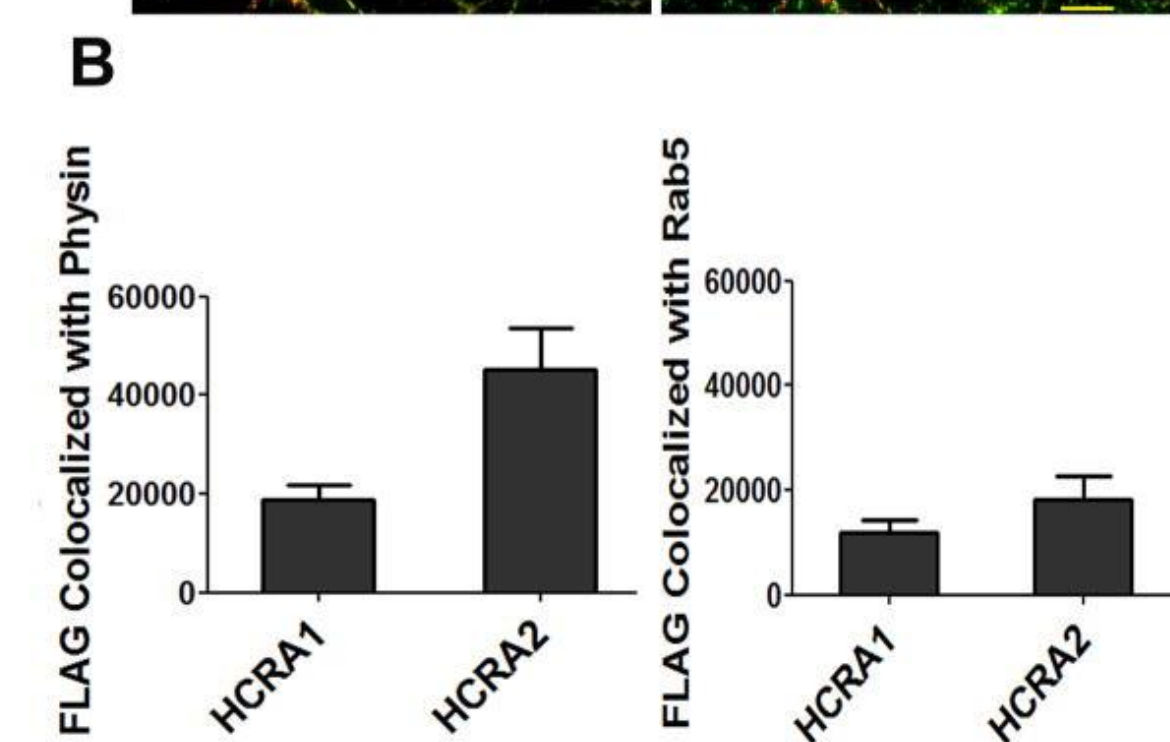
Both HCR-A1 and A2 require the presence of high potassium concentrations (Figure 8), suggesting active neuronal transmission is required for entry through synaptic vesicles (Figure 9). However, A2 appears to enter neurons to a greater extent and rate than A1.



**Figure 8. HCR/A1 and HCR/A2 entry to neurons.** HCR/A1 or HCR/A2 were incubated with rat cortical neurons in low or high potassium buffer for 5 min at 37 ° C. Bound HCR (anti-FLAG) and marker proteins (anti-Rab) were detected by immunofluorescence. (A) A representative field is shown. Figure from Pier *et al.*



**Figure 9. HCR/A1 and HCR/A2 co-localization with synaptophysin 1 (Phy5in) and Rab5 (Rab5).** HCR/A1 and HCR/A2 were incubated with rat cortical neurons in high potassium buffer for 5 minutes at 37 ° C. (A) HCR co-localization (FLAG epitope) with Synaptophysin 1 and Rab 5 (B) Co-localization of HCR/A1 A2 (red) with Rab 5 and Synaptophysin 1 (green) was plotted in fluorescence intensities units (arbitrary). Figure from Pier *et al.*



## 6. CONCLUSION

Purified HCR-A1 and HCR-A2 analyzed using fluorescent microscopy indicated that BoNT/A2 had faster entry into neuronal cells and increased intoxication in comparison with BoNT/A1. However, BoNT/A2's faster entry was not associated with tighter ganglioside binding. BoNT/A1 and BoNT/A2 heavy chain regions were added to rat cortical neurons in varying concentrations of potassium. Marker proteins Rab5 and FLAG were also added to the cortical neurons to depict concentrations of heavy chain regions throughout the neurons. The results indicated a greater intensity of BoNT/A2 in neuronal cells, in the same amount of time, than BoNT/A1. Therefore, BoNT/A2 enters neurons fast than BoNT/A1.

## REFERENCES AND ACKNOWLEDGEMENTS

Borden Lacy, Tepp, Cohen, DasGupta, and Stevens. (1998) Crystal structure of botulinum neurotoxin type A and implications for toxicity. *Nature*. 5:898-902.  
 Benoit *et al.* (2014) Structural basis for recognition of synaptic vesicle protein 2C by botulinum neurotoxin A. *Nature*. 108:108-111.  
 Rossetto, Pirazzini, and Montecucco. (2014) Botulinum neurotoxins: genetic, structural, and mechanistic insights. *Nature Reviews*. 12:535-549.  
 Pier, Chen, Tepp, Lin, Janda, Barbieri, Pellett, Johnson. (2011) Botulinum neurotoxin subtype A2 enters neuronal cells faster than subtype A1. *FEBS Letters*. 585:199-206.