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Biophysical and Structural Studies of CNTNAP2

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Biophysical and Structural Studies of CNTNAP2

by

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Biophysical and Structural Studies of CNTNAP2

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At synapses, cell adhesion molecules provide connection between the presynaptic and postsynaptic membranes through homophilic and heterophilic interactions promoting synapse development, function, and plasticity. The synaptic adhesion molecules contactin 2 (CNTN2), and contactin associated protein-like 2 (CNTNAP2) have an emerging role at synaptic interaction networks. CNTNAP2 is a member of the neurexin superfamily of synaptic cell adhesion molecules; it is localized at the juxtaparanodes of myelinated axons at the Nodes of Ranvier where it mediates interactions between neurons and ensheathing glial cells and recruits potassium channels to the juxtaparanodes. CNTNAP2 is also found at synapses. Association, linkage, gene expression and imaging data highlight the role of CNTNAP2 in several neurodevelopment and neuropsychiatric disorders, including developmental language impairment, Morvan's syndrome, and limbic encephalitis. Cell biology and immunological studies strongly indicate that CNTNAP2 binds to CNTN2. CNTNAP2 and CNTN2 form a macromolecular complex spanning axo-glial and synaptic contacts to maintain neuron-neuron and axo-glial interactions. Currently, it is not known

how CNTNAP2 and CNTN2 interact, nor whether CNTNAP2 binds additional cell surface molecules. High-resolution information on the three dimensional atomic structure of CNTNAP2 is also not known.

The goal during of this master's thesis project was to purify the extracellular domain of CNTNAP2 and several of its fragments, assess whether the extracellular domains of CNTNAP2 and CNTN2 interact directly using solid phase binding assays and obtain crystals of CNTNAP2 fragments. We are the first who did in vitro studies for characterize CNTNAP2 and their partner CNTN2. We found that CNTNAP2 is an endogenous ligand for CNTN2 and associates with nanomolar affinity. The binding of the extracellular domains of CNTNAP2 and CNTN2 are promoted by the presence of Ca^{2+}. These data provide new platform for structural-functional study for CNTNAP2/CNTN2 complex. In the future, we will continue this project to map critical interactions sites on these proteins.

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LIST OF ABBREVIATIONS

UTMB	University of Texas Medical Branch
CNTNAP2	Contactin-associated Protein-like 2
CNTN2	Contactin 2
CNTN1	Contactin 1
RT	Room Temperature
Ig	immunoglobulin like domain
PCT	Pre Crystallization Test

CHAPTER 1

Introduction

According to the current statistics, it is estimated that there are hundreds of millions of people in the worldwide who are affected by human neuropsychological and neurodegenerative disorders (Thakur et al., 2016). Neuropsychological disorders are diseases of the nerves, spinal cord and brain. Structural or chemical abnormalities in the spinal cord, brain or peripheral nerves can cause more than 600 diseases of the nervous system, such as epilepsy, Parkinson's disease, developmental language impairment, autism spectrum disorders, Morvan's syndrome, brain tumors, autoimmune limbic encephalitis etc. (McEwen et al., 2016; Pinatel et al., 2015; Pan et al., 2015; Berg et al., 2014).

Clinical studies have shown that neuropsychological and neurodegenerative diseases are related to each other on a sub-cellular level (Berent et al., 2012). Understanding the connections underlying these diseases is crucial, as therapeutic advances can be developed to target related diseases. Neurodegenerative disorder is an umbrella term, which describes the progressive loss of structure or function of neurons. Despite extensive research (Betlrand et al., 2014), the genetic basis for many neuropsychological and neurodegenerative disorders are poorly understood. Genetic mutations have been associated with many neurodegenerative and neuropsychological diseases; these mutations have often been found in genes that are seemingly unrelated. The respective roles of many of the mutated genes in the pathogenesis of disease are still unclear in many cases (Arking et al., 2008; Chi et al, 2016; Erickson et al., 2016). The goal of this work is to purify and investigate two different proteins that are implicated in neuropsychiatric disorders. As mentioned, CNTNAP2 and CNTN2 play a role in the formation of neuron-neuron and neuron-glia contacts.

The Contactin-associated protein-like 2 (CNTNAP2) is a transmembrane cell adhesion protein (Poliak et al., 1999). It belongs to the neurexin superfamily. The extracellular domain of CNTNAP2 is made up of 8 domains, including 4 Laminin G domains, 2 Epidermal growth factor-like repeats. It also includes a Discoidin/F58C domain and a Fibrinogen-like domain unlike other members of the neurexin family (Fig. 1). CNTNAP2 mutations have been associated with a number of medical conditions including autistic spectrum disorders, Morvan's syndrome, autoimmune limbic encephalitis and language impairment (Alarkon et al., 2008; Pinatel et al., 2015; Penagarikano et al., 2011; Vernes et al., 2011; Irani et al., 2010) (Fig. 2).

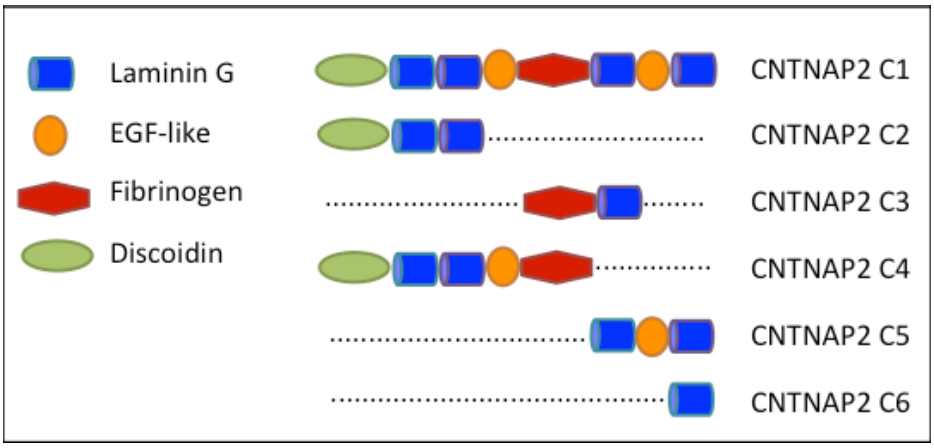

Figure 1. CNTNAP2 encompassing sequential deletion: C1 - full length CNTNAP2; C2 construct lacks Laminin-G3, G4, EGF-like 1 and Fibrinogen like domains, C3 - Laminin-G1, G2, G4, EGF-like 1, 2 and Discoidin-like domains, C4 - Laminin-G3, G4 and EGF-like domains, C5 - Fibrinogen like, EGF-like 2, Laminin-G1, G2 and Discoidin like domains, and C6 consists just Laminin-G6 domain.

Although CNTNAP2 and CNTN2 are related to fundamental biological processes that are involved in the pathology of neurological diseases and disorders (Traka et al., 2003) it is not clear which processes are driven by these proteins. CNTN2, also known as a transient axonal glycoprotein-1 (TAG1) is made up of 6 Ig domains and four Fibronectin like domains (Furley et al., 1990). CNTN2 is expressed by both neurons and myelinated schwann/oligodendrocytes (Traka et al., 2002; Chatzopoulou et al., 2008; Law et al.,

2008). At the nodes of Ranvier, CNTN2 is localized at the juxtaparanodes (Traka et al., 2003; Savvakiet al., 2010; Tzimourakas et al., 2007). Cell-based, and immunological studies show that CNTNAP2 is present at axo-glial and neuron-neuron contacts (Poliak et al., 2003; Betancur et al., 2009).

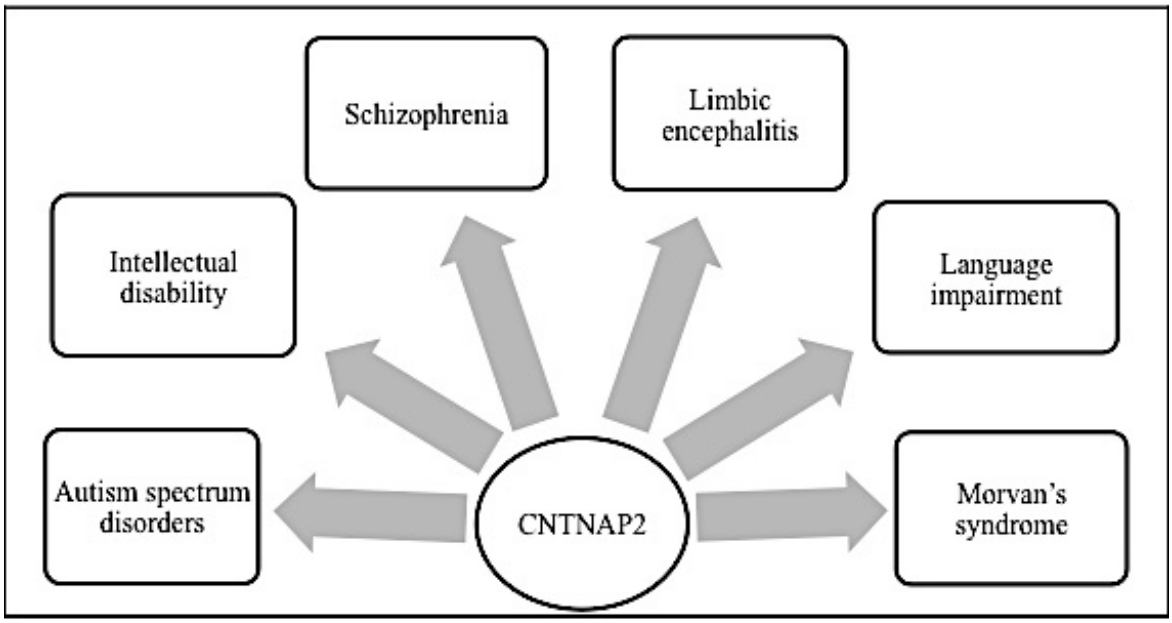

Figure 2. Schematic model describing the role of CNTNAP2 in neuropsychological and neurodegenerative disorders. CNTNAP2 mutations have been associated with a number of medical conditions including autistic spectrum disorders, intellectual disability, schizophrenia, Morvan's syndrome, autoimmune limbic encephalitis and language impairment.

It has been proposed at juxtaparanodes that CNTNAP2 and CNTN2 interact with each other in a cis-complex (side-by-side) on the axonal membrane and recruit another CNTN2 molecule on the opposing glial membrane to form a tripartite trans-synaptic bridge (Poliak et al., 2003; Fig. 3A). It might be that such complex between CNTNAP2 and CNTN2 exists at synapses as well localized in the synaptic clefts connecting presynaptic and postsynaptic membranes, promoting synapse development, function, and plasticity (Melom et al., 2011; Betancur et al., 2009; Toro et al., 2010) (Fig. 3B). **Knowledge gap:** It is not known how CNTNAP2 and CNTN2 interact with each other, and whether CNTNAP2 binds additional cell surface molecules. The three dimensional atomic structure of

CNTNAP2 has also not been determined. Because of the importance of CNTNAP2 in neuropsychological and neurodegenerative diseases **the overall objective** of this project is to 1) identify the mechanism of interaction between CNTNAP2 and its binding partner CNTN2. More specifically, we will determine binding affinity between purified CNTNAP2 and CNTN2 using solid phase binding assays and 2) determine the 3D structure of the N-terminal region of the CNTNAP2 ectodomain or its fragments.

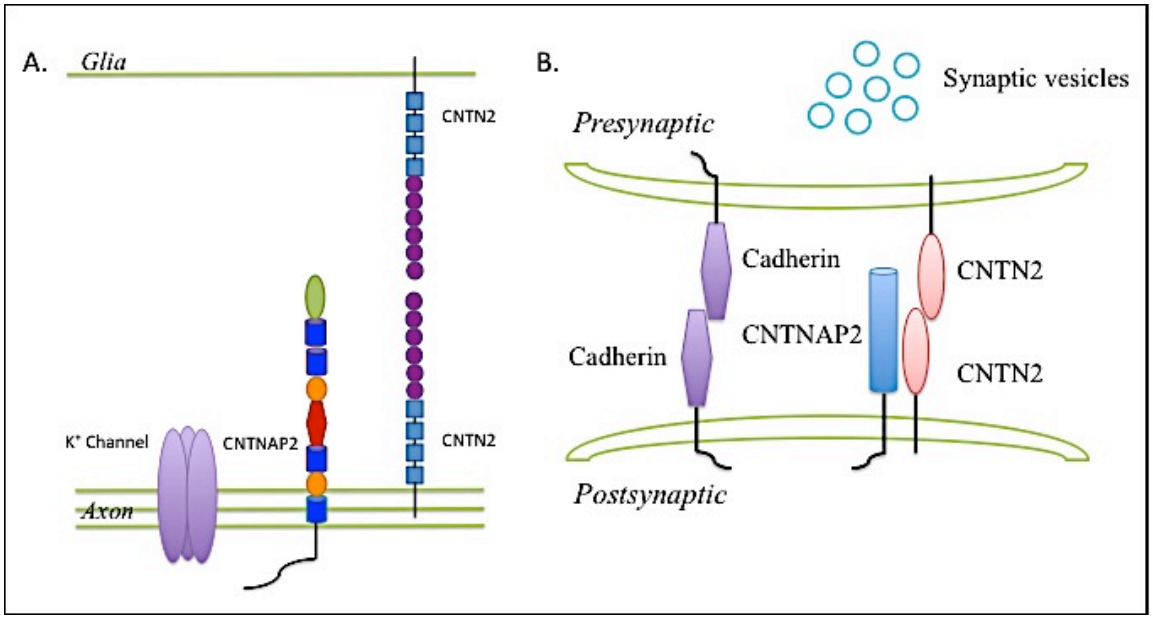

Figure 3. Proposed models describing CNTNAP2 and CNTN2 interactions at axo-glial and neuron-neuron contacts. A. CNTNAP2 and CNTN2 are localized at the juxtaparanodes of myelinated axons. **B.** CNTNAP2 and CNTN2 are localized in synaptic clefts and are thought to form trans-synaptic bridges spanning the synaptic cleft promoting the interaction of presynaptic and postsynaptic membranes.

CHAPTER 2

Methods

EXPRESSION AND PURIFICATION

CNTNAP2 was expressed in the baculovirus/insect cell expression system. For this purpose the human CNTNAP2 and its fragments followed by a C-terminal tag ASTSHHHHHH were produced using baculovirus-mediated overexpression. This was performed in HighFive cells with Insect-XPRESS +l-glutamine medium (Lonza). The medium containing the secreted proteins was concentrated after protease inhibitors were added, then dialyzed overnight (25 mM sodium phosphate, pH 8.0, 250 mM NaCl), and purified with a nickel-nitrilotriacetic acid column (25 mM sodium phosphate, pH 8.0, 500 mM NaCl, eluted with an imidazole gradient). Subsequently, the protein was dialyzed into 50 mM MES, pH 7.0, 100 mM NaCl. Prior to loading on a Mono S column, the protein was incubated with 5 mM $CaCl_2$ for 0.5 hr.

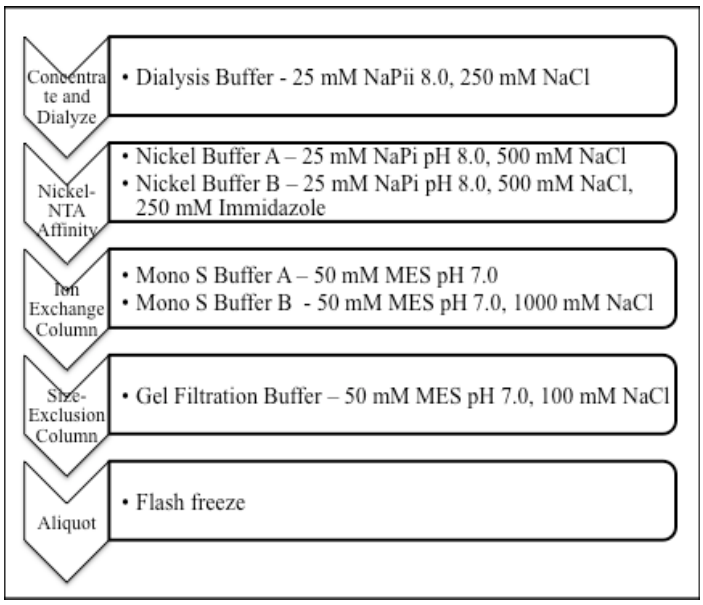

Figure 4. CNTNAP2 purification flow chart. Briefly, medium containing the secreted proteins was concentrated and purified with a Nickel-NTA column. Subsequently, the proteins were further purified on an Ion exchange column followed by size exclusion chromatography.

The protein was purified on a MonoS column equilibrated with 50 mM MES pH 7.0; and subsequently eluted with an NaCl gradient. As a final purification step, the protein was loaded on a Sephadex 200 16/60 size exclusion column (50 mM MES, pH 7.0, 100 mM NaCl) (Fig. 4). The purified CNTNAP2 and its fragments were stored in 50 mM MES, pH 7.0, 100 mM NaCl in flash-frozen aliquots.

SOLID PHASE BINING ASSAY

In order to perform solid phase binding assays to assess the protein interactions (Fig. 5), CNTNAP2 or CNTN2 was labeled with biotin at room temperature. Proteins were dialyzed into PBS buffer (100 mM sodium phosphate, pH 7.0, 100 mM NaCl), then incubated in a 10-fold molar excess of EZ-Link NHS PEG4-Biotin (ThermoFisher Scientific, Cat. #21330) for 45 min and finally dialyzed into 25 mM Tris, pH 8.0, 100 mM NaCl. The labeling efficiency for CNTNAP2 and CNTN2 were typically 6–25 biotin/protein molecule. Each step in the solid phase binding assay was carried out at room temperature. For assays with immobilized CNTN2, 200 ngr of CNTN2 (50 μl) in binding buffer (20 mM Tris, pH 8.0, 100 mM NaCl, 5 mM $CaCl_2$) was incubated in 96-well plates for 2 hr at RT. A series of wells were incubated containing only buffer (no CNTN2) as a control for the background. The wells were washed five times with 300 μl of binding buffer/$CaCl_2$ (20 mM Tris, pH 8.0, 100 mM NaCl, 5 mM $CaCl_2$) for 30 s at 300 rpm or, for $CaCl_2$-free conditions, with binding buffer/EDTA (20 mM Tris, pH 8.0, 100 mM NaCl, 20 mM EDTA) and finally blocked with 300 μl blocking buffer (1% (w/v) gelatin in binding buffer/$CaCl_2$ or binding buffer/EDTA) for 2 hr at room temperature. The wells were then incubated with increasing concentrations of biotinylated CNTNAP2* (C1, C2, C3 C4 and C5) (Fig. 5) in binding buffer/$CaCl_2$ (20 mM Tris, pH 8.0, 100 mM NaCl, 5mM $CaCl_2$, in triplicate) or binding buffer/EDTA (20 mM Tris, pH 8.0, 100 mm NaCl, 20 mM EDTA, in duplicate) for 1 hr at

room temperature, emptied, and washed again three times with binding buffer/$CaCl_2$ or binding buffer/EDTA. For assays with immobilized CNTNAP2, 200 ngr of CNTNAP2 (or fragments) (50μl) in binding buffer/$CaCl_2$ was coated for 1 hr at 150 rpm, and the wells were treated as described above and were blocked with 300 μl blocking buffer containing 1% gelatin. Wells were then incubated with increasing concentrations of biotinylated CNTN2* (0-10 nM; 100 μl /well) in binding buffer/$CaCl_2$+ (in triplicate) for 1 hr at RT. To develop the signal, wells were incubated with anti-streptavidin HRP conjugate (diluted 1:5000 in blocking buffer; 300 μl/well) for 45 min, washed three times with binding buffer/$CaCl_2$ or binding buffer/EDTA, and then incubated with the substrate *o*-phenylenediamine (Calbiochem; 100 μl/well) for 10 min. The reaction was stopped by adding 50 μl/well 500 mM H_2SO_4, and the absorbance was read at 490 nm.

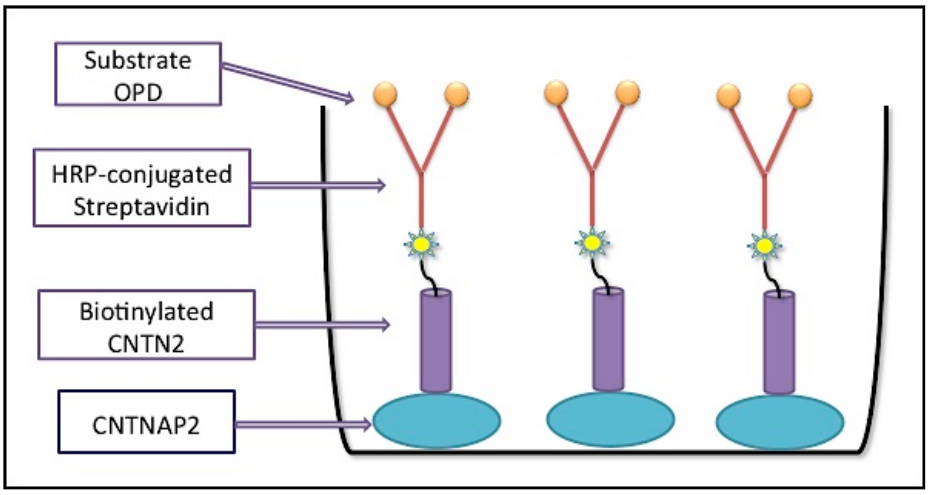

Figure 5. Schematic model describing the solid phase binding assay. The case where CNTNAP2 is immobilized and increasing concentrations of biotinylated CNTN2 are added to the wells is shown.

Data were analyzed with Prism (GraphPad). Specific binding was expressed as total binding in the presence of $CaCl_2$ minus binding in the absence of immobilized bait protein. The K_D was calculated by non-linear regression fitting, using a model for “one-site specific binding”.

FLUORESCENCE-POLARIZATION

To develop a fluorescence-polarization (FP) assay to assess protein interactions, (Fig. 6) CNTNAP2 was labeled with OneQuantTM (5/6) TAMRASE, (Bioscience, Cat. # 786-079) at room temperature. The protein was incubated in labeling buffer (20 mM HEPES pH 8.0, 50 mM NaCl, 5 mM $CaCl_2$) with a 10-fold molar excess of TAMRA for 1hr. Labeled protein was separated from unreacted TAMRA using a spinout column (Catalogue: SpinOUT™ GT-600, 3ml column, Cat. # 786-171, G-Biosciences). The labeling efficiency was determined by measuring the absorbance of the protein–dye conjugate at 280 nm (A280) and was only 0.1 molecule TAMRA per molecule of CNTNAP2. To perform the FP assay, 20 nM TAMRA labeled CNTNAP2 (TMR-CNTNAP2) was transferred in 96 well plates (200 μl/well) at room temperature and titrated with 0.325 to 200 nM cold tracer CNTN2 (200 μl/well). The plate was incubated at room temperature for 10 minutes. A series of wells were incubated with buffer as well as a standard solution (20 nM TAMRA in buffer) for a background control. The plate was read by a microplate reader (BMG Labtech PHERAstar) with a FP-filter (excitation 540nm; emission: 590 nm) and polarization values were recorded.

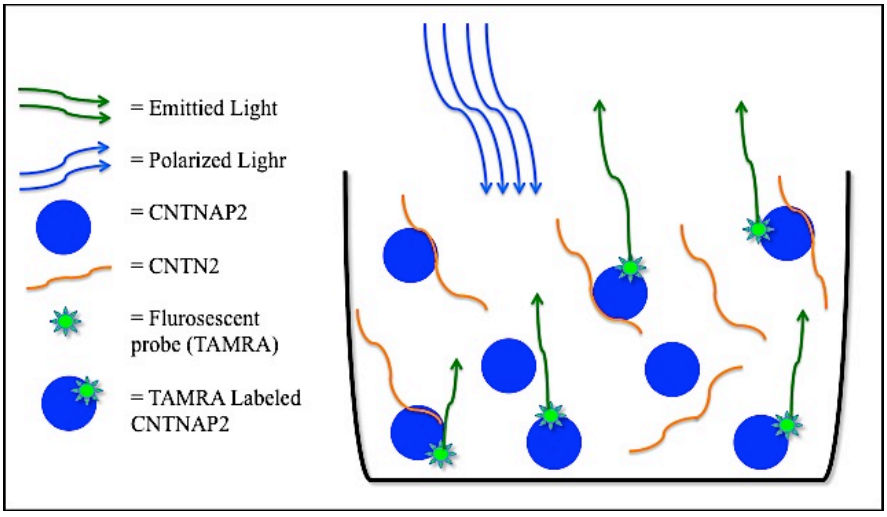

Figure 6. Schematic model describing the Fluorescence-Polarization assay.

CRYSTALLIZATION

Prior to setting up crystallization screens of the CNTNAP2 C2 and C4 constructs, the optimal protein concentration for the experiments was assessed using the Pre crystallization Test (PCT; Hampton Research). Crystallization screens were set up for CNTNAP2 C2 at 5mg/ml and 3mg/ml in 50 mM MES, pH 7.0, 100 mM NaCl buffer and CNTNAP2 C4 at 5mg/ml in 50 mM MES, pH 7.0, 100 mM NaCl buffer. Both proteins were set up at 20°C as well at 5°C respectively (commercial screens from Hampton Research, Molecular Dimensions and Rigaku). A Phoenix crystallization robot (Art Robbins Instruments) was used to set up the screens using a sitting drop format with Art Robbins 96 Intelli-Plates. The crystallization screens were set up by mixing 0.5 µl protein and 0.45 µl crystallization condition in the sitting drop well and putting 45 µl crystallization condition in the reservoir. To induce crystal growth, microseed matrix screening method was also tested, by adding crushed crystals from another protein (0.5 µl) to each crystallization drop.

CHAPTER 3

Results

To carry out our studies we first purified CNTNAP2 and CNTN2, as well as fragments using baculo-virus mediated overexpression in insect cells (Fig.1). The constructs CNTNAP2 C1, C2, C3 and C4 were purified yielding high purity protein preparations as estimated by SDS-PAGE (Fig. 7). The protein yields were respectively CNTNAP2 C1 (8 mgr) , C2 (16 mgr), C3 (1.5 mgr) and C4 (4 mgr). CNTN2 was purified by a lab member.

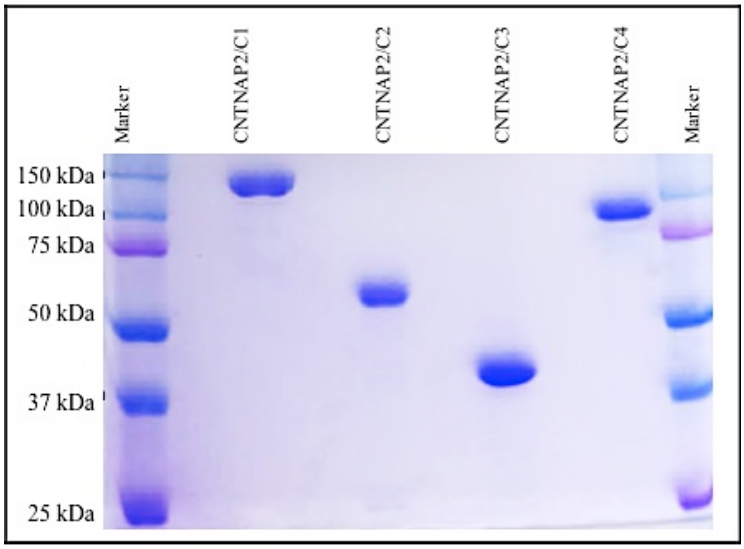

Figure 7. Final purity of CNTNAP2 fragments. Protein constructs (10 µg/lane) were loaded on 10% SDS page

CNTNAP2 BINDS CNTN2 WITH NANOMOLAR AFFINITY

In order to investigate the interaction between CNTNAP2 and CNTN2, we developed and modified a solid phase binding assay. First, we coated a 96 well plate with CNTN2 and titrated with biotinylated CNTNAP2. The interaction between immobilized CNTN2 and labeled CNTNAP2 was in the low nanomolar range (K_D = 3±1 nM) (Fig. 8). In

the reverse assay, immobilized CNTNAP2 bound biotinylated CNTN2 with similar affinity (K_D = 5±1 nM).

To determine if the interaction between CNTNAP2 and CNTN2 was Ca^{2+} dependent, we performed our binding assays with $CaCl_2$ added to the buffer or with EDTA added to ensure Ca^{2+}-free conditions. Our binding assays indicate that the binding of the extracellular domains of CNTNAP2 and CNTN2 are promoted by the presence of Ca^{2+}.

To identify the minimal regions of CNTNAP2 and CNTN2 required for binding, we repeated the solid phase binding assays using the fragments CNTNAP2 C2/C3/C4 and CNTN2 C2/C3 fragments under the same conditions as for the full-length proteins. We also tested the Ca^{2+}- dependence of these interactions.

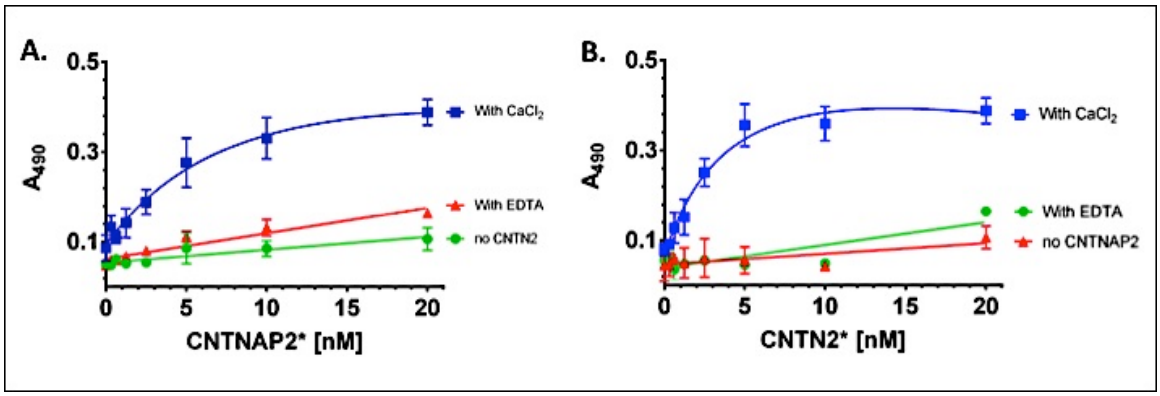

Figure 8. Solid phase binding assay to assess the interaction between CNTNAP2 and CNTN2. A. Increasing concentration of biotinylated CNTNAP2 was incubated with immobilized CNTN2. **B.** Increasing concentration of biotinylated CNTN2 was incubated with immobilized CNTNAP2.

Binding results for individual fragments were not reproducible, so we were not able to map which domains play key roles for CNTNAP2/CNTN2 interaction. Having spent a significant amount of time in trying to develop the approach to be reproducible and quantitative, I have other projects with priority. The fact that we were not able to reproduce results using CNTNAP2 fragments, suggests at least two possible explanations: First, it is possible that the full-length CNTNAP2 domain is required for binding to CNTN2; a second possible scenario is that while designing fragments, the protein fragments might be altered at functionally critical locations. Nevertheless, the importance of these binding experiments

in understanding the mechanistic aspects of these macromolecular interactions, additional studies will be needed.

FLUORESCENCE-POLARIZATION BASED BINDING ASSAY.

To independently confirm the mode of interaction between CNTNAP2 and CNTN2, we tried to develop a fluorescence polarization-based protein interaction assay. Fluorescence-polarization (FP)-based assays are powerful tools to study molecular interactions. We first measured the polarization of TAMRA labeled CNTNAP2 in the presence of increasing concentrations of unlabeled CNTN2. Again I found that the results in the changes in polarization were inconsistent. It is possible that these experiments were highly sensitive to parameters, which I did not consider in my experiments. Further development of such studies is required to test the binding between CNTNAP2 and CNTN2. We could potentially perform an FP-based protein interaction assay using different fluorescence probes such as a FAM.

CRYSTALLIZATION TRIALS OF THE CNTNAP2 C2 and C4 CONSTRUCT

The whole crystal growth process consists of finding the right concentration of a protein for solubility and right crystallization conditions. When a protein concentration is brought above its solubility limit, the solution becomes supersaturated. To find a good starting protein concentration to attempt crystallization of the CNTNAP2 C2 and C4 constructs we used the Pre Crystallization Test (PCT). Based on the PCT test, CNTNAP2 C2 and C4 constructs were screened at 5mg/ml with 5 mM $CaCl_2$. To obtain crystals of CNTNAP2 C2 or C4 we screened an extensive panel of crystallization conditions. Several conditions yielded possible hits (Fig. 9). However, we were not able to reproduce those hits in a confirmation screen. As a next step, we set up the crystallization screens in presence

of 1 mM EDTA at RT and at 4°C, but no crystallization conditions were found. To test whether glycosylation was preventing crystal growth, we deglycosylated the CNTNAP2 C2 fragment using Endoglycosidase H (NEB Catalog # P0702L) which cleaves within the chitobiose core of high mannose and some hybrid oligosaccharides from N-linked glycoproteins. Based on NetNglyc 1.0 server prediction, CNTNAP2 C2 contains 3 potential N-glycosylation sites. Deglycosylation was carried out for 2 hr at room temperature in 50 mM sodium citrate pH 5.5. Deglycosylated CNTNAP2 C2 protein was tested with the PCT test and based in that, the crystallization screens were set up using 3mg/ml protein with 1 mM EDTA or 5 mM $CaCl_2$ at RT as well at 4°Cbut no crystallization conditions were found. Crystallization screens of CNTNAP2 C4 were set up as well, and have not yielded hits.

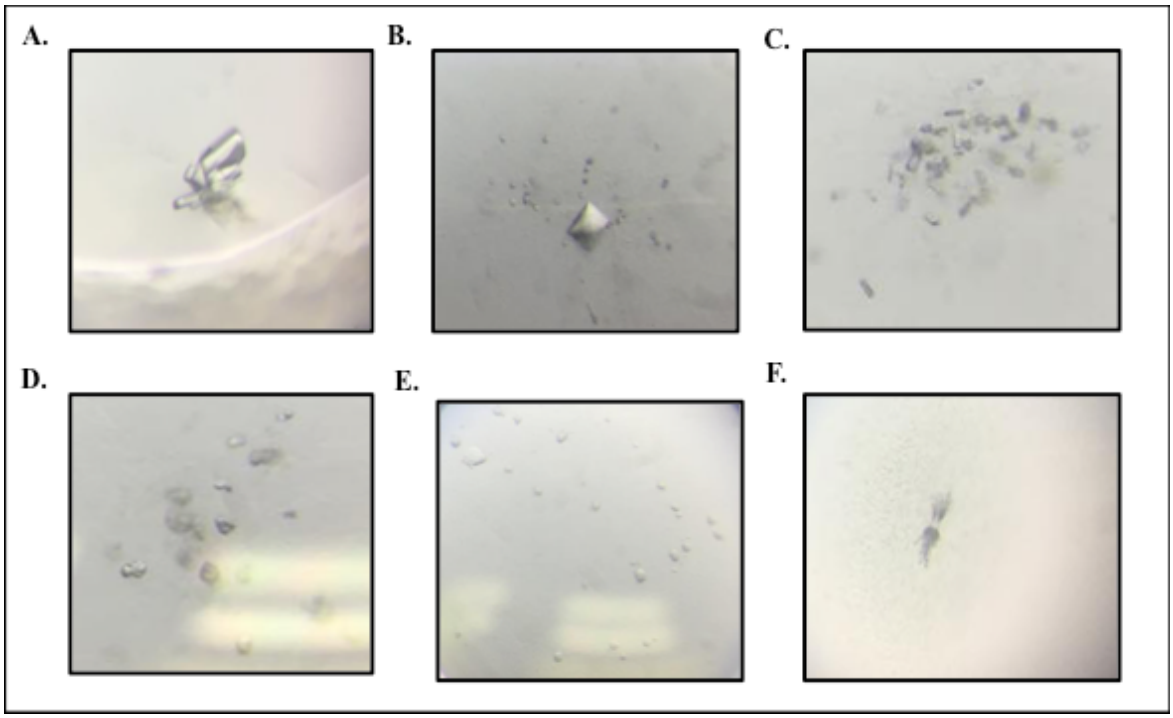

Figure 9. Crystallization screening results obtained with the CNTNAP2 C2 construct. Crystals were obtained in: **A**. 0.2 M Magnesium acetate tetrahydrate, 0.1 M Sodium cacodylate trihydrate pH 6.5 30% v/v (+/-)-2-Methyl-2,4-pentanediol. **B**. 15% v/v Tascimate pH 7.0, 0.1 M HEPES pH 7.0, 2 % (w/v) PEG 3,350. **C**. 10% (w/v) PEG 8000, 0.1 M 100 mM CHES/ Sodium hydroxide pH 9.5, 0.2 M Sodium chloride. **D**. 0.8 M Succinic acid pH 7.0. **E**. 15% (w/v) PEG 20,000, 0.1 M HEPES/sodium hydroxide pH 7.0 buffer. **F**. 50% (v/v) PEG 400, 0.1M Sodium acetate pH 4.5 0.2 M Lithium sulfate.

CHAPTER 4

Discussion

In this research, we characterized the interaction between CNTNAP2 and CNTN2; two cell adhesion molecules that play a role in the formation and maintenance of neuron-neuron and axo-glial contacts (Peles et al., 2000; Bhat et al., 2001; Gordon et al., 2014; Poliak et al., 1999). A number of studies have shown that these two proteins interact in cell-based studies (Perrine et al., 2002; G. Rougon et al., 2000; Kastriti et al., 2015), and that the interaction is specific (Chen et al., 2015; Stogmann et al., 2013).

Using a solid phase binding assay we were able to detect interaction between CNTNAP2 and CNTN2 and determine the equilibrium binding constant. Our data indicate that the recombinant, purified extracellular domains of CNTNAP2 and CNTN2 are sufficient to bind each other directly and with high affinity (K_D ~3 nM). Strikingly, a very recent study using Bio-layer interferometry under different buffer conditions indicated that CNTNAP2 does not bind CNTN2, but interacts weakly with micromolar affinity to CNTN1 (Rubio-Marrero et al., 2015).

Based on our solid phase binding assay, we found that the interaction between extracellular CNTNAP2 and CNTN2 is promoted by Ca^{2+} and can be abolished through the addition of EDTA, which chelates Ca^{2+}. It is known that Laminin G domains, also known as LNS domains (Laminin G, neurexin, sex hormone binding domains) contain calcium binding sites (Fig. 1) and given that CNTNAP2 has 4 such domains and the key Ca^{2+}-interacting residue is conserved it is possible that CNTNAP2 requires Ca^{2+} to interact with CNTN2. We were not able to further characterize the minimal domains of CNTNAP2 and CNTN2 necessary for interaction because of non-reproducible results. It is possible that the assay was not robust enough (high background, technically difficult, bad experimental conditions, protein degradation over time and etc.). Another possible explanation is that the fragments tested simply don't bind because only full-length CNTNAP2 and CNTN2

interact. Alternatively, it is possible that the fragments lose their binding epitopes when taken outside of the context of the full-length protein.

Even though the initial crystallization screens set up for the CNTNAP2 C2 and C4 fragments using the crystallization robot yielded some possible hits, for several we determined that they were salt crystals. For others, we were not able to reproduce the crystallization conditions in order to determine whether the crystals were protein or salt crystals. Though the CNTNAP2 C2 and C4 fragments were produced with high protein purity, as assessed by SDS-PAGE (Fig. 7), no proteins crystals could be obtained. There are several possible reasons for this problem. First, there might be stochastic problems in crystallization process. Second, since crystallization kinetics are different when setting up crystallization screens by robot relative to confirmation screens by hand, it is possible that crystallization hit conditions found by robot don't work for manual crystal screening. Third, it is possible that the proteins we produced were too flexible or heterogenous to crystallize. CNTNAP2 is glycosylated and it is possible that glycosylation interferes with crystal growth in a way that was not prevented by the deglycosylation process described here. In addition, it is possible that smaller fragments need to be tested that are less flexible.

In summary, functionally, we show that CNTNAP2 associates with nanomolar affinity with CNTN2. Though crystals of CNTNAP2 fragments were not obtained, the next step would be to modify the constructs, for example mutating out N-linked glycosylation sites, removing the c-terminal hexa-histidine tag, shortening or lengthening the construct by adding or deleting domains and/or trying CNTNAP2 from different species. The data presented here provide a new platform for structural-functional study for CNTNAP2/CNTN2 complex. In the future, we will continue this project to map critical interactions sites on these proteins.

CHAPTER 5

Future Directions

The fundamental mechanisms of the interactions between transmembrane neurexin superfamily proteins are not well characterized. Our data suggest that CNTNAP2 interacts with CNTN2 with high affinity. However, further studies are needed to confidently assess how these proteins interact. Proteolysis of these proteins will allow us to fragment the protein based on its functional domains, and create new constructs of the individual domains. With these fragments, we can determine which domains are crucial for CNTNAP2-CNTN2 interaction. This will assist us in further studies to determine the structure of CNTNAP2 with x-ray crystallography. Overall, this will increase our understanding of CNTNAP2 and CNTN2's role in neuronal synapse function.

Also, it will be important to investigate the structure of the CNTNAP2-CNTN2 complex as well as experimentally determine whether CNTNAP2 uses similar mechanisms to bind other putative partners.

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