Investigating the role of a $Ca_V2.1$ - Syt7 interaction in Short-term Synaptic Facilitation

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Abstract

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Synaptic facilitation is the enhancement of successive transmissions between neurons over timespans of less than a second. It is a form of synaptic plasticity which has long been observed, yet its molecular mechanisms remain unclear. P/Q-Type Calcium Channels and Synaptotagmin 7 have both been implicated as critical factors in the generation of synaptic facilitation. In this thesis, I prove a direct molecular interaction between $Ca_V2.1$, the $\alpha1$ subunit of P/Q-Type Calcium Channels, and Synaptotagmin 7 *in situ* and in cultured cells. I provide the materials for a more directed study of the binding of these proteins and lay a groundwork for the investigation of a functional role of this interaction, which may serve an instrumental role in the production of facilitation across numerous synapses.

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Dedications and Acknowledgements

This thesis is dedicated to my scholarly role-models: My parents, Dr. Chris Bazinet and Dr. Erica Groshen, as well as my previous mentors, Dr. Ivo Spiegel and Dr. Michael E Greenberg. Their support, encouragement, and wise counsel were indispensable in my scientific and personal growth.

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I would also like to acknowledge the reviews most helpful in my writing of the lit review: (Chen and Jonas, 2017; Jackman and Regehr, 2017; Südhof, 2002)

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Introduction

Considerable evidence is now available to indicate that the brain's ability to modulate connections between neurons underlies learning and memory. The contributions of Eric Kandel's work taught us that alterations to synapses can underlie changes in the behavior of animals as simple as *aplysia*. The Hebbian theory teaches us that activity between neurons can determine the nature of their connection. And the seminal work of Hubel and Wiesel taught us that sensory stimuli critically shaped the development of the function of connections within brain regions.

The primary form of communication between neurons is the release of neurotransmitters across synapses, the junction between neurons. The passage of neurotransmitters across the synapse causes a change in the membrane potential of the post-synaptic neuron, leading the post-synaptic neuron to depolarize in excitatory synapses. Alteration of the signal carried by synapses is known as synaptic plasticity. Synaptic plasticity affects the transmission of electrical potentials between neurons by enhancing or limiting this transmission through various mechanisms altering one or both sides of the connection. Changes to synaptic transmission can last seconds to years with the forms of long-term synaptic plasticity. But with short-term plasticity, synapses throughout the body have the incredible ability to modulate their transmission in timespans of mere milliseconds.

Synaptic facilitation is a form of short-term plasticity that enhances synaptic transmission for less than a second. The reverse phenomenon of facilitation, short-term depression, can be mostly attributed to the depletion of the readily releasable pool (RRP) of neurotransmitter-containing synaptic vesicles. (Zucker and Regehr, 2002) Despite vesicular depletion leading to short-term depression, mechanisms of synaptic facilitation act at numerous synapses throughout the nervous system to increase the amount of neurotransmitter released by successive stimulations.

A Terse History of Synaptic Facilitation

Early in the study of synaptic transmission, physiologists observed that closely spaced stimuli cause a larger synaptic response in the frog neuromuscular junction (NMJ). (Eccles et al., 1941) Facilitation is common in synapses with low initial probability of release (p) of synaptic vesicles. In the NMJ, p can be raised by increasing the external calcium concentration. By raising the p enough, the synapse is transformed from a facilitating synapse to a depressing synapse with a larger initial post-synaptic current (Jackman and Regehr, 2017). The rule-of-thumb that low initial p synapses facilitate while high initial p synapses show short-term depression generally holds.

Early research determined that facilitation reflected an increase in release events as opposed to an enhanced response to an equal number of releases (del Castillo and Katz, 1954). The theory that greater amounts of residual calcium in presynaptic terminals after firing led to larger amounts of neurotransmitter released was proposed by Katz and Miledi (1968). This residual calcium hypothesis was supported by the findings of Dodge and Rahamimoff (1967), who observed that small increases in calcium entry could produce large changes in synaptic strength. Studies of facilitation have largely supported the residual calcium hypothesis of facilitation, which leads to the central question of my thesis: What calcium-dependent factors are responsible for the observed increase in *p* in facilitating synapses?

$Ca_V2.1$ Calcium Channels in Short-term Synaptic Plasticity

One key mechanism required for short-term synaptic plasticity is regulation of P/Q-Type calcium channel by neuronal calcium sensor (CaS) proteins. Voltage-gated calcium channels in eukaryotes are a complex of multiple subunits expressed by separate genes. These channels are typically characterized by the identity of their $\alpha1$ subunit, the central and largest unit. While not sufficient for full function of eukaryotic calcium channels, the $\alpha1$ subunit contains the voltage sensors, gating apparatus and conductive pore. P/Q-Type channels are distinguished from other neuronal calcium channels by their $\alpha1$ subunit, $Ca_V2.1$, which is coded for by the gene CACNA1A. There is strong evidence for the importance of $Ca_V2.1$ channels in facilitation: one CACNA1A knockout study found synaptic facilitation was entirely eliminated from the Calyx of Held, despite $Ca_V2.2$ channels compensating for normal basal transmission (Ishikawa et al., 2005).

One of multiple calcium channel types found in neurons, $Ca_V2.1$ channels have an added feature of regulation by calcium sensor proteins. Repetitive stimulation of $Ca_V2.1$ channels causes calcium (Ca^{2+})-dependent facilitation (CDF) followed by Ca^{2+}-dependent inactivation (CDI) (Lee et al., 2000). CDF and CDI of the calcium current are both reliant on binding of the calcium sensor calmodulin (CaM) binding to $Ca_V2.1$ channels. This interaction with calmodulin has been mapped to a bipartite site upon the channels' c-terminal domain (CTD), which is composed of an IQ-like motif (IM) and a Calmodulin binding domain (CBD) (DeMaria et al., 2001; Lee et al., 1999; Lee et al., 2003). In addition, the Catterall lab has shown that neuronal CaS proteins (e.g CaBP-1, VILIP-2, and NCS-1) regulate $Ca_v2.1$ channels (Leal et al., 2012). In cultured superior cervical ganglion (SCG) neurons, mutations that prevent CaS-dependent facilitation and inactivation of transiently expressed $Ca_v2.1$ channels impair short-term synaptic facilitation and rapid synaptic depression of the postsynaptic response (Catterall et al., 2013). Therefore,

CaS-dependent facilitation and inactivation of $Ca_V2.1$ channels can mediate short-term synaptic facilitation and depression.

The importance of the CaS - $Ca_V2.1$ regulation was interrogated in slice electrophysiology using a genetic knock-in mouse where the IM domain is mutated in a way which prevents CaM from binding it. In hippocampal slices of the Schaffer collaterals of these mutant IMAA mice, our lab found that paired pulse facilitation was cut in half compared to wild-type (Nanou et al., 2016b). Interestingly, the addition of a cell-permeable strong calcium chelator (EGTA-AM) in the IMAA slices completely abolished paired pulse facilitation across the synapse. This suggests that distal or slow calcium sensor proteins, yet to be identified, are responsible for the molecular generation of synaptic plasticity.

CaS proteins are not the only calcium sensors known to interact with $Ca_V2.1$ channels. Located on the intracellular loop between domains II and III of $Ca_V2.1$ (as well as $Ca_V2.2$), the *syn*aptic *pr*otein *int*eraction (synprint) site binds to proteins of the SNAp REceptor (SNARE) complex, including SNAP-25, Synaptotagmin-1 (Syt1) , and Syntaxin-1A (Sheng et al., 1994; Sheng et al., 1997). This interaction allows proximity anchoring of the molecular machinery responsible for vesicle release to the source of Ca^{2+} current. Intriguingly, interaction of SNAP-25 with $Ca_V2.1$ channels initiates faster inactivation of the $Ca_V2.1$ calcium current in response to a train of stimuli, while formation of a complete SNARE complex by binding of Syntaxin-1A and Synaptotagmin-1 relieves this inhibition (Zhong et al., 1999). This evidence provided precedence that the binding of SNARE proteins could affect the function of the channel itself, much like CaS regulation of Ca_V current.

The Enigmatic Role of Synaptotagmin 7

In 2016, a breakthrough paper provided a prime potential molecular mediator of short-term synaptic facilitation. Specifically, slice experiments in a knockout mouse line proved that the calcium sensor Synaptotagmin 7 (Syt7) is necessary for synaptic facilitation at many CNS synapses (Jackman et al., 2016). Across four known facilitating synapses, the Regehr lab found Syt7 knockout (KO) mice showed no paired pulse facilitation. However, more recent results have found that the effects of Syt7 on short-term facilitation are not uniform among CNS synapses. In contrast to the Schaffer collateral, cortical-thalamic tract, mossy fibers, and perforant pathway synapses studied by the Regehr Lab, KO of Syt7 does not impair paired pulse facilitation in the Calyx of Held synapses (Luo and Sudhof, 2017).

These findings came at a time when the roles of Syt7 are still being explored and are relatively uncharted. Its family members Syt1 and Synaptotagmin-2 (Syt2) have been proven to be the major fast release Ca^{2+} sensors across the nervous system (Brose et al., 1992; Chapman, 2002; Südhof, 2002). In contrast, Syt7 was originally discovered as a slow Ca^{2+} sensor for lysosome fusion (Chen and Jonas,

2017). Its roles in endocrine and neuropeptide secretion were studied but its role in neurotransmission was far more nebulous. But Syt7, as a calcium sensor protein, has many factors which make it stand out amongst the 16 other mammalian Synaptotagmins. Syt7, along with Syt1 and Syt4, are the only family members that are found in all metazoans, suggesting crucial molecular roles that may be conserved. It also has the most unique amino acid sequence of all the Synaptotagmins, including a long alternatively spliced region that gives it more than 12 splice variants differentially expressed around the body. In the brain, expression of Syt7 is widespread throughout most brain sections. Finally, it has the strongest affinity to Ca^{2+} within the Synaptotagmin family, paired with slow binding kinetics. The search to understand Syt7's role in neurons has given it many suggested roles in synaptic transmission by various groups.

As mentioned before, the Regehr group has establish that Syt7 is critical for synaptic facilitation in hippocampal and coriticothalamic synapse, and confers frequency invariance to the Purkinje Cell-Deep Cerebellar Nuclei synapse (Jackman et al., 2016; Turecek et al., 2017). Another set of results has identified that Syt7, exclusively among Synaptotagmins, binds CaM and suggests it serves in the replenishment of synaptic vesicles (Liu et al., 2014). Although the Sudhof group did not find any absence of facilitation in their study of the Calyx of Held of Syt7 KO mice, they found that Syt7 played a critical role in asynchronous release at the synapse (Luo and Sudhof, 2017). Given the diversity of Syt7 splice forms and its presence across the plasma membrane of neurons, it is quite possible that Syt7 serves all of these roles and more in neurons (Dean et al., 2012).

Thesis Aim

The importance of both Synaptotagmin 7 and $Ca_V2.1$ channels independently in the generation of short-term facilitation raises the enticing possibility that the channel and calcium sensor may operate in a shared pathway. $Ca_V2.1$ channels interact with SNARE proteins, in particular Syt1, as well as calcium sensor proteins. This suggests that $Ca_V2.1$ channels may have a similar interaction with Syt7, which, in turn, may have a critical function in synaptic plasticity in neurons, allowing for short-term facilitation in neurons which display it. The goal of my thesis research was to develop a foundation of understanding how the $Ca_V2.1$ channels and Syt7 interact molecularly. With more time, the functional purpose of the relationship between these two pre-synaptic players may illuminate the unclear mechanism of synaptic facilitation.

Synaptotagmin 7 Interacts with $Ca_V2.1$ Calcium Channels in Brain Tissue

Introduction

The pilot experiment of my studies was an inquiry into whether a molecular interaction between these specific calcium sensor and calcium channel would be observed in brain samples. The prospect of Syt7 and $Ca_V2.1$ channels operating together in a functional pathway toward the creation of synaptic facilitation became plausible after discovering that these proteins do associate in neurons. For this simple question, I performed my first immunoprecipitations.

Methods

Mouse Brain Lysate Co-Immunoprecipitation

Whole brain tissue samples were taken from wild-type C57BL/ 6 mice at or over the age of P21. Brains were manually homogenized on ice in 320 mM Sucrose, 5 mM Tris pH 7.4 with sigmaFAST proteinase inhibitor cocktail added. Tissue debris was pelleted by centrifugation at low speed for 10 minutes at 4°C and discarded. The supernatant was layered atop an equal volume 1.2 M Sucrose, 5 mM Tris pH7.4 with proteinase inhibitors added in order to create a sucrose gradient. Solubilized protein was isolated in an interface layer of approximately .8M Sucrose between solutions by high speed ultra-centrifugation for 30 minutes at 4°C. Concentrated protein in the interface layer was extracted and resuspended in 800 mM Sucrose, 5 mM Tris pH 7.4 with proteinase inhibitors added. Protein was then pelleted by high speed centrifugation in a benchtop centrifuge at 4°C for 20 minutes. Sucrose solution were aspirated from protein pellets and protein was resuspended in RIA buffer (25 mM Tris, 150 mM NaCl, 1 mM EDTA, 2% Triton X-100, adjusted to pH 7.4 with NaOH). Protein was solubilized in Triton-containing solution by rotating end-over-end at 4°C for 1-2 hours. Remaining solid debris was removed from solution by high speed centrifugation in a benchtop centrifuge at 4°C for 30 minutes. Protein concentration was measured using a Bichinonic acid (BCA) assay and samples were aliquoted to contain 50 mg of protein each.

Samples were then pre-cleared with Protein G-coated Dynabeads with a short 30-minute wash at 4°C. After removal from pre-clear beads, samples were incubated overnight with no antibody, control antibodies or antibody specific to $Ca_V2.1$ (in house antibody described in (Westenbroek et al., 1995). The following day, the sample-primary antibody mixtures were mixed with washed fresh Protein-G coupled magnetic dynabeads and allowed to mix while rotation for 30 minutes at room temperature. The beads with protein were then washed five times with RIA buffer with appropriate concentration of free

calcium. Antibody-bound sample protein was then eluted from beads using RIA buffer, NuPage 4x LDS Sample Buffer and 120 mM Dithiothreitol (DTT), incubated for 15 minutes at 70°C and run on 4-20% SDS-Page gels. The protein gels were transferred onto nitrocellulose membranes, blocked in 5% Milk, 1% BSA TBS-T then incubated with commercial antibodies to stain for the presence of Syt7, Syt1, and $Ca_V2.1$.

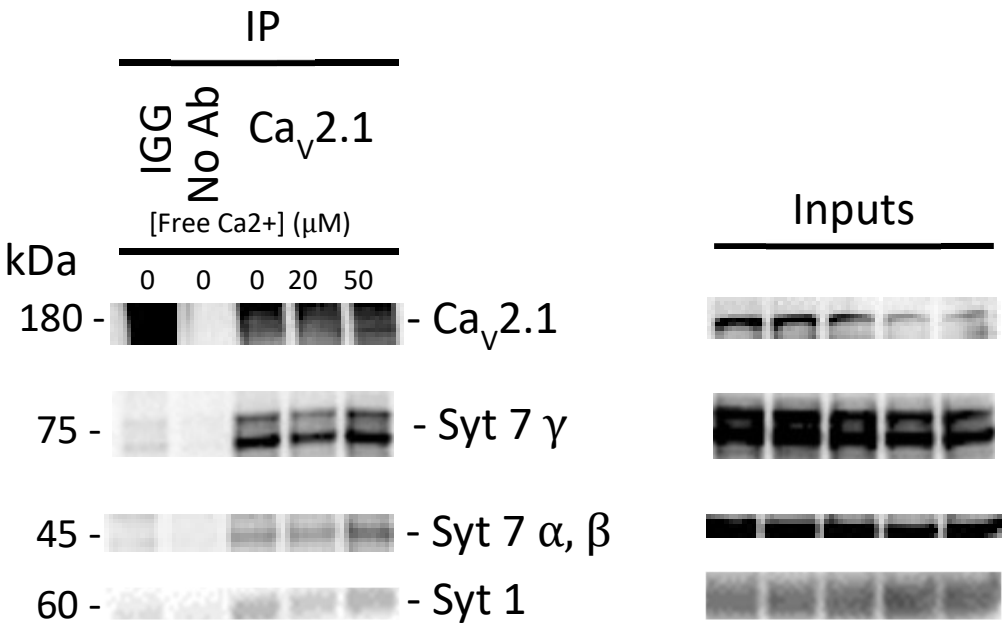
After applying secondary antibodies, ECL and imaging, blots were analyzed by quantifying pixel density in Image Lab software. First, the signal intensity in IP bands were compared to corresponding input bands. Then the ratios were normalized to the 0 μM free Ca^{2+} $Ca_V2.1$ IP condition ratio.

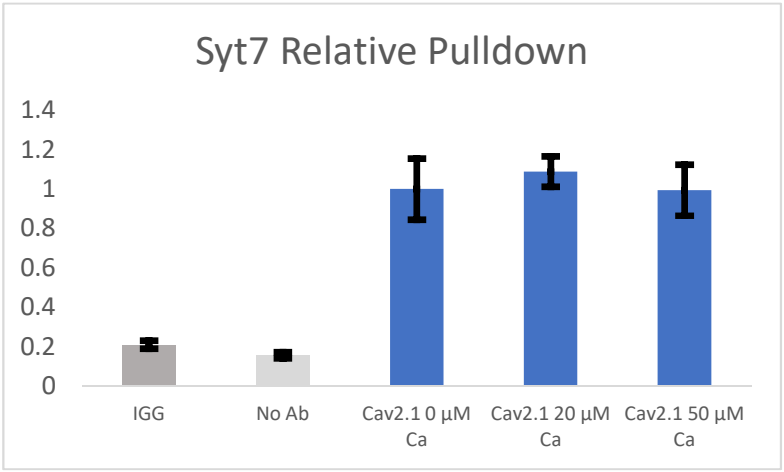
Results and Discussion

Syt7 was pulled down from whole brain protein using a $Ca_V2.1$- specific antibody, suggesting an interaction in neuron membranes. (Figure 1) The immunoprecipitations were carried out in RIA solutions containing 1 mM EDTA with additional $CaCl_2$ added according to Maxchelator to bring the approximate free Ca^{2+} of the solution to Ca^{2+} concentrations similar to active zones during excitation, 20 and 50 μM. Surprisingly, the interaction between Syt7 and $Ca_V2.1$ does not seems to have a strong calcium dependence. At free Ca^{2+} concentrations of 0, 20, and 50 μM, Syt7 was successfully pulled down. Syt7 staining in both lysate and pulldown consistently contained bands of three different molecular weights, approximately 45, 75 and 80 kDa, respectively. The size of 45 kDa is consistent with the size of Syt7 α, the predominant neuronal isoform of the calcium sensor. The larger molecular weights, consistently observed over repeats, are likely to be Syt7γ or Syt7 carrying post-translation modification.

Previous results showed that Syt1 binds to $Ca_V2.1$ and $Ca_V2.2$ synprint peptides. Our results confirmed that Syt1 can also be pulled down from *in situ* samples. The calcium-dependency of Syt1 pull-down was variable, suggesting a mix of rbA and BI isoforms of $Ca_V2.1$ as per the results of Kim and Catterall (1997).

Figure 1. Co-immunoprecipitation of Syt7 and Syt1 with $Ca_V2.1$ in Whole Brain Lysate





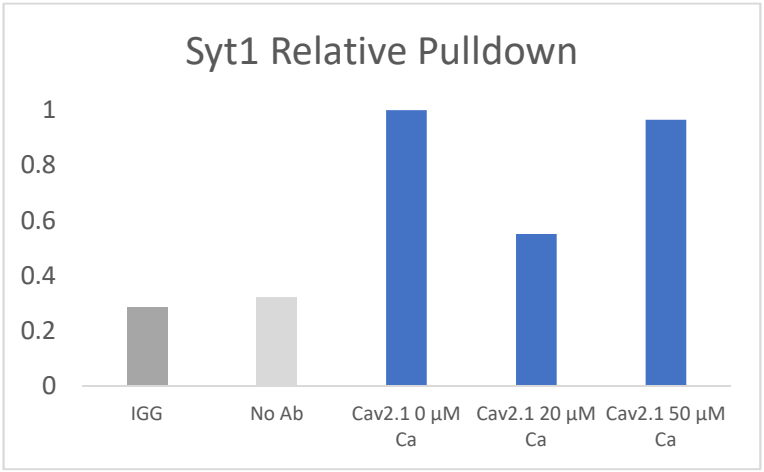


FIG 1. Mouse brain lysate protein precipitated with $Ca_V2.1$- specific antibody is strongly enriched for the presence of Syt7 and Syt1 across three different buffered concentrations of free Ca^{2+}. Syt7, n = 3, Syt1, n=2

Synaptotagmin 7 Interacts with $Ca_V2.1$ Calcium Channels While Co-Expressed in Cultured Non-Neuronal Cells

Introduction

The results in brain lysate sample are encouraging, but do not provide strong enough evidence to prove a functional relationship alone. For one, the active zone in which calcium channels reside are densely populated with hundreds of other proteins acting in the release, replenishment, or recycling of synaptic vesicles. (Müller et al., 2010) The interconnected molecular machinery of the SNARE complex, RIM proteins, and PKC, among others, are intertwined through numerous bindings and calcium-dependent interactions. A simple precipitation of the calcium channels in mild detergents yields hundreds of proteins, some of which may not directly bind to the channel itself. Thus, stronger evidence for a direct link between Syt7 and $Ca_V2.1$ would need to be gathered from outside of the pre-synaptic complex.

To this end, I next moved to replicate my findings in mice brain samples in transfected cultured mammalian cell lines. The pervasive human embryonic kidney (HEK) cell lines do not express the numerous proteins of a neuron's active zone, so I ventured to replicate the findings of my whole brain studies in transfected HEK cells.

The system of transfected cells gave an emergent property to this iteration of the experiment. With separate expression constructs for the three primary neuronal isoforms of Syt7, experiments determining whether each of the three isoforms interact with $Ca_V2.1$ independently became possible.

Methods

Cloning

Synaptotagmin 7 (Syt7) alpha, beta, and gamma mouse cDNA expression constructs were obtained from the Lab of Edwin Chapman, originally produced in the lab of Katsuhiko Mikoshiba. (Fukuda et al., 2002) Primers were designed using the Ensembl sequence for Syt7 mouse cDNA and were produced as follows: 5' – ATATTAGCTAGCATGTACCGGGACCCG – 3' (5' Syt7 cds end with NheI restriction site and buffer bases added) and 5' – ATTATAGGGCCCTCAGGCTTTCAGCTG – 3' (3' Syt7 cds end with ApaI restriction site and buffer bases added). Using the Q5 High-fidelity DNA Polymerase kit, Syt7 alpha, beta, and gamma cds inserts were generated using the original expression constructs as templates. All three cds inserts were digested then cloned into the Invitrogen mammalian expression vector pC3.1 (+) using the NheI and ApaI restriction sites. Clones were validated by Sanger sequencing from GENEWIZ and

positive staining and size verification of protein produced by tsA-201 cells transfected with experimental clones using the Polyjet transfection system.

Cell Culture and Protein Expression

tsA201 cells were thawed from laboratory -80°C stocks and grown in Hyclone 1:1 DMEM/F12 media, 10% FBS, Penicillin/Streptomycin on 100mm Corning cell culture petri dishes. Cells were split and passaged every 2-3 days until p40 or poor health was displayed.

For transfection, the Signagen Polyjet protocol was followed: cell dishes 70-90% confluent were washed with 1x PBS and incubated in a smaller volume of serum-free media for a period of approximately one hour. Expression constructs for Cav subunits $\alpha 1$, $\beta 2a$, and $\alpha 2\delta$ and expression contructs for Synaptotagmin 1 or Synaptotagmin 7 alpha, beta, and gamma isoforms were mixed with Polyjet transfection reagent in serum-free medium and incubated at room temperature for 10 minutes. DNA-Polyjet mixture was then added to cells and left to incubate for 12-18 hours. Transfection media was then replaced with standard serum-containing medium and cells were left to generate protein for an additional 36 hours.

48-72 hours after transfection, cells were harvested from dishes using dishes using a plastic scraper and pelleted by a medium speed spin at 4°C for 20 minutes. Cells were washed with ice-cold proteinase inhibitor-containing PBS and re-suspended in lysis buffer (50 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 320 mM Sucrose, 1 mM PMSF, with proteinase inhibitor cocktail added) by trituration. Nuclear fraction and insoluble debris were pelleted by a low speed spin in a benchtop centrifuge at 4°C for 5 minutes. Supernatant was transferred to new tubes and then spun at a high speed in a benchtop centrifuge at 4°C for 30 minutes in order to pellet remaining protein. Supernatant was removed from pelleted protein and then pelleted protein was washed with additional lysis buffer and then spun for an additional 30 minutes at 4°C. Lysis buffer wash was then removed and pellet was re-suspended in RIA buffer (25 mM Tris, 150 mM NaCl, 1 mM EDTA, 2% Triton X-100, adjusted to pH 7.4 with NaOH) to solubilize protein.

Immunoprecipitation

Samples were pre-cleared with Protein G-coated Dynabeads with a short 30 minute wash at 4°C. After removal from pre-clear beads, samples were incubated overnight with no antibody, control antibodies or antibody specific to $Ca_V2.1$ (in house antibody described in (Westenbroek et al., 1995). The following day, the sample-primary antibody mixtures were mixed with washed fresh Protein-G coupled magnetic Dynabeads and allowed to mix while rotation for 30 minutes at room temperature. The beads

with protein were then washed five times with RIA buffer with appropriate concentration of free calcium. Antibody-bound sample protein was then eluted from beads using RIA buffer, NuPage 4x LDS Sample Buffer and 120 mM Dithiothreitol (DTT), incubated for 15 minutes at 70°C and run on 4-20% SDS-Page gels. The protein gels were transferred onto nitrocellulose membranes, blocked in 5% Milk, 1% BSA TBS-T then incubated with commercial antibodies to stain for the presence of Syt7, Syt1, and $Ca_V2.1$.

After applying secondary antibodies, ECL and imaging, blots were analyzed by quantifying pixel density in Image Lab software. First, the signal intensity in IP bands were compared to corresponding input bands. Then the ratios were normalized to the 0 μM free Ca^{2+} $Ca_V2.1$ IP condition ratio.

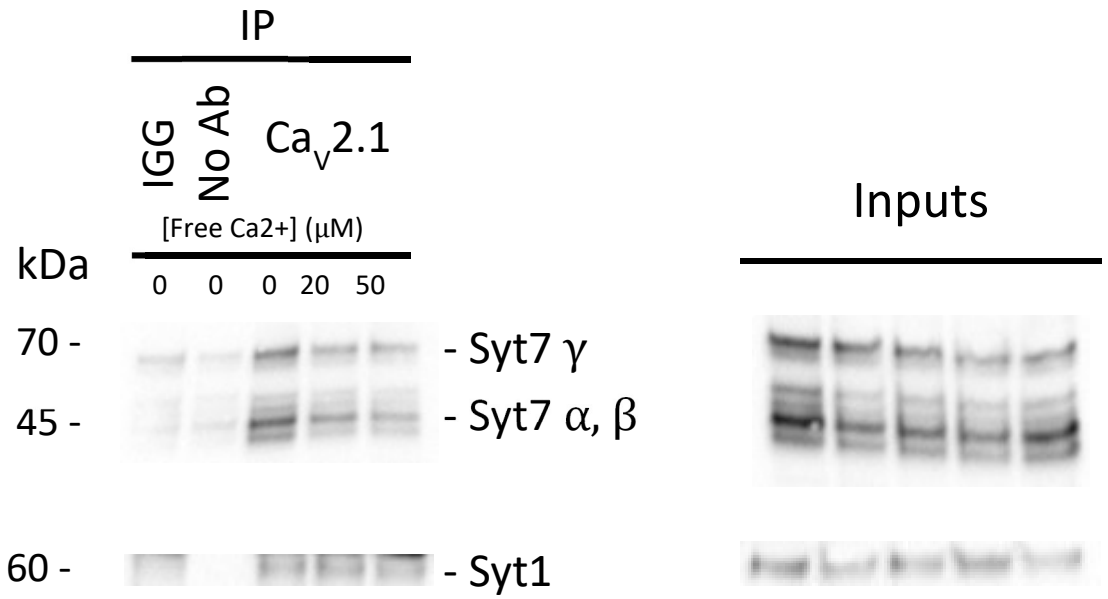
Results and Discussion

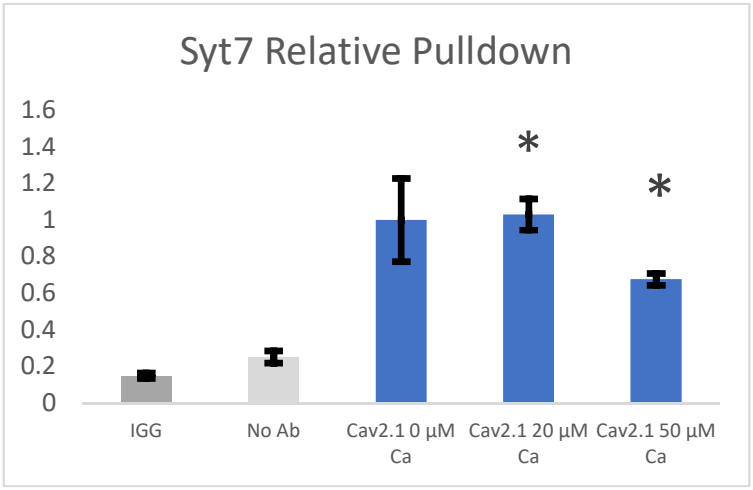
This time in transfected HeK cells, Syt7 was pulled down using a $Ca_V2.1$- specific antibody, suggesting a molecular interaction between the two proteins. (Figure 2) Given the lack of other active zone proteins expressed, this lends credence to the hypothesis that $Ca_V2.1$ and Syt7 bind directly. Once again, a calcium-dependency of this binding is not evident. With a degree of variability, Syt7 binds to the $Ca_V2.1$ at all the concentrations of free Ca^{2+} tested.

As a positive control, another condition of cells was transfected with $Ca_V2.1$ channels along with Syt1. In this condition, Syt1 was also successfully co-precipitated, supporting the validity of this model, given prior results.

In additional experiments, experimental conditions of cells transfect with $Ca_V2.1$ channels and just a single isoform of Syt7, all three tested isoforms (α, β, & γ) independently co-precipitated with $Ca_V2.1$. (Suppl. Fig.) This indicates that the interaction between $Ca_V2.1$ and Syt7 is not isoform-specific.

Figure 2: Co-immunoprecipitation of Syt7 and Syt1 with $Ca_V2.1$ from co-transfected tsa201 cells





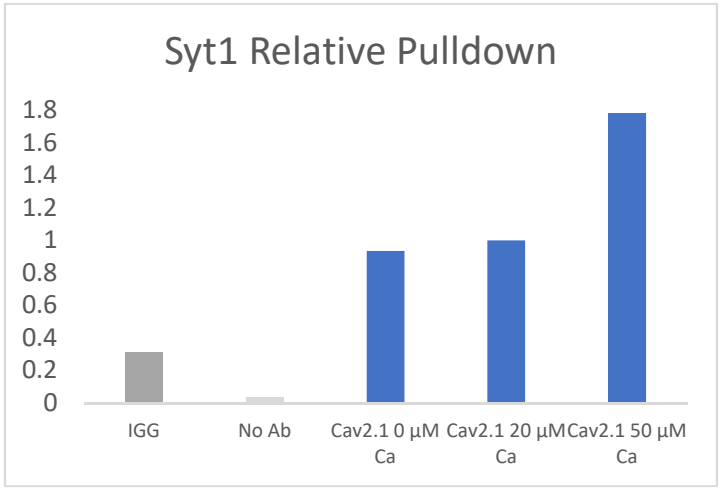


FIG 2. Syt7 is pulled down by $Ca_V2.1$-specific antibodies in co-expressed HEK cell lysates. In cells co-expressing Syt1 and $Ca_V2.1$, Syt1 is pulled down by $Ca_V2.1$-specific antibodies. Syt7, n = 3, Syt1, n=2

Future Direction: Determining Binding Domain and Conditions of Synaptotagmin 7 and $Ca_V2.1$ *in vitro*

Introduction

With my evidence for a molecular interaction between Syt7 and $Ca_V2.1$, the next aim of my project is to definitively prove a direct interface between the two proteins and isolate the domain of the $Ca_V2.1$ protein which binds Syt7. While the $Ca_V2.1$ protein is a massive 190 - 250 kDa, (depending on the isoform) past research has given us a very likely candidate domain within an intracellular region of the protein. As mentioned before, $Ca_V2.1$ has a synprint site on the intracellular loop between the II and III homologous domains. This site, found on residues 722-1036 on the rat $Ca_V2.1$ sequence, has been shown to bind to SNARE proteins SNAP25, syntaxin, and, most importantly, Synaptotagmin 1 (Syt1) (Kim and Catterall, 1997). The direct binding of Syt1 suggests that family member Syt7 may share the same binding site.

There is an alternative possible binding site for Syt7 on the intracellular regions of $Ca_V2.1$. As discussed before, ample evidence from our lab has proven that CaM directly binds the IM and CBD sites on the C-terminal domain (CTD) of $Ca_V2.1$. (Lee et al., 1999) Syt7 also binds CaM in a calcium-dependent manner, a unique property among Synaptotagmins (Liu et al., 2014). This raises the potential for a tri-partite interaction between $Ca_V2.1$, Syt7, and CaM, which would presumably occur at the IM or CBD sites.

For the purposes of proving a direct interaction and identifying the binding domain *in vitro*, I set about cloning fusion peptides containing the synprint and CTD sites, as well as Syt7 itself.

Methods

Fusion Protein Construct Preparation

Fusion constructs for bacterial expression of GST-SNAP25, GST-Synatxin 1A, GST-Synaptotagmin 1 as described in Sheng et al. (1996) and Kim and Catterall (1997) were obtained from laboratory stocks. For the cloning of GST-Calmodulin (CaM): A construct containing the cDNA of rat gene *Calm2* was used as template for an insert amplification using the following primers: 5’ – AGCTACGTCGACATGGCTGAT CAGCTGAC – 3’ (CaM cds forward edge with SalI site added) and 5’ – TGCACCGCGGCCGCTCATTTTGCAGTCATCAT – 3’ (CaM cDNA reverse edge with NotI site added) For the cloning of GST-Synaptotagmin 7: the previously made pc3.1-Synaptotagmin 7 alpha expression construct was used as template for an insert amplification using the following primers with the Q5 High-fidelity

DNA Polymerase kit: 5’ – TCAGATGTCGACATGTACCGGGACCCG – 3’ (Syt7 cds forward edge with SalI site added) and 5’ – ATTATCGCGGCCGCTCAGGCTTTCAGCTGG – 3’ (Syt7 cds reverse edge with NotI site added). Both CaM and Syt7 inserts were cut with SalI and NotI and ligated into empty GE Healthcare Life Sciences vector pGEX-4T2 cut in the same fashion.

For the cloning of MBP-Synprint and MBP-CTD, residues of the rat $Ca_V2.1$ channel intracellular regions were chosen based on previous publications. Based on (Sheng et al., 1998), the residues chosen for the synprint fusion protein were residues 722 – 1036. Based on (Lee et al., 2003), the residues chosen for the CTD fusion protein were residues 1848 – 2050. The following primers were designed to amplify, using the Q5 High-fidelity DNA Polymerase kit, the cDNA of these regions and add NdeI and SalI restriction sites to the respective ends of both inserts: 5’ – GCGTACCATATGGCCCAGGAACTCACC – 3’ (Synprint forward with NdeI site added) 5’ – ATTTATGTCGACGGCAGGCTCCCCGGT – 3’ (Synprint reverse with SalI site added) 5’ – CCGTCCATATGTTGATGGCTCTGATCCG – 3’ (CTD forward with NdeI site added) 5’ – TATTAAGTCGACTGCTGGGAGGCGGGG – 3’ (CTD reverse with SalI site added) Both Synprint and CTD inserts were cut with NdeI and SalI and ligated into empty New England Biolabs vector pMAL-c5X cut in the same fashion.

Clones were validated by colony PCR and Sanger sequencing ordered from GENEWIZ. Standard sequencing primers 5GEX and 3GEX were used for pGEX constructs while the following primers were used in the sequencing of pMAL constructs: 5’ – GGTCGTCAGACTGTCGATGAAGCC 3’ – TGTCCTACTCAGGAGAGCGTTCAC.

All validated constructs were transformed into competent BL21 cells and stored at -80°C in glycerol stocks.

Fusion Protein Production

Glycerol stocks of expression construct-transformed cells were spiked into small volumes LB-ampicillin and incubated for 12-18 hours whilst shaking at 37°C. A larger volume of LB-ampicillin was spiked with a fraction of the preculture and then grown until the OD600nm of the culture was 0.9-1.1. Protein expression was then induced by the addition of 100 μM IPTG and cells were incubated shaking at 11°C for 16 hours. After induction, cells were harvested by centrifugation at a medium speed at 4°C for 10 minutes. Cells were washed three times in ice cold PBS and centrifuged at a medium speed at 4°C for 5 minutes. Then, cells were resuspended in smalls volumes of proteinase inhibitor-containing PBS and incubated with lysozyme for one hour on an ice tray upon an orbital shaker.

Sample were then sonicated 20 times on a 50 duty cycle %, 5 output control. Solubilized protein was then separated from cell debris by high speed centrifugation at 4 at a medium speed at 4°C for 30 minutes. The resulting supernatant was directly used for binding experiments without purification.

Expression Test

Crude supernatants prepared for western blot by the addition of 120 mM DTT and 2x NuPage LDS sample buffer. Following an incubated for ~10 minutes at room temperature, samples were run on 4-20% SDS-Page gels. The protein gels were transferred onto nitrocellulose membranes, blocked in 5% Milk, 1% BSA TBS-T then incubated with commercial antibodies to stain for the presence of GST, MBP, Syt7, and Syt1.

Binding Assay

Fusion protein samples were mixed according to experimental condition, along with washed amylose resin, to gently mix for 12 – 16 hours at 4°C. Amylose-protein complex was then loaded into chromatography columns, allowed to drain, and washed three times with protease inhibitor-containing PBS. Protein was then eluted from resin using TBS buffer containing 10 mM Maltose. Eluted protein was then prepared for western blot by the addition of 120 mM DTT and 2x NuPage LDS sample buffer. Following an incubated for ~10 minutes at room temperature, samples were run on 4-20% SDS-Page gels. The protein gels were transferred onto nitrocellulose membranes, blocked in 5% Milk, 1% BSA TBS-T then incubated with commercial antibodies to stain for the presence of GST and MBP.

Materials

Figure 3: Synprint, CTD and SNARE fusion proteins are recognized by antibodies for protein identity and MBP or GST expression

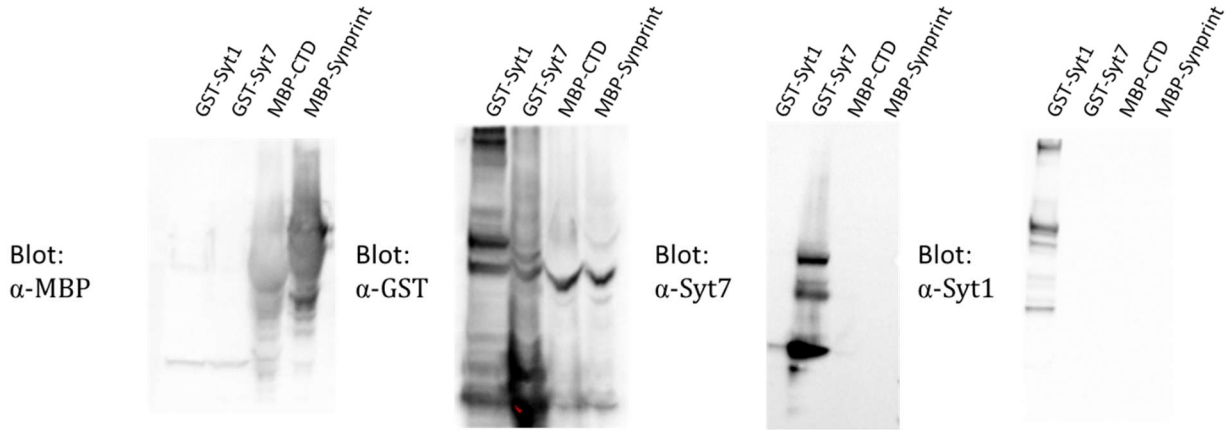


Fig 3: Fusion constructs positively stain for MBP, GST, and Synaptotagmin expression.

Discussion

The materials for this important binding assay were completed by me and I was able to verify the constructs with the above expression tests. Limited time only allowed one repeat of the binding assay itself, and sadly, the results were muddled and inconclusive. The materials I have produced will hopefully allow for the completion of this binding assay. When completed, the experiment has the potential to confirm a direction interaction, isolate the binding domain, or identify exactly which intermediaries or calcium concentrations are sufficient for the binding of Syt7 and $Ca_V2.1$.

The results of this experiment will likely suggest different functional roles for this interaction. The binding partners of the synprint, IM and CBD sites have similar functions in their interactions with $Ca_V2.1$ that are distinct from the other group. Following this logic, an interaction at the synprint site would suggest $Ca_V2.1$ plays an anchoring role for a primarily vesicular role of Syt7 in transmission. An interaction at the CTD sites of IM or CBD suggests that an effect of Syt7 on $Ca_V2.1$ calcium channel currents is more likely.

Future Direction: Investigating Transfected P/Q-Type Calcium Channels for Electrophysiological Effects of Synaptotagmin 7 Co-expression

Introduction

With evidence supporting our hypothesis of an interaction between $Ca_V2.1$ calcium channels and Syt7, the overriding question of my inquiry quickly becomes the functional significance of this interaction. One potential function of this interaction, which would contribute to short-term facilitation, is modulation of the $Ca_V2.1$ calcium current. The precedent of SNAP25 altering the inactivation properties of $Ca_V2.1$ demonstrates that SNARE proteins can alter the physiological properties of bound calcium channels. (Zhong et al., 1999) Therefore, our first direction in the investigation for a functional effect was searching for any alterations of channel currents.

Methods

tsa201 cells were thawed from laboratory -80°C stocks and grown in Hyclone 1:1 DMEM/F12 media, 10% FBS, Penicillin/Streptomycin on 100mm Corning cell culture petri dishes. Cells were split and passaged every 2-3 days until p40 or poor health was displayed.

For transfection, cells were split onto 6-well plates and grown until 70-90% confluent. Using the Mirus transfection system, cells were then transfected with expression constructs for Cav subunits $\alpha 1$, $\beta 2a$, and $\alpha 2\delta$ in addition to an expression construct of Synaptotagmin 7 alpha (experimental) or an empty version of the same expression vector, one well per condition. 18-24 hours after transfection, cells were split from their 6-well plate onto 10 35mm petri dishes for each condition. 40 – 72 hours later, cells were recorded using Patchmaster software. Recordings were performed in the following solutions: Extracellular: 10 mM $CaCl_2$ or 10 mM $BaCl_2$, 150 mM Tris, 1 mM MgCl 2 (305 mos M) Intracellular: 120 mM N-methyl-D-glucamine, 60 mM Hepes, 1 mM $MgCl_2$, 2 mM Mg-ATP, and 0.5 mM EGTA (295 mos M). Data would then be exported to IGOR for analysis had the experiment proceeded properly.

Status

All materials and data of this aim have been transferred to members of the Catterall lab who will further pursue these studies.

Future Direction: Understanding the Contribution of Facilitation to Circuit Function and Animal Behavior

How the loss of short-term synaptic facilitation in the Syt7 KO mouse affects the behavior of the animal has yet to be determined. Our lab's transgenic IMAA mouse has impaired facilitation at synapses and displays deficits in learning and memory. (Nanou et al., 2016a) Therefore, the possibility that the physiological phenotype of the Syt7 KO mouse may also show a learning deficit is considerable. In the future, these two mouse lines could be crossed to explore whether heterozygotes of both mutations, for which independently do not present phenotypes, may produce a behavioral phenotype. The original scope of my project included using the Syt7 homozygous KO mouse and the Syt7 & IMAA heterozygous mice, context-dependent fear conditioning and the Barnes circular maze tests to determine if observed functional phenotypes causes any deficits in spatial learning in these animals. These experiments on the Syt7 KO could shed light on how short-term synaptic physiological phenotype alter an animal's learning and memory, whereas the double het experiments will illustrate if $Ca_V2.1$ and Syt7 share a common pathway in animal learning.

Conclusion

My results suggest that Synaptotagmin 7 and the α1 subunit of PQ-Type Calcium Channels, $Ca_V2.1$, have a molecular interaction, which is likely direct. I have created materials to conclusively prove the direct nature of this binding, as well as elucidate the site where this binding occurs upon $Ca_V2.1$. The critical roles of Syt7 and $Ca_V2.1$ for synaptic facilitation in certain synapses give the exciting possibility that this interaction may be instrumental in the mechanism of facilitation. The exact function of this interaction will require additional experiments to identify. Understanding the binding domains and properties of this interaction will allow future research via the possibility of disrupting the interaction through targeted mutation, which may be the key for discovering the functional role of the interaction.

Syt7's suggested roles in the literature, RRP replenishment or asynchronous release sensing, may be independent of its relationship to $Ca_V2.1$. However, if the anchoring of Syt7 to $Ca_V2.1$ is supportive to these roles, disrupting the interaction experimentally may yield insight into the mechanisms of this pathway and validate the importance of the interaction. The ongoing study into an effect of Syt7 activity on $Ca_V2.1$ calcium channel function could also be a key mechanism in facilitation. With extensive study of Syt7 being performed on many fronts, the enigmatic mechanisms of synaptic facilitation may soon be revealed. Perhaps, the pathway explored in this thesis may be a contribution towards those exciting discoveries.

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