

**Vesicle-associated membrane protein (VAMP)/synaptobrevin-2
protein mutants functionality test in synaptic vesicle fusion**

Sophia Praggastis

Committee Members:

Dr. Jingshi Shen, MCDB, Thesis Advisor

Dr. Xuedong Liu, CHEM

Dr. Jerry Rudy, PSYCH

Dr. Ravinder Singh, MCDB

University of Colorado at Boulder

Department of Molecular, Cellular, and Developmental Biology

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I. Abstract

Release of neurotransmitters to complete synaptic transmission is dependent upon the concerted action of the SNARE and SM (Sec1/Munc18) protein families. The SNARE complex, formed during synaptic vesicle fusion, is made up of syntaxin-1, SNAP-25, and synaptobrevin/VAMP. This process is catalyzed by the SM protein Munc18. We are interested in determining how the SNARE complex and VAMP2 are involved in the fusion of synaptic vesicles during the process of synaptic transmission, as little about these mechanisms is known. Previous studies suggested that without functional VAMP2 protein Munc-18 is no longer capable of stimulating the trans-SNARE complex. Based upon this previous research two motifs within VAMP2 were targeted and a series of mutants designed, upon which functionality screens were performed. Our results show that nonfunctional VAMP2 mutants impair both endocytosis and exocytosis of neurotransmitters. Nonfunctional VAMP2 greatly reduces the frequency of spontaneous neurotransmitter release, and greatly reduces the amplitude of evoked neurotransmitter release.

II. Introduction

In order to function successfully, neurons must pass signals to specific target cells through synapses via vesicle fusion. Synaptic vesicle fusion is mediated by two different branches of proteins, the SNARE and Sec1/Munc18 (SM) families. SNARE-mediated synaptic transmission between neurons is required for memory, learning, and computational ability (Bear et al., 2007, pg 40). Located on the surface of the lipid membrane, the SNARE complex is a four-helical bundle made up of syntaxin-1, SNAP-25, and synaptobrevin/VAMP (Zhou et al., 2013). All SNAREs contain a conserved region of 60-70 amino acids within their cytosolic domain called the SNARE motif, which homo-dimerizes. V-SNAREs on the vesicular membrane “zip together” with t-SNAREs on the target membrane, becoming tightly bonded, and in the process cause the opposing membranes to fuse.

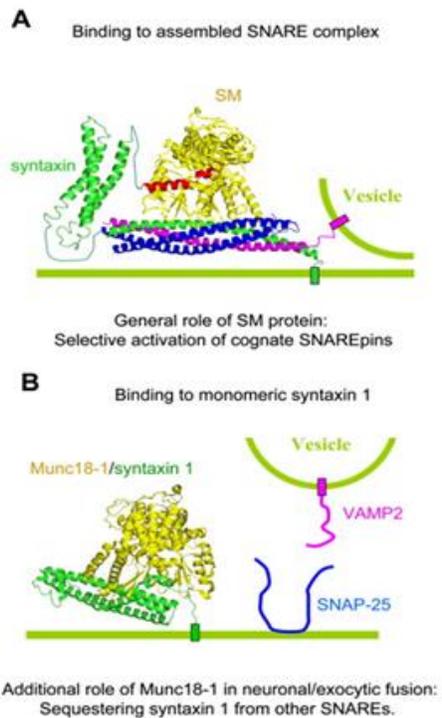


Figure 1. Model for the General Mechanism of Fusion and SM Function. Yellow-SM, Green-Syntaxin, Blue-SNAP-25, Purple-VAMP2 (From Shen et al., 2007)

Thus, the neurotransmitters within the vesicle can be released into the target cell (Karp, 2010, pg. 296-297). The importance of SNARE protein complex function is exemplified by the fact that it is the target of two bacterial toxins, botulism and tetanus. These toxins cleave neuronal SNAREs, blocking the release of neurotransmitters and causing paralysis (Karp, 2010, pg. 296).

Previously, it was determined that Sec1/Munc18 (SM) proteins were required for synaptic transmission, in particular Munc18-1 was determined to be essential to neurotransmitter release. Munc18-1 accelerates vesicle fusion by directly interacting with v- and t-SNAREs of the trans-SNARE complex, increasing specificity and assisting zippering of the SNARE complex. The delivery of lipid cargo between membranes requires a fusion event which can be examined by using a Fluorescence Resonance Energy Transfer (FRET) based lipid mixing assay. Our group has used FRET to visualize fusion of liposomes in which neuronal SNARE

proteins have been reconstituted at appropriate surface densities (Scott et al., 2002). In these studies, performed by Shailendra Rathore, a post-doctoral research scientist in Dr. Jingshi Shen’s lab, v-

liposomal fusion events were examined using the FRET pair NBD-PE (fluorophore) and Rho-PE (quencher). These lipid chromophores become diluted as the target and vesicular membranes fuse, and dequenching occurs as the chromophores are no longer in close proximity. This dequenching can be an indication of successful membrane fusion. Stimulation of Munc18 is greatly reduced when mutations in VAMP2 are present, but with no effect to the core SNARE catalytic function (Figure 2). Two specific motifs of VAMP2 were found to be required for successful stimulation of vesicle fusion and lipid mixing – S61/E62 and S75/Q76 (Shen et al., 2007).

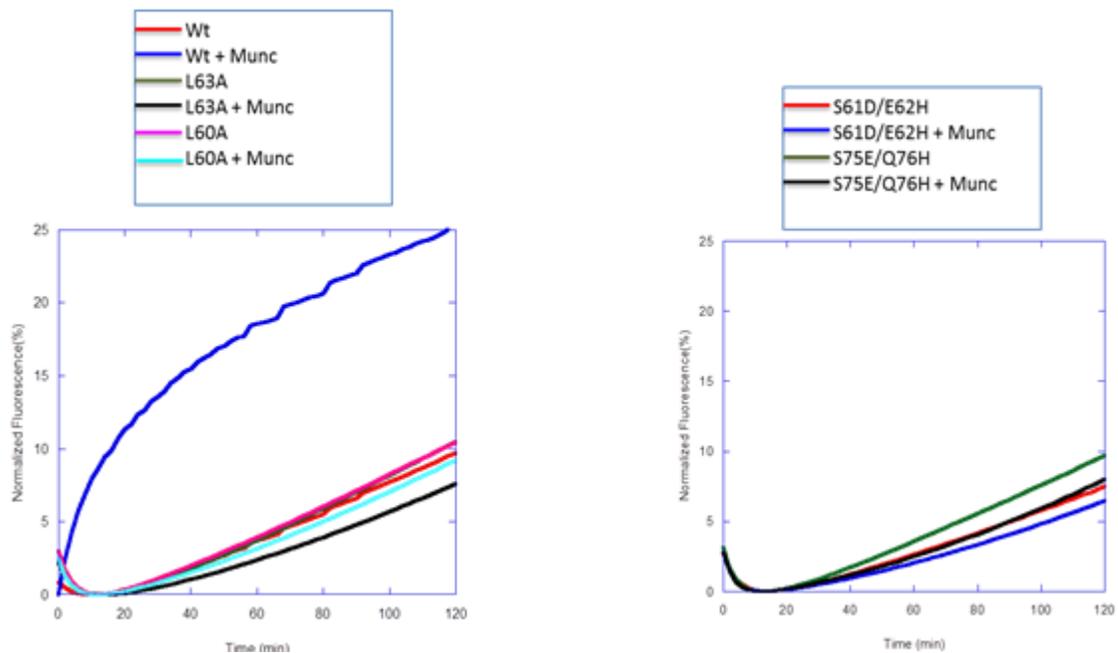


Figure 2. FRET analysis of v-liposome fusion. Munc18 activated fusion of t-SNARE liposomes containing WT or mutant synaptobrevin/VAMP2. (From Shailendra Rathore)

VAMP2, a component of the SNARE complex, has been implicated in the process of storage vesicle fusion (Papini et al., 1995). VAMP2 complexes with syntaxin-1 and SNAP 25 from the presynaptic membrane, as well as a synaptic vesicle membrane protein (VAMP/synaptobrevin; Taubenblatt et al., 1999). In the absence of VAMP2, neurotransmitters are not transported across the synaptic membrane (Taubenblatt et al., 1999). When synaptic transmission does not proceed properly, it can result in a range of mental disorders. We are interested in determining how VAMP2 is involved in the fusion of synaptic vesicles in the process of synaptic transmission, as a protein within the SNARE

complex. To answer this question, we have performed a complementation screen to determine the functional domains of VAMP2 in vesicular fusion.

III. Materials and Methods

Primary Neuronal Cell Cultures

Neuronal cultures were obtained from postnatal wild type mice on day 1 or 2 following birth. Neurons were plated on 18 mm coverslips in 12 well dishes, or 5 coverslips in a 60 mm Petri dish. Coverslips were first cleaned by soaking in concentrated nitric acid (70%); the coverslips were further sterilized by washing in MilliQ water several times and exposure to ultraviolet light for 30 minutes, and finally coated with poly-L-lysine. The brain was removed, placed in the dissection/dissociation medium, and dissected. Hippocampal and cortex neurons were dissociated by trypsin (5 mg/mL for 5 min at 37°C). DNAase solution was added and incubated. The cell density was calculated and cells were plated at 1×10^5 cells per mL in plating medium. Cells were incubated in the cell culture incubator at 37 °C with 95% air and 5% CO₂. Cells were maintained in Neurobasal medium supplemented with B-27, 2 mM glutamine, and pencillin/streptomycin. See protocol in (Beaudoin, 2012). All mice were handled according to the NIH guidelines for Animal Care & Use, and the procedures were approved by the Institutional Animal Care and Use Committee at the University of Colorado-Boulder.

Plasmid Construction and production of lentiviruses

From the University of Colorado Functional Genomics Facility a VAMP2 shRNA silencing lentiviral vector was obtained. This vector targets a sequence within the 3'UTR of the mouse VAMP2 gene (5'-CCGACCACAATCTGGTTCTTT-3'). The pLKO-puro lentivirus vector (Sigma-Aldrich Co. LLC) along with three packaging plasmids (VSVG, pSPAX, and pAdvantage) were cotransfected into HEK293T cells at 0.5 ug, 5 ug, and 1 ug per 100 mm cell culture respectively, using the transfection reagent PEE25. Cell culture supernatants were collected every 24 hours following transfection for 3 days and used directly in infection of neurons or stored at -80°C. Neuronal cultures were infected DIV 6 by adding 20 uL of viral suspension per well, and examined using electrophysiology DIV 16-20.

Plasmid Sequence

The vector TRC2- pLKO-puro lentivirus was used, which is 7,518 bp in length. In order to create the VAMP2 rescue the puroR gene (an antibiotic gene) within this vector was selectively removed using BamH1 and Kpn1 splicing factors, and was replaced with the mouse VAMP2 gene sequence. In this rescue the mouse VAMP2 gene which had been cloned in is driven by the hPGK promoter, allowing for the simultaneous expression of the shRNA VAMP2 and the VAMP2 mouse gene. All mutant constructs were designed using site-directed mutagenesis, and verified using sequencing. The transgene was then delivered by the lentivirus. This particular vector allows for the production of stable lentiviral particles, as well as for transfection of the cell culture. The 3'UTR was used as the targeting region for the shRNA in order to conserve the coding region of VAMP2.

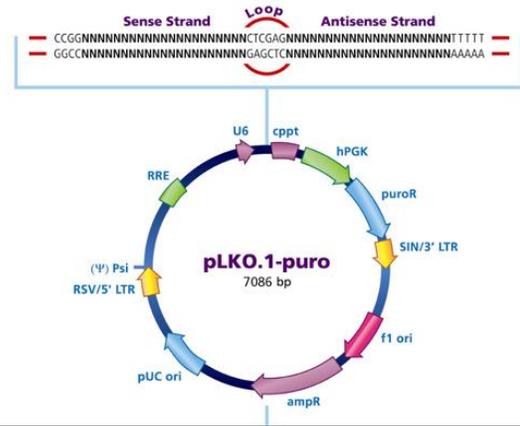


Figure 3. Schematic of the pLKO.1-puro vector. pLKO is a lentiviral vector that contains a puroR resistance gene inducible by a hPGK promoter (From Sigma-Aldrich Co. LLC).

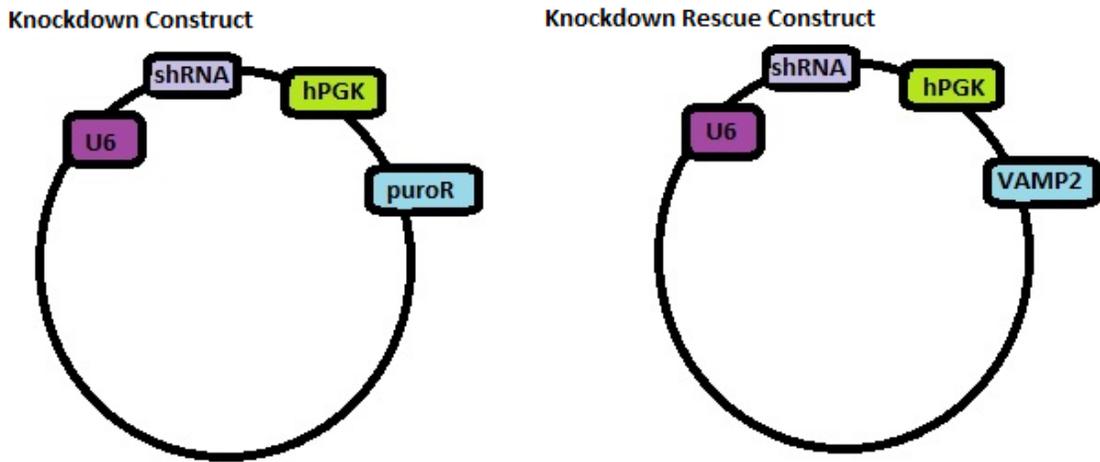


Figure 4. Schematic of pLKO lentiviral Knockdown construct and Knockdown Rescue construct. In both of these constructs the cppt gene is replaced with a shRNA coding region, driven by the U6 promoter. In the Knockdown Rescue construct the puroR gene is replaced with the WT VAMP2 sequence and transcription is independently driven by the hPGK promoter.

There were two controls:

TurboGFP Control

The TurboGFP control (Sigma-Aldrich Co. LLC) is used as a positive control in transfection/infection. This pLKO construct contains a TurboGFP tag driven by the CMV promoter.

Non-Mammalian shRNA Control

The Non-target shRNA control (Sigma-Aldrich Co. LLC) is used as a negative control. It is a version of the pLKO construct containing an shRNA insert which does not align with any BLAST product. This control allows us to examine the potential effects of infecting cells with a shRNA on gene expression.

There were four mutants of interest:

VAMP2 WT

5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80
SATAATAPPAAPAGEGGPPAPPPNLTSNRRLQQTQAQVDEVVDIMRVNVDKVLERDQKLELDDRADALQAGASQFETSAAKL

VAMP2 L63A Mutant

5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80
SATAATAPPAAPAGEGGPPAPPPNLTSNRRLQQTQAQVDEVVDIMRVNVDKVLERDQKLEADDRADALQAGASQFETSAAKL

VAMP2 L60A Mutant

5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80
SATAATAPPAAPAGEGGPPAPPPNLTSNRRLQQTQAQVDEVVDIMRVNVDKVLERDQKLELDDRADALQAGASQFETSAAKL

VAMP2 S61D/E62H Mutant

5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80
SATAATAPPAAPAGEGGPPAPPPNLTSNRRLQQTQAQVDEVVDIMRVNVDKVLERDQKLEHLLDDRADALQAGASQFETSAAKL

VAMP2 S75E/Q76H Mutant

5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80
SATAATAPPAAPAGEGGPPAPPPNLTSNRRLQQTQAQVDEVVDIMRVNVDKVLERDQKLELDDRADALQAGAEHFETSAAKL

Titration Assay

The titration assay was performed using the GFP control, which contained the viral construct with a GFP tag, but no targeting shRNA inserted. Following transfection into the HEK293T cells, these cells were examined under a fluorescent microscope at 1:1, 1:10, 1:100, 1:1000, 1:10000, and 1:100000 dilutions in order to determine the titer of the virus. Titer was calculated using the following equation: $\{(\# \text{ cells at starting time})(\text{dilution factor})(\text{percent infection})\}/(\text{vol virus solution expressed in mls})$. The titer of GFP virus is normally 1×10^8 IU/ml. Once the titer is calculated we further concentrate the supernatant about 10X by ultracentrifuge at 25k rpm for 2hr. Neuronal cultures are plated at 1×10^5 cells per ml, the titer of the virus is used to calculate the volume of virus needed to infect 100% of cells at a ratio of one virus per cell, then increased 10 fold due to the difficulty infecting neuronal cultures. Titer must be calculated each time an infection is performed since it can be variable. If only 80% of the neuronal cell culture is infected a Western Blot will no longer be reliable.

Western Blot

Immunoblotting was used to examine the knockdown efficiency and gene expression within the rescue. Neuronal cells which had been previously infected were separated at DIV 16-20 on 8% SDS-PAGE gels. These membranes were stained using monoclonal anti-VAMP2 antibodies, monoclonal anti-syntaxin antibodies, or anti-Bip antibodies. VAMP2 was detected using the secondary antibody goat Fluor 488-conjugated (Life Technologies), Syntaxin was detected using the secondary antibody goat rhodamine-conjugated (Jackson ImmunoResearch). Chemiluminescence was detected using enhanced chemiluminescence (ECL) after the membrane was exposed to photographic film.

FM4-64 labeling and Live Imaging

FM4-64 labeling of recycling vesicles in live neurons and live imaging was performed following the described protocol in (Beaudoin, 2012, pg. 1744). Cultured neurons were incubated in Tyrode's solution, high KCL Tyrode's solution containing 10mM FM4-64, and Tyrode's solution containing Advasep-7. Cells were fixed using 10mM FM4-64X and 4% paraformaldehyde. These cells were

examined using a confocal imaging system, FM4-64 can be excited between 510-570 nm and emission collected at 560-750 nm.

Electrophysiology Recordings

Electrophysiology Recordings were performed in whole-cell patch-clamp mode using a Multiclamp 700B amplifier (Molecular Devices) to examine synaptic currents. Evoked synaptic transmission was stimulated by concentric bipolar electrodes placed 100-150 μm from the neuron, which delivered one millisecond current injections. The resistance of pipettes varied between 3 and 5 Ohms, after the whole cell was patched resistance was adjusted to between 8 and 10 Ohms. The frequency, duration, and magnitude of extracellular stimuli were controlled by an Isolated Pulse Stimulator (World Precision Instruments). The whole cell pipette solution contained 135 mM CsCl, 10 mM HEPES-CsOH (pH 7.25), 0.5 mM EGTA, 2 mM MgCl₂, 0.4 mM NaCl-GTP, and 4 mM NaCl-ATP. The bath solution contained 140 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 0.8 MgCl₂, 10 mM HEPES-NaOH (pH 7.4), and 10 mM Glucose. IPSCs and EPSCs were isolated by adding either the GABA_A receptor blocker picrotoxin (50 μM) or the glutamate receptor blockers CNQX (20 μM) and AP-5 (50 μM) (all from Sigma) to the bath solution. In order to block action potentials the spontaneous mEPSCs and mIPSCs were analyzed using tetrodotoxin (TTX). Electrophysiological data were analyzed using the pClamp 10 software (Molecular Devices), statistical analysis were performed using Student's t-tests. VAMP2 rescue mutants were compared to WT control neurons.

IV. Results

In order to determine the effects of VAMP2 mutations on synaptic transmission, we expressed four different mutant VAMP2 constructs in VAMP2 knockdown neuronal cultures. First, VAMP2 was silenced in mouse neuronal cultures using lentiviral constructs containing shRNAs. To determine the proper concentration of lentivirus necessary to infect mouse neuronal cultures, a GFP Titration Assay was performed (Figure 3). The titration assay is necessary to accurately determine the titer of the virus as neuronal cell cultures are more difficult to infect and the titer can vary. A GFP control was used during transfection, containing a GFP tag but no shRNA silencing elements.

As shown in Figure 3, the lentivirus concentrations are increased 10 fold, and the neurons are typically infected at an adjusted titer so that there is ~100% infection rate. The levels of transfection are comparable to that of the other constructs used in our experiments.

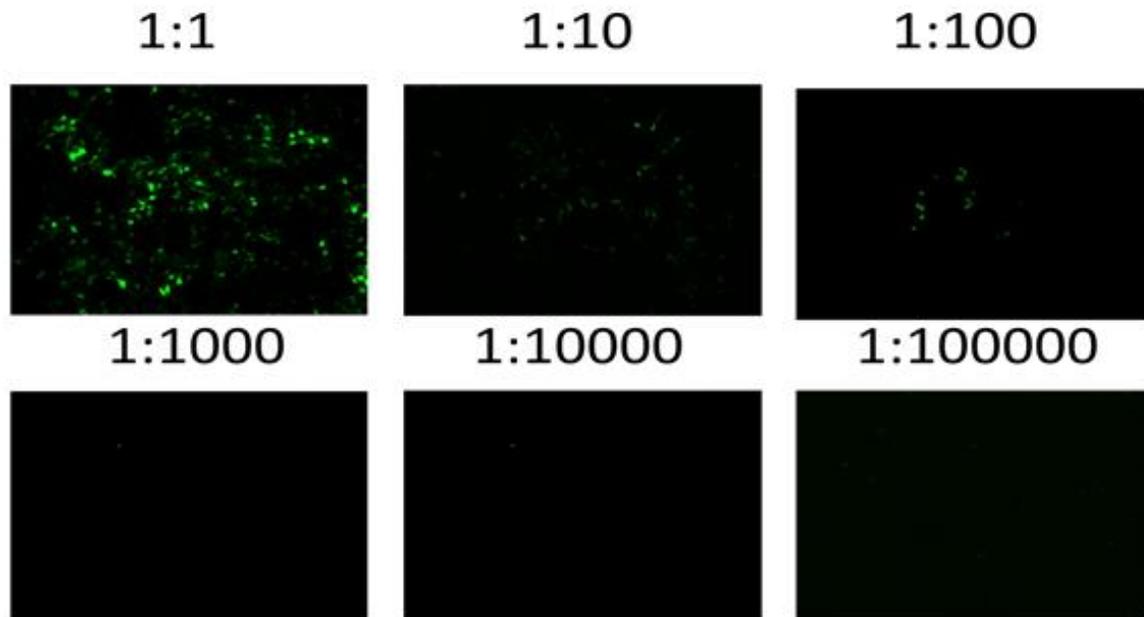


Figure 3. GFP Titration Assay. HEK293T cells were transfected with the GFP control and levels of fluorescence were analyzed to determine virus titer in order to determine the viral concentration needed to infect neuronal cultures.

Knockdown efficiency of multiple VAMP2 knockdown constructs was determined by immunoblotting. The WT form of VAMP2 was compared to a GFP control construct, a shRNA control without any targeting shRNA, and three separate knockdowns. V1 contains a shRNA sequence within the 3'UTR, while V2 and V4 contain the shRNA targeting region within their coding regions. V1 is a more reliable knockdown to use because the shRNA targeting region has no effect on the construct's coding region and is not predicted to cause interference. Incorporating the shRNA targeting region into the 3'UTR is also more efficient, if the shRNA targeting region is incorporated within the coding region of the protein it is necessary to perform a mutagenesis. The data illustrates that no VAMP2 protein is present in V1, V2, or V4 (Figure 4). Also no unexpected interference results from the addition of a GFP tag or the use of shRNA on gene expression. Syntaxin was detected in order to ensure synaptic formation was normal, and that the shRNA was specifically targeting VAMP2. Bip was used as an indication of ER stress, and acted as a loading control.

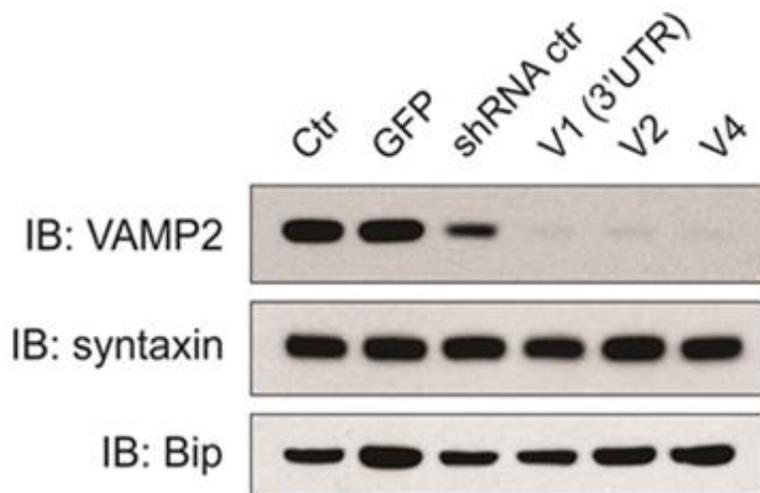


Figure 4. Analysis of Knockdown efficiency. Knockdowns were resolved with western blot using indicated antibodies. WT VAMP2 (ctr), construct with GFP marker (GFP), shRNA control lacking targeting shRNA (shRNA ctr), VAMP2 Knockdown Rescue encoding shRNA in 3'UTR (V1), VAMP2 Knockdown Rescue encoding shRNA (V2), VAMP2 Knockdown Rescue encoding shRNA (V3).

Once knockdown cell lines were obtained, we expressed the mutant constructs of interest to perform the complementation assay. The expression level of VAMP2 rescue mutants was examined using immunoblotting. VAMP2 knockdown with four different rescue constructs was compared to wildtype VAMP2 expressed at endogenous levels (CTR). A wild type rescue construct as well as four rescue mutant constructs, two with single point mutations (L63A and L60A) and two with double mutations (S61D/E62H and S75E/Q76H) expression levels were compared. The expression level of VAMP2 rescue mutants was examined via immunoblotting, and each cell line was determined to have similar VAMP2 protein levels as the WT control. (Figure 5). Syntaxin was used to ensure synapse formation was normal, and that the shRNA was targeting specifically VAMP2.

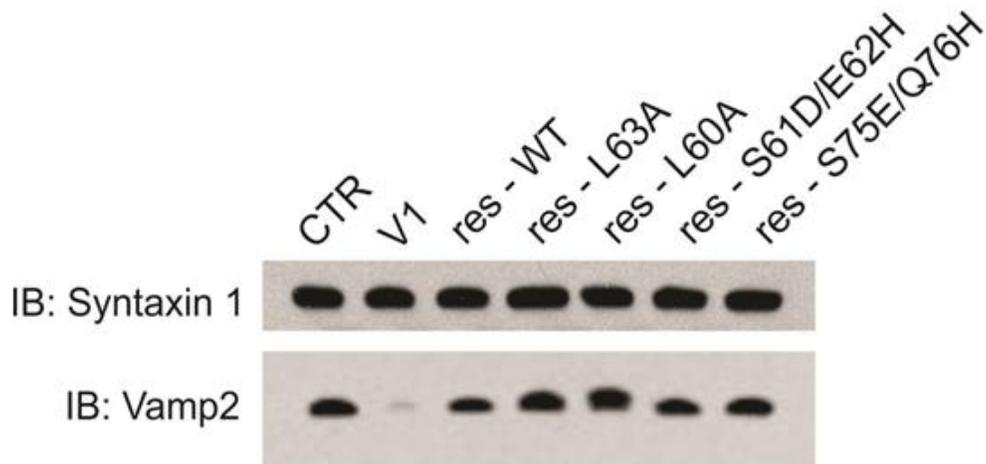


Figure 5. Expression of VAMP2 protein in Knockdown Rescue constructs. Resolved with western blot using indicated antibodies. WT VAMP2 (CTR), Knockdown (V1), Knockdown Rescue WT VAMP2 (res-WT), Knockdown Rescue L63A, Knockdown Rescue L60A, Knockdown Rescue S61D/E62H, Knockdown Rescue S75E/Q76H

The effects of mutant VAMP2 expression on endocytosis during synaptic transmission were analyzed using FM4-64 dye. FM dyes are amphiphilic styryls which insert into one leaflet of the lipid bilayer through a lipophilic tail. During endocytosis the dye becomes entrapped within vesicles and fluoresces, allowing it to be used as a marker of synaptic transmission.

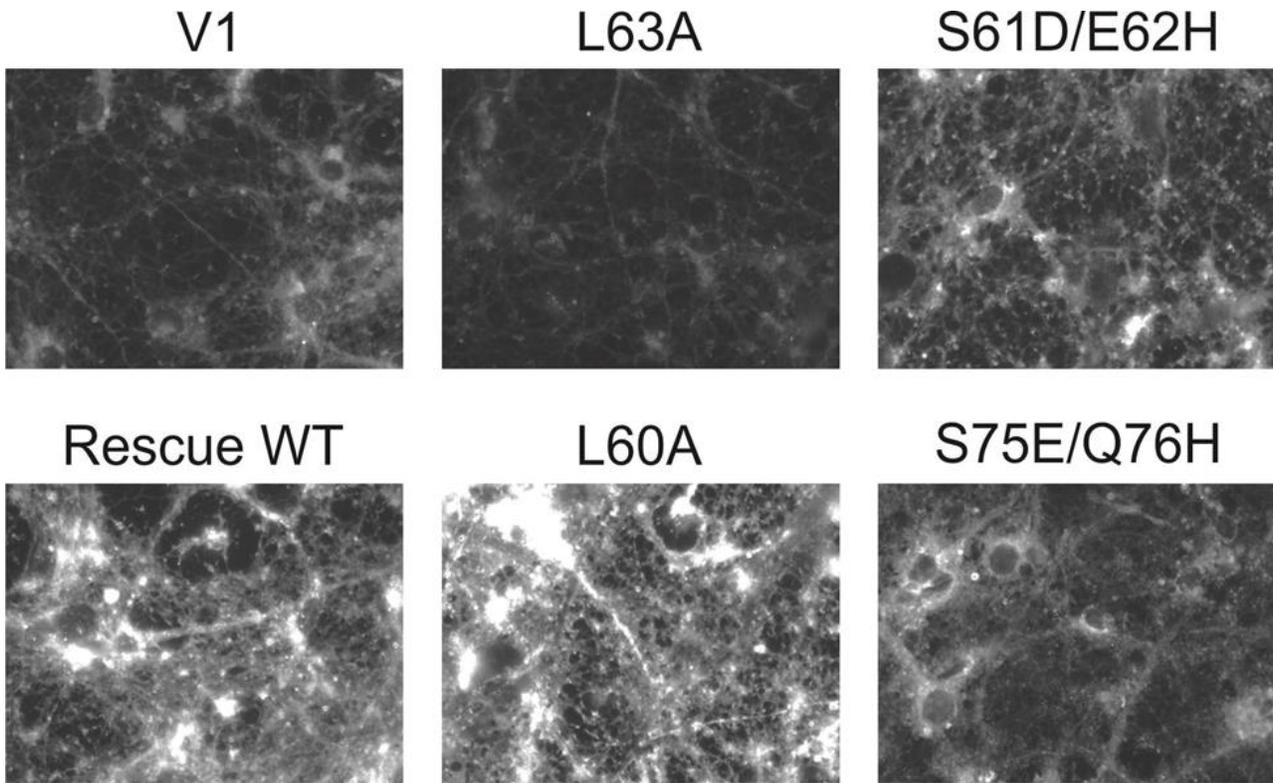


Figure 6. Analysis of Synaptic Transmission using FM4-64. The effect of the knockdown and VAMP2 rescue mutants on synaptic transmission can be detected using fluorescence levels.

Following exposure to the FM dye the cells are washed and any remaining FM dye is present within vesicles which have budded off from the plasma membrane. In VAMP2 knockdown cells there is very little fluorescence, while the Rescue WT shows significant fluorescence. Cells expressing the L60A mutant protein show comparable levels of fluorescence to those of the Rescue WT, illustrating that this mutant's mutation has little effect on the functionality of VAMP2. S61D/E62H and S75E/Q76H show partial fluorescence when compared to the Rescue WT, and therefore most likely have partially functional VAMP2 proteins. L63A, on the other hand, shows a very low level of fluorescence comparable to that of the VAMP2 knockdown V1. The L63A mutation is significant because it has completely ablated VAMP2 functionality, thus affecting the fusion process (Bolte et al., 2004).

Next, we examined the effects of the L60A and L63A VAMP2 mutations on neurotransmitter release/exocytosis using electrophysiology recordings. The S61D/E62H and S75E/Q76H VAMP2 mutants were not examined as they demonstrated only partial effects on synaptic endocytosis. I was

able to assist Dr. Chong Shen, a post-doctoral research scientist within Jingshi Shen's lab, in some aspects of this work.

The spontaneous neurotransmitter release was examined by monitoring miniature excitatory postsynaptic currents (mEPSCs). The VAMP2 knockdown greatly diminished the frequency of spontaneous neurotransmitter release (Figure 7), and could only be successfully rescued by the VAMP2 WT. Both the L63A and L60A VAMP2 mutants were unable to restore the frequency of spontaneous neurotransmitter release back to a wildtype level. There was no effect on the amplitude of these neurotransmitter releases. This indicates that these mutations are detrimental to VAMP2 protein's functionality in regulating exocytosis during neurotransmitter release. In order to ensure these mutations had a universal effect on the mechanism of transmission, miniature inhibitory postsynaptic currents (mIPSCs) were also examined (Figure 8). Similarly, the L63A and L60A mutants demonstrated greatly reduced frequency of neurotransmitter release with little effect on the amplitude. Since both the mEPSCs and mIPSCs were affected by the VAMP2 knockdown as well as the VAMP2 mutants, it appears that these mutations universally affect the mechanism of SNARE mediated synaptic transmission.

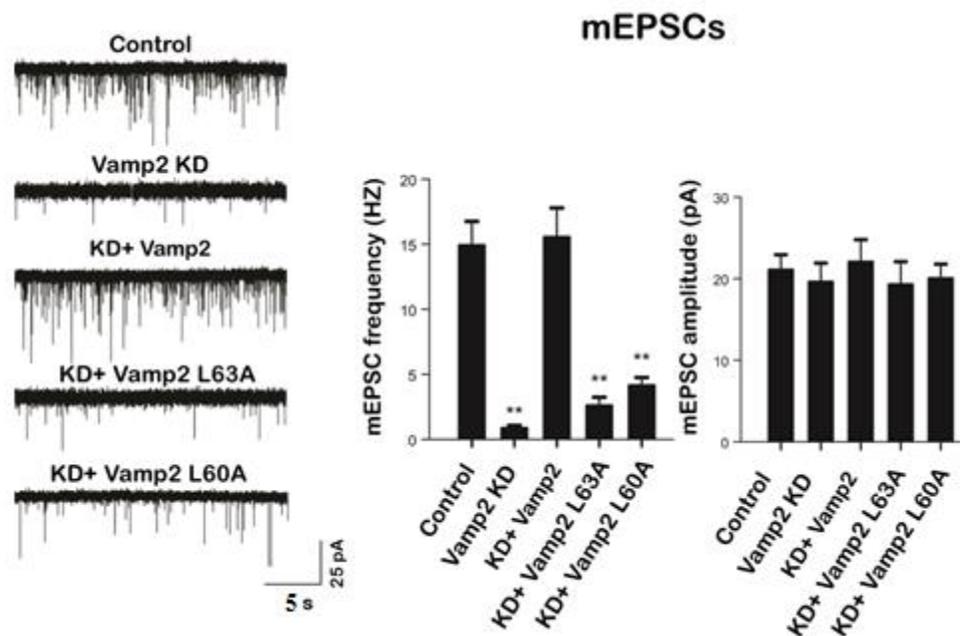


Figure 7. Electrophysiological analysis of VAMP2 impact on spontaneous neurotransmitter release by monitoring miniature excitatory postsynaptic currents. Test of the rescue ability of VAMP2 mutants. (From Chong Shen)

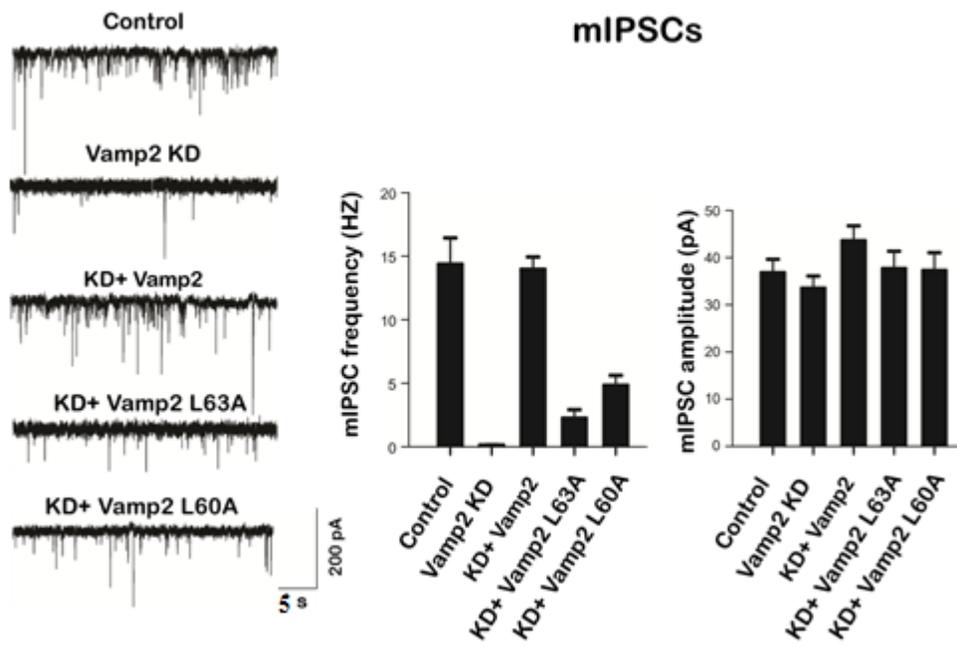


Figure 8. Electrophysiological analysis of VAMP2 impact on spontaneous neurotransmitter release by monitoring miniature inhibitory postsynaptic currents. Test of the rescue ability of VAMP2 mutants. (From Chong Shen)

We also investigated the effects of the VAMP2 mutations on evoked neurotransmitter release. Action potential-evoked neurotransmitter release in the cultured neurons was triggered using electrical stimulus, then currents were quantified (Figure 9). The amplitude of these evoked EPSCs was greatly reduced by the L63A and L60A mutants. Neither of these mutants was capable of rescuing the VAMP2 knockdown, which demonstrated a greatly reduced amplitude as compared to the wildtype. The wildtype VAMP2 was capable of rescuing the VAMP2 knockdown and successfully restored evoked neurotransmitter release. The evoked IPSCs were affected in a similar manner (Figure 10); the VAMP2 knockdown greatly reduced the amplitude of the evoked IPSCs and was only successfully rescued by the VAMP2 wildtype. Neither mutant was capable of rescuing the knockdown phenotype.

These results indicate that the loss of functional VAMP2 protein inhibits endocytosis, as well as spontaneous and evoked neurotransmitter release.

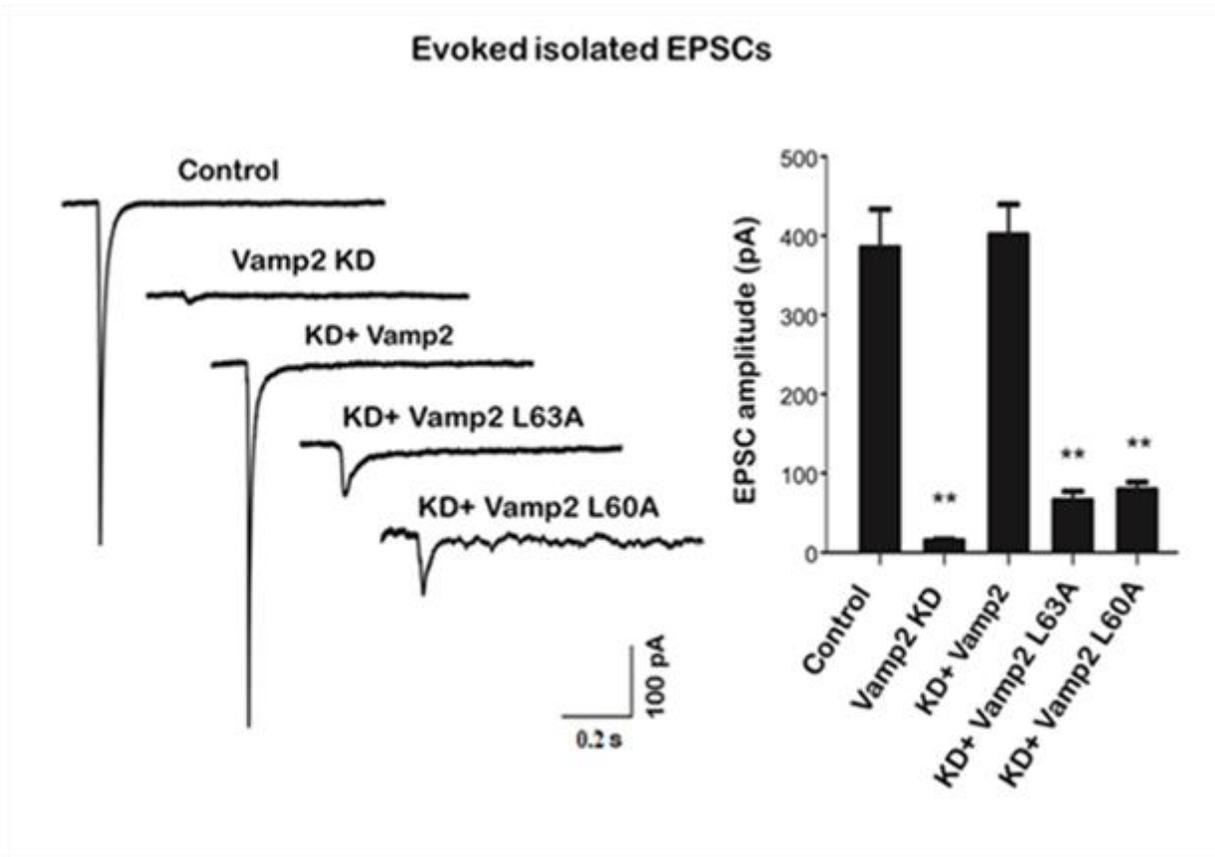


Figure 9. Electrophysiological analysis of VAMP2 impact on evoked neurotransmitter release by monitoring excitatory postsynaptic currents. Test of the rescue ability of VAMP2 mutants. (From Chong Shen)

Evoked isolated IPSCs

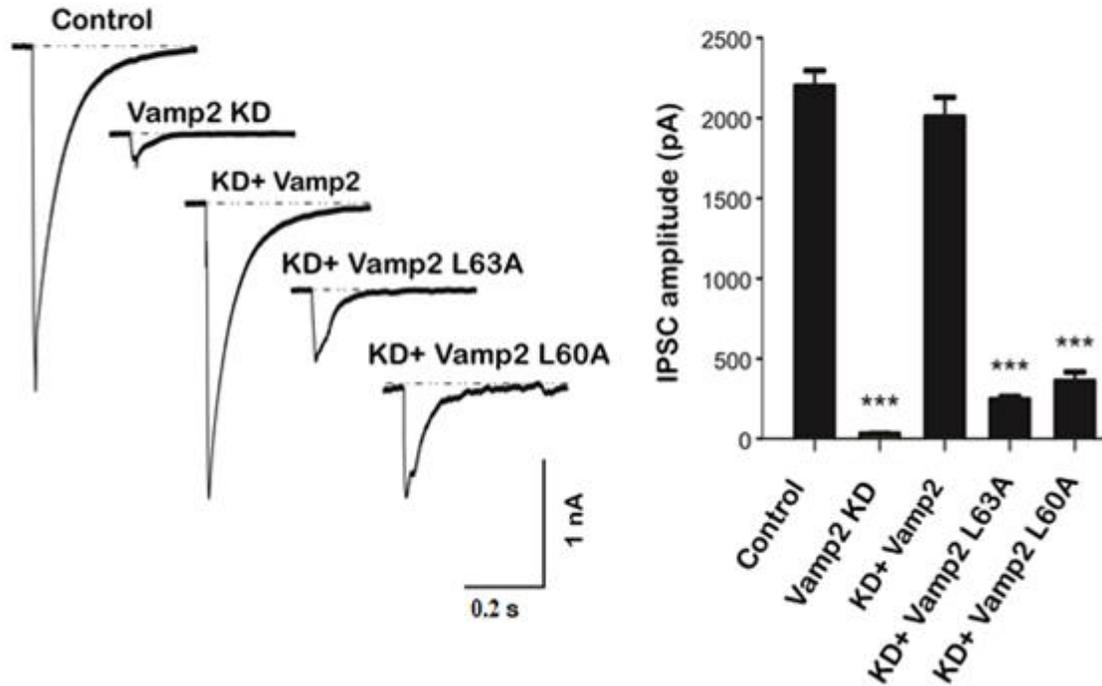


Figure 10. Electrophysiological analysis of VAMP2 impact on evoked neurotransmitter release by monitoring inhibitory postsynaptic currents. Test of the rescue ability of VAMP2 mutants. (From Chong Shen)

V. Discussion

In order to function properly, neurons must transmit signals to target cells via vesicle fusion in the neuronal synapse. VAMP2 is a necessary component of the SNARE complex and is required for vesicle fusion to take place. The previous identification of two essential motifs in the v-SNARE VAMP2 (S61/E62 and S75/Q76) that appear to be indispensable in the Munc18-1/trans-SNARE interaction lead to further examination of VAMP2 and its potential implications in neurotransmitter

release. Mutations within these two motifs disrupt the catalytic role of Munc18-1 in SNARE complex zippering, but do not impact the formation of the trans-SNARE complex (Shen et al., 2007).

The purpose of this research was to examine what residues of the VAMP2 protein are involved in the fusion of synaptic vesicles during synaptic transmission. We were able to successfully design and test an effective series of knockdowns and rescue mutants to examine the effects of knockdown and mutant VAMP2 expression in mouse neuronal cultures on SNARE mediated synaptic transmission. Once the efficiency and level of expression was verified using immunoblotting the knockdown (V1), rescue WT, and four rescue mutants implications in endocytosis were examined using FM4-64 dye. Of the four mutants L63A demonstrated the most intriguing results, as it reduced synaptic endocytosis to similar levels as the VAMP2 knockdown. L60A appeared to have little effect on endocytosis, while the S61D/E62H and S75E/Q76H mutants only had partial inhibition of synaptic endocytosis. Our electrophysiological data also indicate that the VAMP2 L60 and L63 residues are required for neurotransmitter release.

Previous work in the field of vesicular fusion has shown that the SNARE complex is required for neuronal transmission *in vivo*. Recently a correlation between genetic variations in VAMP2 and Idiopathic Generalized Epilepsy (IGE) has been observed in humans. IGE is believed to be dependent on genetic variations which impair synaptic processes (Yilmaz et al., 2013). To determine the function of VAMP2 in vesicular fusion in mice, I am interested in creating a VAMP2 knockdown mouse model. This model could be created by injecting a VAMP2-specific CRISPR sequence into mouse embryos, and then analyzing the neurological phenotype in adult animals. Based upon this previous research I expect that VAMP2 knockdown mice will display a phenotype similar to IGE patients.

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