UNIVERSITY OF CALIFORNIA, SAN DIEGO

Caspr2: Possible Synaptogenic Cell Adhesion Molecule

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by

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ABSTRACT OF THE THESIS

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Trinh Thuy Do

Master of Science in Biology

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Professor Palmer Taylor, Chair

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Synapses are crucial for communication among neurons in the central nervous system. Contactin-associated protein-like 2 (Caspr2) is a neuronal protein that is a member of the neurexin superfamily and is found in the juxtaparanodal regions of myelinated axons. Caspr2 has also been found in synapses and therefore is also thought to function as a cell adhesion molecule. As such, it should also induce synaptogenesis *in vitro* similar to the interaction between neurexins (located presynaptically) and neuroligins (located postsynaptically). Neurexins and neuroligins are calcium-dependent

cell adhesion proteins that mediate the signaling across synapses and have the ability to affect the neuronal network by promoting the formation of excitatory or inhibitory synapses. Mutations in neurexins and neuroligins have been implicated with autism and autism spectrum disorders, among other neurodevelopmental disorders. As a member of the neurexin superfamily, Caspr2 shares the same increased risk for autism. Here I describe a co-culture experiment with rat hippocampal neurons and Caspr2-expressing HEK293 cells to test for synapse formation and its location in particular synapse sub-types.

Introduction

Autism spectrum disorders

Autism spectrum disorders are a spectrum of developmental, psychiatric conditions that are characterized by abnormal social interactions, restricted interests and repetitive behavior, and an inability to communicate (Bailey *et al*, 1996; Lord *et al*, 2000). Autism spectrum disorders encompass autism, Asperger's syndrome, and pervasive developmental disorder not otherwise specified. Autism is the most recognized form of autism spectrum disorders and is defined as a complex neurodevelopmental disorder that appears during the first three years of a child's life. Information processing is altered in autistic patients where nerve cells and their synapses do not connect and organize normally.

Both genetic and nongenetic factors contribute to the etiology of autism (Folstein and Piven, 1991). The prevalence of autism spectrum disorders is about 1 in every 150 children while autism specifically is about 1 – 2 in every 1,000 people with males being four times as likely to be diagnosed with this disorder as females (Arking *et al*, 2008; Kim *et al*, 2008). A third to a half of autistic people do not develop enough natural speech to carry a normal daily conversation and autistic children have less babbling and/or word combinations and their gestures are less often integrated with words.

Despite the fact that autism and autism spectrum disorders are largely hereditarily based (70 – 90%; Folstein and Rosen-Sheidley, 2001; Alarcon *et al*, 2008), autism spectrum disorder susceptibility genes have not been completely identified due to the heterogeneity of the syndromes. There have been correlations drawn from statistical analyses of whole genome scans that link regions on chromosomes 7q22 – 32 and 17q21

to autism spectrum disorders, but the actual allele(s) remain(s) unknown (Ashley-Koch *et al*, 1999; Philippe *et al*, 1999; Auranen *et al*, 2002; Cantor *et al*, 2005).

Neurexins and neuroligins

Neurexins and neuroligins play an essential role in synaptic contact differentiation and synaptic transmission, though they are most well known for their possible autism etiology. Neuroligins-1 to -4, neurexins-1α to -3α, and -1β to -3β are the ten primarily expressed adhesion molecules in mammals (Ushkaryov and Sudhof, 1993; Ushkaryov *et al*, 1994; Ichtchenko *et al*, 1996; Bolliger *et al* 2001; Rowen *et al*, 2002; Tabuchi and Sudhof, 2002) and are responsible for synaptogenesis, synapse maturation, function, and plasticity (Dalva *et al*, 2007).

Neurexins are a family of transmembrane proteins and were first discovered as receptors to the presynaptic neurotoxin α-latrotoxin, triggering substantial neurotransmitter release (Ushkaryov *et al*, 1992), but they can also bind to leucine-rich repeat transmembrane proteins (De Wit *et al*, 2009; Ko *et al*, 2009) and postsynaptic neuroligins as a presynaptic cell adhesion molecule (Boucard *et al*, 2005). α-Neurexins, the larger of the two isoforms of neurexins, contains multiple copies of laminin, neurexin, sex-hormone-binding globulin (LNS) domains and epidermal growth factors (EGF) as compared to only one LNS domain in β-neurexins (Ushkaryov *et al*, 1992; Ushkaryov *et al*, 1994; Ullrich *et al*, 1995; Rowen *et al*, 2002; Tabuchi and Sudhof, 2002; Reissner *et al*, 2008). Neuroligins all resemble the acetylcholinesterase structure with its α- and β-hydrolase folds (Comoletti *et al*, 2007, Fabrichny *et al*, 2007, Arac *et al*, 2007). The different isoforms of neurexins and neuroligins localize in different areas of the brain.

Alternative splicing of both the neurexins and neuroligins allows potentially for the thousands of isoforms to exist with separate capabilities to determine synapse differentiation and specificity due to their highly selective binding. As calcium-dependent cell adhesion molecules, neurexins and neuroligins are able to induce both excitatory and inhibitory signaling in neurons. The interaction between α-neurexins and neuroligin2 stimulate GABAergic inhibitory synapses while β-neurexins and neuroligin1 induce formation of glutamatergic excitatory synapses (Boucard *et al*, 2005; Chih *et al*, 2006; Graf *et al*, 2006; Kang *et al*, 2008).

Studies have shown that neuroligin-expressing non-neuronal cells have the ability to induce presynaptic differentiation in neurons (Scheiffele *et al*, 2000) and neurexin-expressing non-neuronal cells are capable of inducing postsynaptic differentiation in co-cultures (Graf *et al*, 2004). And so, the interaction between neurexins and neuroligins can create pre- and postsynaptic terminals in the nervous system and their ability to control inhibitory and excitatory synaptogenesis depends on the different splice variants and subtypes involved.

Contactin associated protein-like 2

Contactin associated protein-like 2 (Caspr2) has recently been discovered as the newest member of the neurexin superfamily and the mammalian homolog of the *Drosophila* Neurexin IV gene (Poliak *et al*, 1999). Through chromatin-immunoprecipitation, the first intron of Caspr2 was discovered to have a fragment that bound to FOXP2, a transcription factor gene that causes monogenic forms of speech when mutated (Fisher & Scharff, 2009). In developing cerebral cortexes, FOXP2 binds to

the Caspr2 promoter and regulates its expression such that high FOXP2 levels were matched inversely with low Caspr2 expression (Vernes *et al*, 2008; Fisher & Scharff, 2009; Whitehouse *et al*, 2011).

As a 2.5Mb neuronal membrane gene (encompassing approximately 1.5% of chromosome 7; Stein *et al*, 2011), Caspr2 is well known for its association with other neuropsychiatric and neurodevelopmental disorders such as attention deficient hyperactive disorder (Elia *et al*, 2010), Tourette syndrome (Verkerk *et al*, 2003), and schizophrenia (Friedman *et al*, 2008) when they occur together with other risk factors. Furthermore, several single-nucleotide polymorphisms in the Caspr2 gene in the exon 13 – 15 region have also been discovered to be linked with autism and specific language impairment when mutated (Alarcon *et al*, 2008; Arking *et al*, 2008; Bakkaloglu *et al*, 2008; Stephan, 2008; Vernes *et al*, 2008; Burbach and Van der Zwaag, 2009; Stein *et al*, 2011; Whitehouse *et al*, 2011).

Through analysis of its expression using Northern blots, Caspr2 was found to be predominately expressed in the human central nervous system and demarcates the subdomains in myelinated nerves (Bel *et al*., 2009). Caspr2, unlike Caspr, contains a PDZ domain-binding motif at its C-terminal stabilizes the localization with Shaker-like voltage-activated potassium channels in juxtaparanodal regions (Poliak *et al*, 1999, 2001; Abrahams *et al*, 2007) and is located beneath the compact myelin helping to maintain the structure of the nodes of Ranvier (Fig. 1). In a study involving the deletion of Caspr2 in mice, the functionality of the potassium channels was disrupted, suggesting that Caspr2 is necessary to maintain its function (Poliak *et al*, 2003).

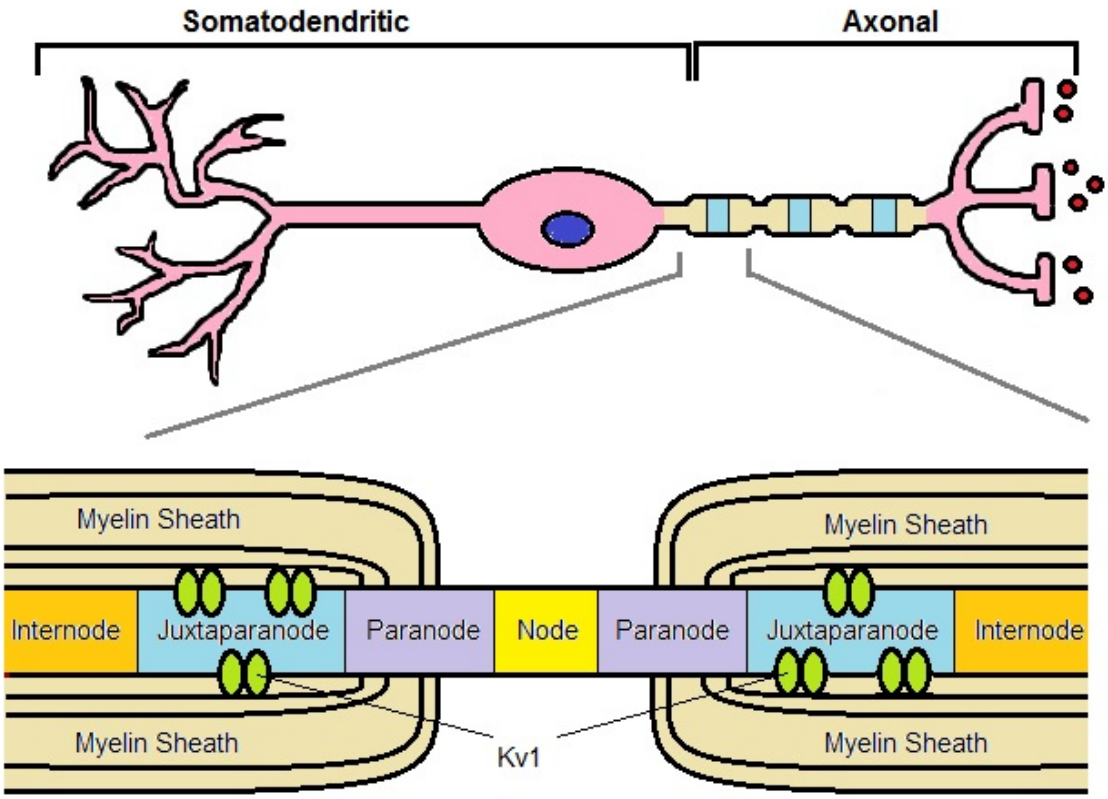


Figure 1: Anatomical structure of neurons
The Shaker-like voltage-gated potassium channels (Kv1) are located on the juxtaparanodes of neurons. The juxtaparanode is the region of the axon in between the paranode and internode underneath the myelin sheath. Caspr2 is able to localize with the Kv1 channels in neurons.

Caspr2 can interact with transient axonal glycoprotein 1 (TAG-1), a glycosyl-phosphatidyl-inositol-anchored cell adhesion molecule, that is expressed in opposing glial and axonal membranes via its extracellular domain (Traka *et al*, 2002, 2003; Bel *et al*, 2009). In a study involving Caspr2- and TAG-1 deficient mice, the positioning of the potassium channels at the juxtaparanodes was altered (Traka *et al*, 2002; Poliak *et al*, 2003; Bel *et al*, 2009). However, the underlying mechanism involved in the co-clustering of the potassium channels and Caspr2 at the juxtaparanodes have yet to be determined (Bel *et al*, 2009).

Studies have shown that Caspr2 transcripts appear heavily in areas of the brain responsible for executive function; more specifically in developing frontotemporal-subcortical circuits (Alarcon *et al*, 2008; Tan *et al*, 2010). Caspr2's expression is restricted to the soma and axons in neurons with little to no expression in the dendrites due to the fact that it is recognized by the endocytic pathway and colocalizes with EEA1, a marker found in early endosomes. The internalization of Caspr2 in dendrites during neuronal maturation gives it its polarized expression and is due to its cytosolic juxtamembrane region (Bel *et al*, 2009). On the other hand, the axonal targeting of Caspr2 does not depend on its internalization and the endocytic pathway.

There is a point mutation in wild-type Caspr2 (D1129H) that is a deleterious variation at a conserved site on chromosome 7. This nonsynonymous mutation normally results in a higher risk of autism (Bakkaloglu *et al*, 2008). From unpublished work performed in the Taylor lab, there is evidence that this mutation causes an improper expression of Caspr2 in transfected HEK cells and partially retains it in the endoplasmic reticulum. However, little else is known about this protein.

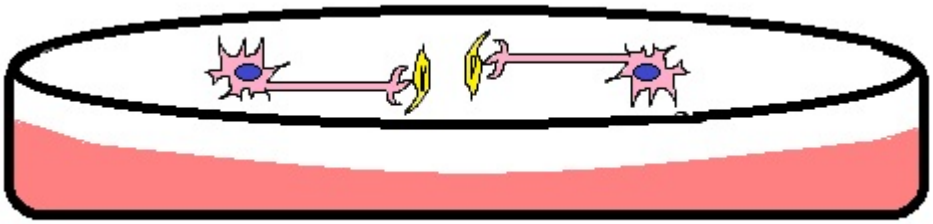


Figure 2: Co-culture design
Caspr2-CFP transfected HEK293 cells (yellow) are plated on top of rat hippocampal neurons (pink) two days before immunostaining analysis with antibodies. Areas of contact between Caspr2 and neurons, whether with dendrites (not shown) or axons, will be analyzed for recruitment of synaptic markers in order to determine the induction of excitatory or inhibitory synapses.

Co-culture assays

Although many cell adhesion molecules in the central nervous system have been identified, the exact mechanisms underlying synaptogenesis have not been determined due to the complexities of the trans-synaptic interactions and bidirectional nature of the signals (Waites *et al*, 2005**;** Biederer and Scheiffele, 2007). However, in a co-culture system where primary neurons are plated with non-neuronal cells transfected with the protein of interest, the complexities of trans-synaptic signals can be greatly reduced such that a single neuronal protein is presented in a non-neuronal plasma membrane (Biederer and Scheiffele, 2007). When interactions occur with primary neurons, the responses can be measured in a number of ways in order to examine the effects of synaptic differentiation, neuronal growth, and axon guidance.

Using this co-culture system, neuroligin1 (NL1) was shown to be capable of inducing presynaptic differentiation and synaptic vesicle formation (Scheiffele *et al*, 2000). SynCAM1, an immunoglobin domain protein, and Netrin-G ligand, a leucine-rich repeat protein has also demonstrated similar results (Biederer *et al*, 2002; Kim *et al*, 2006). Postsynaptic differentiation also was triggered using neurexin1 using this system (Graf *et al*, 2004). This co-culture system is extremely useful in identifying synaptogenic abilities of adhesion molecules and does not put a limit on the type of synaptic protein that can be examined.

As a synaptic cell adhesion molecule and a member of the neurexin superfamily, Caspr2 should be able to induce synaptic differentiation in neurons. Mixed co-culture

experiments with Caspr2-cyan fluorescent protein (Caspr2-CFP) labeled proteins transfected in HEK293 cells will be plated with rat hippocampal neurons in order to observe its characteristics with synaptogenesis, whether pre- or post-synaptic formation. The responses of the neurons to Caspr2 will be measured using immunofluorescent staining and imaging when the neuronal protein is brought into close contact with the primary neuronal cell *in vitro* (Fig. 2). After understanding Caspr2's role in synaptogenesis, the D1129H point mutation will be observed in terms of how it affects those abilities, if at all.

Results

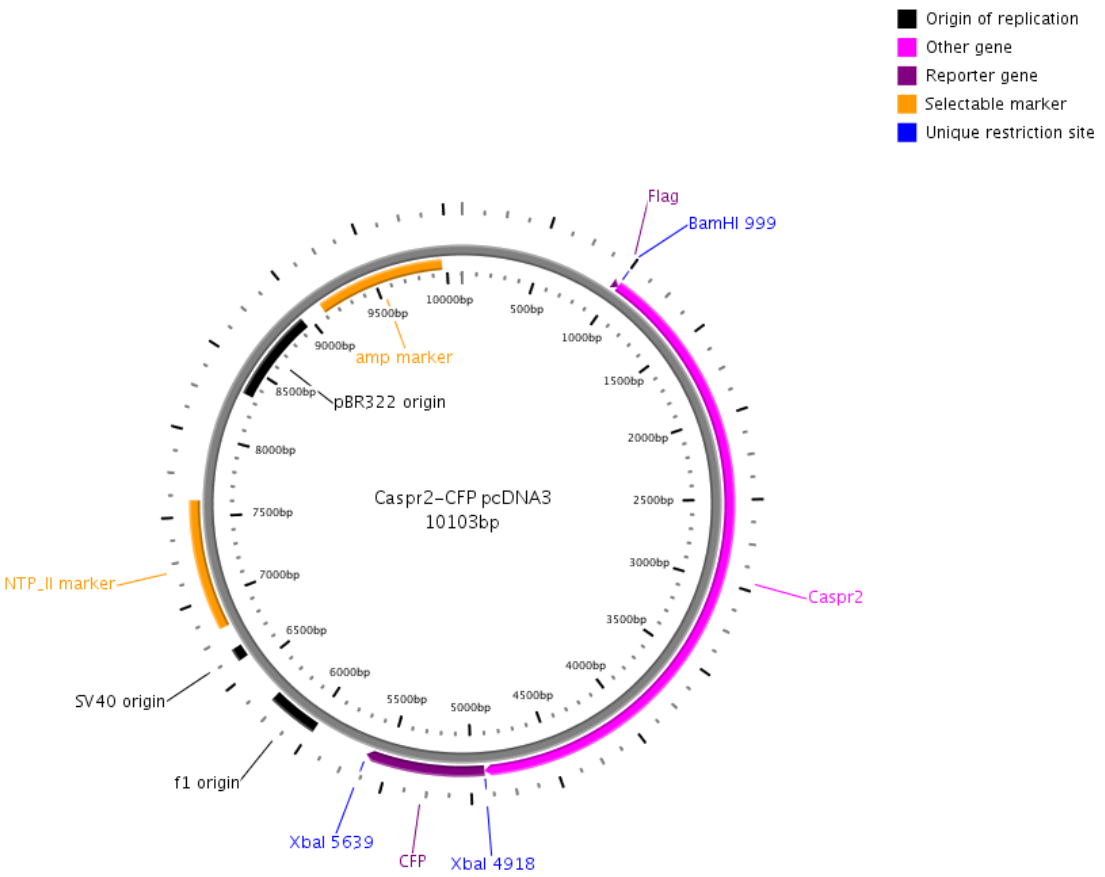
Subcloning CFP

The cDNA sequence of CFP was confirmed through sequencing and amplified by PCR with the primer pairs CFP start-*Xba*I and CFP stop-*Xba*I (Table 1). The PCR product was digested with *Xba*I and subcloned into the wide-type Caspr2 vector. The samples exhibiting the correct orientation of CFP with respect to the wild-type Caspr2 vector also contained an accidental deletion of the thymine nucleotide located in the stop codon. In order to introduce the thymine back into the sequence, a two-step mutagenesis was performed where the forward and reverse primers (CFP (T) start-*Xba*I and CFP (T) stop-*Xba*I; Table 1) were amplified separately via PCR and later combined in a 1:1 ratio to carry out the rest of the amplification. Once again, the PCR products were digested with *Xba*I and subcloned into the wide-type vector. The transformants were plated and isolated for sequencing to ensure that the stop codon was fully functional. With confirmation that the thymine nucleotide was incorporated, the entire Caspr2-CFP vector was sequenced (Fig. 3). Its expression, along with neuroligin1 (positive control in synaptogenesis during co-culture assays) was verified with a Western blot (Fig. 4) before conducting co-culture experiments.

Table 1: Synthetic oligonucleotide primers used in this study
Sequences in bold and italics denote sites of restriction enzymes.

Name	Primer sequence
CFP start-*Xba*I	ATTGCT***TCTAGA***ATGGTGAGCAAGGGCGAGGAGCTGTTC
CFP stop-*Xba*I	ATGA***TCTCTAGA***TTACTTGTACAGCTCGTCCATGCC
CFP (T) start-*Xba*I	GACGAGCTGTACAAGTAA***TCTAGA***GGGCCC
CFP (T) stop-*Xba*I	GGGCCC***TCTAGA***TTACTTGTACAGCTCGTC

A.



B.

Met SALLILALVGAAVADYKDDDDKLAAAPSTSQKCDEPLVSGLPHVAFSSSSSISGSYSPGYAKINKRGGAGGWSPSDSD
HYQWLQVDFGNRKQISAIATQGRYSSSDWVTQYRMLYSDTGRNWKPYHQDGNIWAFPGNINSDGVVRHELQHPIIARYV
RIVPLDWNGEGRIGLRIEVYGCSYWADVINFDGHVVLPYRFRNKKMKTLKDVIALNFKTSESEGVILHGEGQQGDYITLEL
KKAKLVLSLNLGSNQLGPIYGHTSVMTGSLLDDHHWHSVVIERQGRSINLTLDRSMQHFRTNGEFDYLDLDYEITFGGIPF
SGKPSSSSRKNFKGCMESINYNGVNITDLARRKKLEPSNVGNLSFSCVEPYTVPVFFNATSYLEVPGRLNQDLFSVSFQF
RTWNPNGLLVFSHFADNLGNVEIDLTESKVGVHINITQTKMSQIDISSGSGLNDGQWHEVRFLAKENFAILTIDGDEASAV
RTNSPLQVKTGEKYFFGGFLNQMNNSSHSVLQPSFQGCMQLIQVDDQLVNLYEVAQRKPGSFANVSIDMCAIIDRCVPNH
CEHGGKCSQTWDSFKCTCDETGYSGATCHNSIYEPSCEAYKHLGQTSNYYWIDPDGSGPLGPLKVYCNMTEDKVWTIVS
HDLQMQTPVVGYNPEKYSVTQLVYSASMDQISAITDSAEYCEQYVSYFCKMSRLLNTPDGSPYTWWVGKANEKHYYWG
GSGPGIQKCACGIERNCTDPKYYCNCDADYKQWRKDAGFLSYKDHLPVSQVVVGDTDRQGSEAKLSVGPLRCQGDRNY
WNAASFPNPSSYLHFSTFQGETSADISFYFKTLTPWGVFLENMGKEDFIKLELKSATEVSFSFDVGNGPVEIVVRSPTPLN
DDQWHRVTAERNVKQASLQVDRLPQQIRKAPTEGHTRLELYSQLFVGGAGGQQGFLGCIRSLRMNGVTLDLEERAKVTS
GFISGCSGHCTSYGTNCENGGKCLERYHGYSCDCSNTAYDGTFCNKDVGAFFEEGMWLRYNFQAPATNARDSSSRVDN
APDQQNSHPDLAQEEIRFSFSTTKAPCILLYISSFTTDFLAVLVKPTGSLQIRYNLGGTREPYNIDVDHRNMANGQPHSVNI
TRHEKTIFLKLDHYPSVSYHLPSSSDTLFNSPKSLFLGKVIETGKIDQEIHKYNTPGFTGCLSRVQFNQIAPLKAALRQTNA
SAHVHIQGELVESNCGASPLTLSPMSSATDPWHLDHLDSASADFPYNPGQGQAIRNGVNRNSAIIGGVIAVVIFTILCTLV
FLIRYMFRHKGTYHTNEAKGAESAESADAAIMNNDPNFTETIDESKKEWLIGSRMVSKGEELFTGVVPILVELDGDVNGHK
FSVSGEGEGDATYGKLTLKFICTTGKLPVPWPTLVTTLTWGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGN
YKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNYISHNVYITADKQKNGIKANFKIRHNIEDGSVQLADHYQQNTP
IGDGPVLLPDNHYLSTQSALSKDPNEKRDHMVLLEFVTAAGITLGMDELYK Stop

Figure 3: DNA construct

(A). Caspr2-CFP pcDNA3 plasmid with the restriction enzyme sites used to clone in Caspr2 and CFP.

(B). The protein sequence of the Caspr2-CFP pcDNA3 plasmid. Texts in black represent the starter peptide, green is the flag sequence, and grey text represents a linker region. Orange denotes the Caspr2 sequence and texts in blue denote the CFP sequence in the pcDNA3 vector.

D1129H Mutant Construct

To extend our experimental approach, we wanted to test the effect of the mutation D1129H on Caspr2's ability to create synapses in neurons. Thus, this mutation was subcloned from a Caspr2 vector already containing it using the restriction enzymes *Bsr*GI and *Pme*I. The change in amino acid translation from the wild-type Caspr2 vector is denoted by the bolded and underlined purple letter in Fig. 3B. The expected mass of Caspr2-CFP is approximately 190kDa. From other experiments conducted in our lab, we know that Caspr2-D1129H is partially retained in the endoplasmic reticulum. This causes a reduced amount of carbohydrate processing with respect to the wild-type protein, thus explaining the slightly smaller size of this mutant.

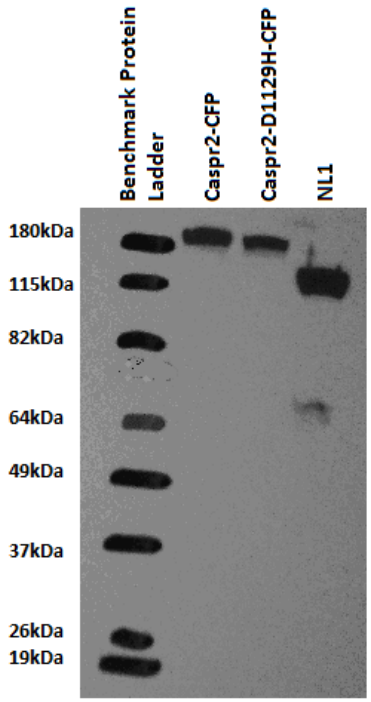


Figure 4: Protein expression of Caspr2-CFP, Caspr2-D1129H-CFP, and NL1
Western blot containing proteins from HEK293-transfected cells hybridized with anti-flag primary and anti-mouse IgG HRP secondary antibodies. The approximate size of Caspr2-CFP and Caspr2-D1129H is 190kDa. Neuroligin1 has an expected molecular weight of about 110kDa.

Co-cultures

Song and colleagues (1999) reported that neuroligin1 colocalizes with PSD-95 and NMDA-R1, proteins that are found in postsynaptic densities, and is specific for excitatory synapses (1999). The neuroligin1 co-cultures were conducted to serve as a positive control for the Caspr2-CFP experiments. The antibodies used for the immunostaining were anti-flag (binds to flag-neuroligin1-transfected HEK293 cells), synapsin (detects presynaptic vesicles due to its implication in the regulation of neurotransmitter release in synapses), DAPI (which served as a mounting medium as well as a stain for HEK293 nuclei), vGAT (shows induction of presynaptic inhibitory vesicles by detecting the vesicular GABA transporter), vGLUT (shows induction of postsynaptic excitatory vesicles by detecting the vesicular glutamate transporters), and MAP2 (stains dendritic processes due to its role in stabilizing microtubule growth in dendrites).

In a co-culture experiment involving flag and synapsin antibodies, both fluoresced on the neuroligin1-transfected HEK293 cell (Fig. 5A-D). As a presynaptic vesicle marker, synapsin should only have fluoresced in axonal terminals. However, this was not the case and there was no difference between the staining for flag and synapsin, indicating a possible error with the secondary antibody staining (Fig. 5B, 5D). Ideally, with proper staining, the markers for synapsin would fluoresce on the neuronal cells in close proximity to the HEK. If synaptogenesis induction occurred, the additive color produced from the flag and synapsin staining should only appear at places where neuronal axons contact the HEK cell.

In another co-culture experiment involving flag, vGAT, and MAP2 antibodies, only fluorescence for flag can be seen on the HEK cells. As an inhibitory presynaptic marker, vGAT should not and was not fluorescing on the HEK cells (Fig. 5F), but should fluoresce on the neurons instead. However, two specks (indicated by arrows in Fig. 5F and H) did appear. It could be possible that these two specks were bound to neuronal cells on a different plane. Ultimately, this co-culture experiment served as a negative control for neuroligin1, confirming that neuroligin1-transfected HEK293 cells do not induce presynaptic inhibitory synapses.

When stained with flag, vGLUT, and MAP2, neuroligin1-transfected HEK cells induced a large number of presynaptic excitatory differentiations, as expected (Fig. 5I-L). When merging images stained with vGLUT and flag together, the fluorescent intensity increased in the HEK cells, indicating contact between axonal terminals and the HEK cells.

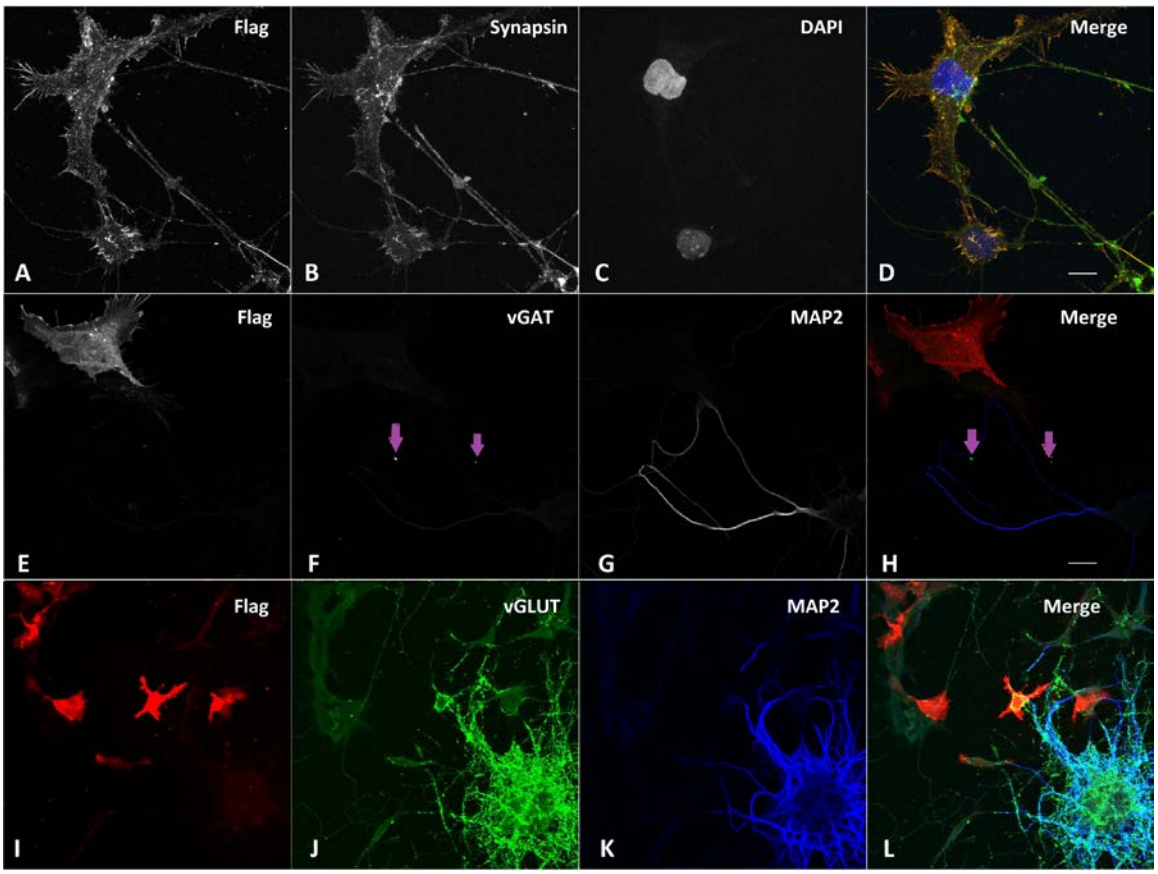


Figure 5: NL1 co-cultures
Hippocampal neurons plated with NL1 transfected HEK293 cells at DIV 6 and immunostained for synaptogenesis at DIV 8. Immunostaining for the each of the transfected cells is displayed in red (A), (E), and (I), synapsin, vGAT, and vGLUT in green (B), (F), and (J), and DAPI and MAP2 in blue (C), (G), and (K). (A-C) and (E-G) are shown in black and white to show higher contrast.

As a negative control, untransfected HEK293 cells were also co-cultured with hippocampal neurons. Regardless of the combination of antibodies that can be used, no additive colors should be present on the cells and no induction of synaptic differentiation of any kind should be seen. Done correctly, all the co-cultures should just show the HEK nuclei stained with DAPI and not making any contacts with a random display of fluorescent markers.

Several co-culture experiments with Caspr2-CFP have been carried out; however no conclusive data were obtained. Much like the co-culture experiment with neuroligin1, the secondary antibody for synapsin also labeled the HEK cells as well, producing two almost identical images (Fig. 6A-B). As a result of the same labeling from the secondary antibody for flag and synapsin, nothing could be concluded regarding synaptogenesis. The only information that could be obtained from this co-culture was that the addition of the CFP sequence did not disrupt the expression of Caspr2 when transfected, further confirming the results of the Western blot. Due to the lack of timing and unpredicted complications, there remains an unanalyzed Caspr2-CFP co-culture experiment that was immunostained using three different sets of antibodies: 1). synapsin, and MAP2, 2). vGAT, and MAP2, and 3). vGLUT, and MAP2.

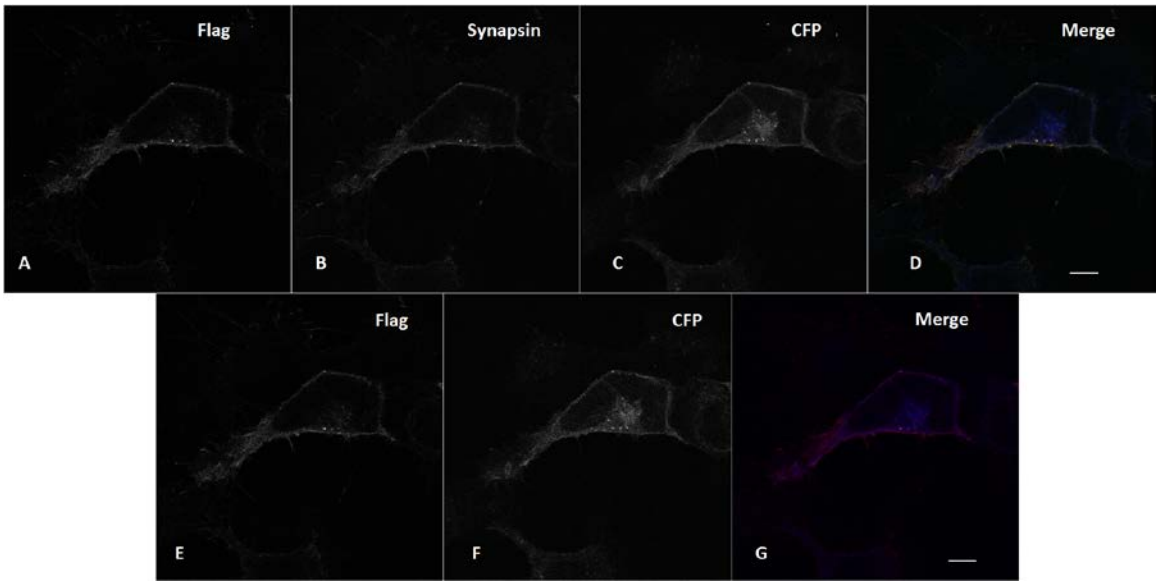


Figure 6: Caspr2-CFP co-culture
Hippocampal neurons plated with Caspr2-CFP transfected HEK293 cells and immunostained with CFP in red (A) and (E), flag in green (B) and (F), and synapsin in blue (C).

In addition to the unanalyzed set of Caspr2-CFP co-cultures, another set of co-cultures involving the Caspr2-D1129H-CFP mutant was also carried out under the same conditions and immunostained with the same set of antibodies.

Discussion

Caspr2-CFP co-cultures

Through these co-culture experiments, we hope to gain a better knowledge regarding the role of Caspr2 in the central nervous system. This can lead to a greater understanding of the loss-of-function mutations that this protein is so commonly associated with in neuropsychiatric disorders. Eventually, the gap of knowledge regarding the etiology of autism and other related disorders can be bridged.

As a postsynaptic protein capable of inducing presynaptic differentiation, neuroligin1 is able to contribute to the remodeling of processes throughout adulthood. Neuroligin1 can be induced to create new presynaptic differentiation or stabilize existing synapses. And so, it is possible that other synaptogenic proteins like neuroligin1 have the machinery to form and remodel synapses in the central nervous system.

And so, co-culture experiments involving Caspr2 were conducted in order to determine its role in synaptogenesis. As another means to visualize Caspr2 in those co-cultures, CFP was subcloned to create a Caspr2-CFP construct. The D1129H mutation was also subcloned using two unique restriction enzymes in order to create a Caspr2-D1129H-CFP construct. By creating this mutant construct, the effects of the mutation on synaptogenesis can be compared directly to the results that would be obtained from Caspr2-CFP. The expression of Caspr2 and the D1129H mutant was confirmed through a Western blot, indicating not only that the sequences of both construct were correct, but also that the addition of the CFP sequence did not disrupt their expression.

In the first neuroligin1 co-culture experiment, staining with synapsin caused a false positive result of colocalization on the HEK cells, indicating a second co-culture experiment had to be performed. In the second co-culture, vGAT did not stain neuronal axons properly and only two specks appeared. The third co-culture experiment with neuroligin1 showed colocalization on the neuroligin1-transfected HEK cells, as predicted when staining with vGLUT. Having obtained the expected excitatory presynaptic differentiation in neuroligin1 co-cultures and no synaptogenesis in the untransfected HEK co-cultures, the results for Caspr2-CFP can then be analyzed. With the synaptogenesis results of wild-type Caspr2, the data from the D1129H mutant in co-cultures can then be analyzed in order to determine if that point mutation alters synaptogenic abilities.

Had the results from the last Caspr2-CFP co-culture experiments been examined, the first set of antibodies would have yielded an HEK cell labeled from the CFP tag and MAP2 staining the dendritic processes within close proximity of the cell. The synapsin antibody would stain the axons and produce colocalization on the HEK cell if Caspr2 is capable of presynaptic differentiation. On the other hand, if Caspr2 acts more like a neurexin protein based on its anatomical structure to create postsynaptic differentiation, the staining with synapsin would not produce colocalization.

If Caspr2 is shown to induce presynaptic vesicle formation from colocalization of synapsin and flag, staining with vGAT would show colocalization on the HEK cell if Caspr2 stimulates inhibitory synapse formation. Alternatively, if the presynaptic vesicles are excitatory, no colocalization would appear unless stained with a vGLUT antibody.

It is possible that Caspr2 stimulates the induction of postsynaptic differentiation instead, making these last three co-culture experiments somewhat uninformative. If this was the case, all three sets of co-culture experiments would not show colocalization on the transfected HEK cells. However, the absence of colocalization could indicate improper staining technique and cannot be confirmed without conducting further co-culture experiments staining with postsynaptic markers such as gephyrin and PSD-95. Depending on whether Caspr2 creates excitatory or inhibitory synaptogenesis with dendrites, colocalization for PSD-95 or gephyrin respectively, should appear on the transfected HEK cells.

The co-cultures with the D1129H mutant were all immunostained with the same antibodies as the Caspr2-CFP co-cultures and so the analysis and interpretation of the results remain the same. If the D1129H mutant causes retention in the endoplasmic reticulum in transfected HEK cells, it would seem likely that the function of Caspr2 is altered from the inability to be expressed on the surface. It is possible then, that this improper expression would lead to different synaptogenic results, leading to the underlying reason behind this mutation’s increased risk for autism.

Materials and Methods

DNA Constructs

The CFP protein of the vector was sequenced, amplified via PCR, and subcloned into the Caspr2 wild-type expression vector using the *Xba*I restriction enzyme site. Transformation of Caspr2-CFP into *E. coli* DH5α cells and selection of the transformants were performed on LB plates supplemented with 50µg/ml ampicillin. Several colonies were isolated, purified using Qiagen Miniprep Purification Kit, and sent for sequencing to confirm the orientation of the CFP insert. Construction of the Caspr2-D1129H-CFP expression vector was carried out in the same manner with *Pme*I and *Bsr*GI restriction enzymes.

Cell Culture

Dissected rat hippocampal neurons from embryonic day 18 were ordered from BrainBits® and plated onto poly-D-lysine-coated coverslips in 12-well plates using Neurobasal media supplemented with glutamic acid, glutamine, penicillin/streptomycin, and B27 at a cell density of 30,000 cells/well.

The HEK293 cells were maintained in a DMEM media supplemented with 10% fetal bovine serum and 1% glutamine. Roche FuGENE® 6 Transfection Reagent was used to transfect Caspr2-CFP, neuroligin1, and Caspr2-D1129H-CFP into HEK293 cells for protein expression. Upon becoming confluent, the cells were split into a DMEM media that was supplemented with 10% fetal bovine serum, 1% glutamine, and 1% gentamicin. For co-culture experiments with HEK293 cells and neurons, the HEK293 lines were conditioned to Neurobasal media supplemented with B27, glutamine, and

penicillin/streptomycin. Six to seven days after plating the neurons, Caspr2-CFP transfected HEK cells, neuroligin1 transfected HEK cells (positive control for synaptogenesis), and untransfected HEK293 cells (negative control) were plated on top of the neurons at the same cell density.

Western blot analysis

Confluent plates of Caspr2-CFP, Caspr2-D1129H-CFP, and neuroligin1 transfected HEK293 cells were lysed and spun at 14,000rpm, 4°C, for 10 minutes with lysis buffer. The cell lysates were solubilized in a 4X SB buffer and boiled for 5 minutes and ran in a 10% acrylamide 12-well SDS gel. After transferring the blot to a nitrocellulose membrane, it was incubated overnight in a blocking solution consisting of 5% milk in PBS. The membrane was then incubated with the primary antibody (anti-flag) for 1 hour at room temperature with gentle rocking. Following several washes to the membrane with 1X phosphate-buffered saline (PBS), it was incubated with the secondary antibody (anti-flag HRP mouse) for another hour. Protein expression from the blot was detected using chemiluminescence with Thermo Scientific Pierce ECL Western Blotting Substrate.

Antibodies

The primary mouse monoclonal antibodies used for immunostaining were synapsin1 (1:500; Chemicon) for the detection of synaptic vesiscles, vGlut1 (1:5,000; Chemicon) for synaptic vesicles of excitatory presynaptic terminals, vGat (1:1,000; Synaptic Systems) for the synaptic vesicles of inhibitory presynaptic vesicles, PSD-95

(7:500; Affinity Bioreagents) for postsynaptic sites of excitatory synapses, flag (1:800) for the detection of the Caspr2, Caspr2-D1129H-CFP, and NL1 protein tags, map2 (1:1000, Abcam) for dendritic markers, and gephyrin (1:500; Cederlane) for the postsynaptic sites of inhibitory synapses. These antibodies were used in various combinations during the co-culture experiments. The secondary antibodies used were monoclonal Alexa Fluor® 633 goat anti-mouse which binds specifically to PSD-95, polyclonal Alexa Fluor® 568 goat anti-rabbit which binds to all the other primary antibodies, and Rhodamine Red-X goal anti-chicken which binds to map2 antibodies.

Immunocytochemistry and Imaging

Two to three days after plating the HEK cell lines, the co-culture plates were fixed with 4% formaldehyde in 1X PBS with a pH of 7.4 $\pm$ 0.2 for 20 minutes at room temperature. The fixed cells were incubated in a detergent containing 0.5% saponin, 1% bovine serum albumin (BSA), and 10mM EDTA for 5 minutes, blocked with 2% normal goat serum (NGS), 0.5% BSA, and 50mM glycine for 30 minutes, and incubated with primary and secondary antibodies prepared with a 1:5 diluted mixture of the blocking solution for one hour each. The cells were mounted in DAPI and analyzed with a confocal fluorescent microscope.

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