Capture Assay for Botulinum Neurotoxin Type A Utilizing the Neuronal Receptor Protein SV2c

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ABSTRACT

Recent biochemical and molecular genetic studies have established synaptic vesicle glycoprotein 2c (SV2c) as the protein receptor for Botulinum Neurotoxin Type A (BoNT/A). The luminal domain loop of SV2c, between transmembrane domains 7 and 8, has been shown to be the location of BoNT/A binding. Utilizing this information, we have shown through GST pull-down experiments that we can detect binding of BoNT/A to SV2c using an immunoassay. This can be done with either the rat or human forms of the SV2c protein.

Upon specific binding of BoNT/A to its receptor protein, we can introduce our FRET peptide SNAPtide® and detect enzymatic cleavage. We describe the reaction conditions and the detection limits using both rat and human forms of the SV2c binding region as well as multiple FRET substrates to determine the optimal conditions for this detection assay.

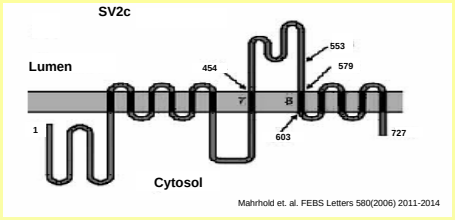
There are three steps in the interruption of synaptic transmission by botulinum toxin. The first is binding to neuronal cells; the second is translocation of the enzymatic light chain out of the endosome; and finally cleavage of synaptosomal proteins to inhibit neurotransmitter release. With this assay we can monitor two of the three steps of toxin activity, binding and cleavage. In the future, we hope to establish this as a new functional assay for the detection and activity of Botulinum Neurotoxin Type A and demonstrate a direct correlation to the gold standard, the mouse bioassay.

INTRODUCTION

Multiple assays have been developed to detect botulinum toxin based solely on antigenic properties and/or enzymatic activity. Now with the recent identification of SV2c as the protein receptor for BoNT/A[1,2] new detection methods can be attempted that combine multiple steps of the disease process of botulinum toxin. We can utilize the SV2c receptor domain to obtain specific binding of BoNT/A followed by exposure to a FRET peptide to detect this binding through specific endopeptidase activity.

The specific binding domain of SV2c for BoNT/A has been shown to be the luminal domain loop between transmembrane domains 7 and 8.[1,2] The amino acid sequence of this loop for both rat and human is shown in Figure 1. There are several amino acid differences between the two represented by black and green letters. Similar binding affinity of BoNT/A to each has been observed. We have purified both as GST fusions and utilized them in capture assays. Three strategies have been tested including a direct GST pull down assay of BoNT/A using glutathione sepharose resin bound with GST-SV2c and assays using GST-SV2c immobilized to anti-GST antibodies that are covalently attached to either magnetic Dynabeads or Protein G coated resin. See Figure 2 for an illustration of the strategies.

Our goal for this research is to establish an assay which demonstrates binding and catalytic activity and that can compete with the mouse bioassay.



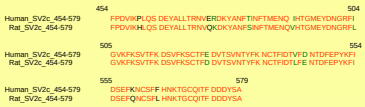


Figure 1: SV2C structure and alignment

The binding domain for Botulinum Neurotoxin Type A has been shown to be in the luminal loop, amino acids 454-579. In the alignment between Human and Rat SV2c the green amino acids are conserved changes while the black represent amino acid differences in the proteins.

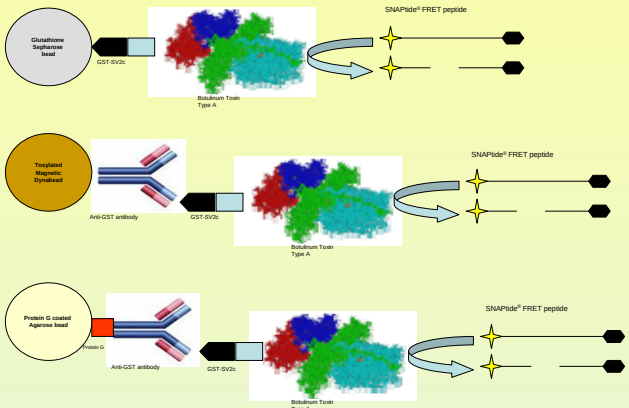


Figure 2: Strategies for capturing BTA utilizing GST-SV2c

MATERIALS

SNAPtide® substrates (Product # 520), Botulinum Neurotoxin Type A (Product #130A) and Botulinum Neurotoxin Type B (Product #136) are products of List Biological Laboratories. Plasmid encoding binding fragment of human and rat SV2c was provided by A. Rummel and S. Mahrhold. Glutathione Sepharose resin is from GEHealthcare. The Dynabeads® MyOne™ Tosylactivated are supplied by Invitrogen. Immobilized Protein G resin supplied by Pierce.

METHODS and RESULTS

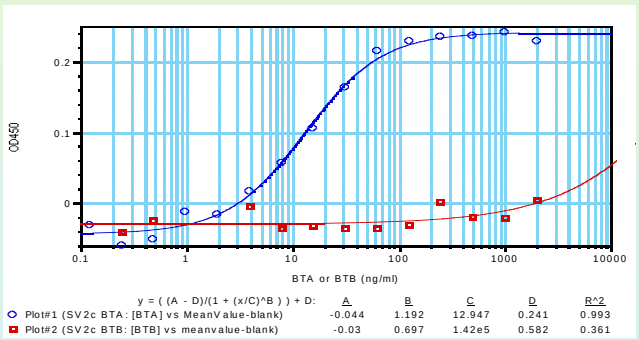


Figure 3: "Sandwich" ELISA assay showing specificity of BTA for binding to GST-SV2c.

Microtiter plates (96 wells) were coated with 2µg of GST human SV2c in 1x carbonate buffer overnight at 4ºC. Plates were blocked with 1% BSA. Botulinum Neurotoxin Type A or B was applied to the wells and incubated for 1 hr at 37ºC. Toxin was detected using one of the following pair of antibodies: horse anti BoNT/A / anti horse HRP or rabbit anti BoNT/B / anti rabbit HRP. The assay was developed using One Step Turbo ELISA TMB from Pierce. These data indicate that the binding to SV2c is specific for Type A neurotoxin. The 50% binding point occurs at approximately 13ng/ml BoNT/A.

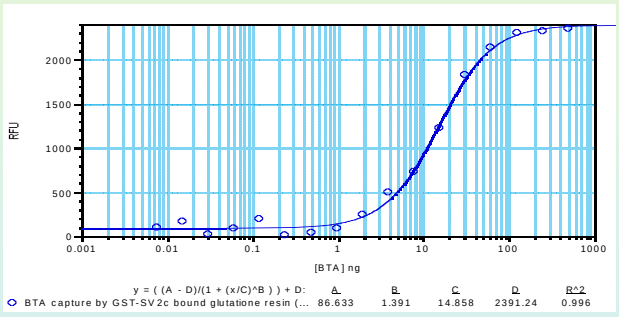
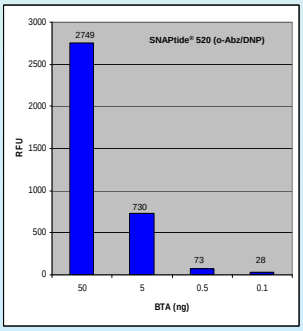


Figure 4: GST Pull Down Assay

Glutathione sepharose resin (20µl) was applied to each well of an Acrowell 96 well filter plate, Pall #5020. GST human SV2c (50µg) was added to each well and allowed to bind to the resin overnight at 4ºC. After SV2c binding, the resin was washed and dilutions of BoNT/A was added to the plate. Toxin was incubated overnight at room temperature. Unbound toxin was washed away and SNAPtide® reaction buffer (50 mM HEPES, pH 8, 5 mM DTT, 1 mM $ZnCl_2$, 0.2% Tween 20 and 20µM SNAPtide® 520) was added to each well. The plate was incubated at 37ºC for thirty minutes and then overnight at room temperature. The content of the filter plate were centrifuged into a black 96 well receiving plate and read using SPECTRAmax GEMINI XS fluorescence microplate reader (Molecular Devices). The excitation and emission wavelengths were set to 321 nm and 418 nm, respectively, for the oAbz/DNP substrate. The 50% binding point is ~15ng BTA using the GST pull down assay with SNAPtide® cleavage for detection.



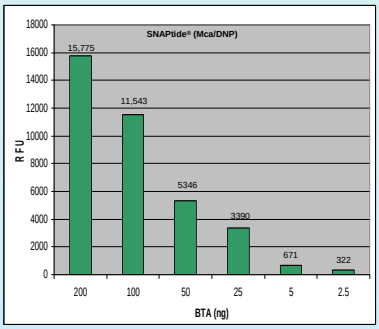


Figure 5: Dynabead Experiment

My One™ tosylactivated magnetic Dynabeads (5mg) were mixed with 200µg of anti GST antibody (BioDesign #K03540G) and covalently attached following the product instructions. After covalent attachment the beads were mixed with 400µg of GST human SV2c and incubated overnight at 4ºC with mixing. The Dynabeads were washed and divided into 12 tubes. Varying concentrations of Botulinum Neurotoxin Type A were added to each tube. Toxin binding was held overnight at room temperature with mixing. Unbound toxin was washed away and SNAPtide® reaction buffer (50 mM HEPES, pH 8, 5 mM DTT, 1 mM $ZnCl_2$, 0.2% Tween 20 and 20µM SNAPtide® 520 or 10µM SNAPtide® Mca/DNP) was added to each tube. The reactions were mixed at 37ºC for thirty minutes and then overnight at room temperature. The reaction mixtures were decanted from the tubes and placed into a black 96 well plate. The beads were washed several times with 50mM HEPES, pH 8 buffer and added to the wells. The plate was read using a SPECTRAmax GEMINI XS fluorescence microplate reader (Molecular Devices). The excitation and emission wavelengths were set to 321 nm and 418 nm, respectively, for the oAbz/DNP substrate and 325 and 398 for the Mca/DNP substrate. These data, with the background subtracted, demonstrate a sensitivity of ~0.5ng of BoNT/A with the oAbz/DNP substrate and at least 2.5ng with the Mca/DNP.

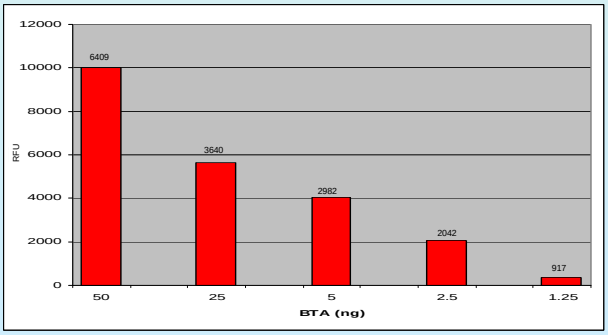


Figure 6: Immobilized Protein G Experiment

Immobilized Protein G resin (0.4ml) was washed and crosslinked to 100µg of anti GST antibody (BioDesign #K03540G) using the the Seize X Protein G Immunoprecipitation kit from Pierce. After crosslinking, the resin was washed using the Gentle Ag/Ab binding and elution buffers from Pierce (#2102). GST human SV2c (200 µg) was added to the protein G resin and incubated overnight at 4ºC. The bound resin was washed and divided into 8 spin columns. Dilutions of BoNT/A were added to each column and incubated for 4 hours at room temperature. Unbound toxin was washed away and SNAPtide® reaction buffer (50 mM HEPES, pH 8, 5 mM DTT, 1 mM $ZnCl_2$, 0.2% Tween 20 and 20µM SNAPtide® 520) was added to each column. The reactions were mixed at 37ºC for thirty minutes and then overnight at room temperature. The reaction mixtures were centrifuged from the columns and placed into a black 96 well plate. The resin was washed several times with 50mM HEPES, pH 8 buffer and added to the wells. The plate was read using a SPECTRAmax GEMINI XS fluorescence microplate reader (Molecular Devices). The excitation and emission wavelengths were set to 321 nm and 418 nm, respectively, for the oAbz/DNP substrate. The data from points 0.5, 0.25 and 0 toxin gave high backgrounds and are not shown here. We are currently investigating the reason for the high background.

REFERENCES:

1) Mahrold et al. FEBS Letters. 2006 April 3, 580(8):2011-4.

2) Dong et al. Science. 2006 April 28; 312(5773):592-6