Title
Presynaptic protein ubiquitination and synaptic transmission

Author
Vojnovic, Dijana

Publication Date
2018

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA

Los Angeles

Presynaptic protein ubiquitination and synaptic transmission

A thesis submitted in partial satisfaction

of the requirements for the degree Master of Science

in Physiological Science

by

Dijana Vojnovic

2018

ABSTRACT OF THE THESIS

Presynaptic protein ubiquitination and synaptic transmission

by

Dijana Vojnovic

Master of Science in Physiological Science

University of California, Los Angeles, 2018

Professor Felix Erich Schweizer, Co-Chair

Professor Gina Rochelle Poe, Co-Chair

Ubiquitination of a target protein is an important post-translational modification. There is evidence for the involvement of ubiquitination of presynaptic proteins in synaptic transmission. Vesicle associated membrane protein (VAMP2) is a presynaptic protein that is fundamental for vesicle docking and fusion and here we test the hypothesis that ubiquitination of VAMP2 is important for synaptic transmission. To test this hypothesis, we downregulated expression of endogenous VAMP2 and allowed for the expression of mutant VAMP2 that cannot undergo ubiquitination. Using electrophysiological recordings in cortical neuronal cultures from autapses (synapses made by a neuron onto itself), we found that knocking down endogenous VAMP2 reduces synaptic transmission and this is rescued with exogenous VAMP2. Our findings indicate that E1 inhibition alters synaptic transmission in control

neurons, but not in neurons rescued with mutant VAMP2 with a knock down background. Our findings support our hypothesis that ubiquitination of VAMP2 modulates synaptic transmission.

The thesis of Dijana Vojnovic is approved.

Scott H. Chandler

Gina Rochelle Poe, Committee Co-Chair

Felix Erich Schweizer, Committee Co-Chair

University of California, Los Angeles

2018

TABLE OF CONTENTS

LIST OF FIGURES

ACKNOWLEDGEMENTS

I would like to thank my mentor, Dr. Felix Schweizer, and the rest of the Schweizer lab for their guidance and help during completion of my thesis project. I would like to acknowledge my committee members for their assistance with thesis preparation and the approval process. I would like to thank my boyfriend, friends and family for their love, support and encouragement.

INTRODUCTION

Ubiquitination is a multifaceted protein modification that adds ubiquitin, a small protein composed of a single polypeptide chain of 76 amino acids, to a target protein through a covalent bond. Ubiquitination of a target protein involves a highly regulated three step enzymatic cascade by the E1 activating enzyme, E2 conjugating enzyme, and the E3 ligase (Figure 1). Ubiquitin activation of a substrate is an ATP dependent process and conjugation results in the transfer of ubiquitin from the E1 to the E2 enzyme. The E3 ligase interacts with the E2 ubiquitin complex and the substrate, assisting or directly transferring ubiquitin to a target protein. During ubiquitination, an ispopeptide bond is formed between the carboxyl group of ubiquitin and the epsilon-amino group of the target protein's lysine residue or occasionally on the N-terminus of a target protein (Hershko and Ciechanover 1992). A single ubiquitin can be added to a substrate in a process called monoubiquitination or multiple ubiquitin proteins can be added as lysine-linked chains in a process called polyubiquitination (Glickman and Ciechanover 2002). The complexity and diversity of ubiquitination as a protein modification is evident because of the existence of one to two E1s interacting with many E2s and hundreds of E3s. The pathway is also regulated by deubiquitinating enzymes (DUBs) which are believed to counter the role of ubiquitination by removing ubiquitin and ubiquitin chains from target proteins. DUBs, hundreds of which have been discovered, make the ubiquitination pathway reversible and create a pool of ubiquitin that can be re-used in future ubiquitination events (Weissman 2001).

Ubiquitination was originally recognized as a signal for protein degradation by the proteasome (Hershko and Ciechanover 1992), but it is now believed to be involved in various other pathways including receptor trafficking, inflammation, and response to DNA damage (Winget and Mayor 2010). It is classically believed that monoubiquitination is involved in protein sorting, trafficking, activity, and localization, while polyubiquitination is believed to

be involved in protein signalling and degradation by the proteasome or by autophagy (Ronai 2016). Recent studies contradict this belief, revealing that monoubiquitination can also trigger the degradation of substrate proteins (Braten et al. 2016). Braten et al. (2016) found that monoubiquitination is more widespread than originally believed and that certain substrates are degraded by the proteasome after monoubiquitination. Although the mechanisms associated with different types of ubiquitination still need elucidation, it is clear that ubiquitination is a complex and versatile modification important for maintaining cell homeostasis.

Ubiquitination as a post-translation modification acts as a signal, determining protein stability, activity, location in the cell and whether it binds to other proteins (Welchman RL 2005; Xu and Jaffrey 2011). Ubiquitin binding domains (UBDs) are believed to translate ubiquitin signals into cascades important for cell physiology by forming weak non-covalent bonds to ubiquitin. Up to twenty families of UBDs have been identified and a multitude of human proteins are estimated to contain UBDs (Dikic, Wakatsuki, and Walters 2009). Ubiquitin and ubiquitin-like proteins (UBLs) are believed to modify the activity of proteins in a reversible manner and this is believed to be mediated through association with UBDs (Hurley, Lee, and Prag 2006). Similar to ubiquitin, UBLs bind transiently to thousands of substrates, controlling the protein's binding partners and protein-protein interactions (Hochstrasser 2009). UBLs are also believed to act as modifiers in ubiquitin signalling by binding to ubiquitin machinery through UBDs (Winget and Mayor 2010). UBDs have a higher affinity for polyubiquitin chains and selectivity is based on the chain linkage and length; this specificity further adds to the complexity of regulation and the diversity of ubiquitin as a cellular signal (Dikic, Wakatsuki, and Walters 2009).

The role of ubiquitination as a post-translational modification is particularly interesting, as it may rapidly and reversibly regulate presynaptic protein turnover in response to synaptic activity (Chen et al. 2003). Chen et al. (2003) found that the number of ubiquitin-

conjugated proteins in presynaptic terminals rapidly decreases in response to stimulation with high potassium and in the presence of extracellular calcium. The decrease in presynaptic protein ubiquitination was not sensitive to proteasome inhibition and included the endocytic protein, epsin 1. Knock down of mammalian *fat facets*, a DUB, using RNA interference (RNAi) resulted in ubiquitination of epsin 1 after depolarization. Stimulation may trigger deubiquitination of epsin 1, resulting in increased endocytosis and alterations in the dynamics of exocytosis. Ubiquitination of epsin 1 is thought to impair its ability to bind clathrin adaptor proteins, potentially acting as a post-translational modification that rapidly alters the protein's conformation and modifies its protein-protein interactions through UBDs. Ubiquitination may rapidly regulate turnover of presynaptic proteins in response to synaptic activity, increasing endocytosis and altering dynamics of vesicle fusion events leading to neurotransmitter release (Chen et al. 2003).

Ubiquitination of presynaptic proteins has been association with modifications in synaptic transmission. At the neuromuscular junction (NMJ) of *Drosophila,* pharmacological and genetic inhibition of the proteasome led to an increase in DUNC-13, a vesicle priming protein important for vesicle fusion (Betz et al. 1997; Richmond, Davis, and Jorgensen 1999; Speese et al. 2003). The increase in DUNC-13, the *Drosophila* analogue of Munc-13, was accompanied by defects in evoked synaptic transmission (Speese et al. 2003). In hippocampal neuronal cultures from mice with SCRAPPER (an E3 ligase knock-down) there was an increase in spontaneous vesicle fusion measured by mini excitatory postsynaptic currents (mEPSCs). The increase in mEPSCs was also observed in mice with Rab-3 interacting molecule 1 (RIM1) overexpression (Dobie and Craig 2007; Yao et al. 2007). RIM1 is a presynaptic protein important for vesicle priming and calcium channel localization to the active zone (Kaeser et al. 2012; Kaeser et al. 2011). The alterations in synaptic transmission in these E3 ligase knock-down SCRAPPER mice were rescued with knock-down of RIM1 or

by exogenous SCRAPPER expression (Yao et al. 2007). These studies provide evidence for the role of ubiquitination of presynaptic proteins in modulating synaptic transmission.

Studies in our lab were the first to look at ubiquitination as a post-translational modification and whether changes in presynaptic protein turnover alter synaptic transmission (Rinetti and Schweizer 2010). We found evidence for the role of ubiquitination in rapidly regulating presynaptic protein turnover and synaptic transmission. In hippocampal neurons, there was an increase in spontaneous synaptic transmission in response to addition of MG131, a proteasome inhibitor, and ziram, an E1 ligase inhibitor (Chou et al. 2008). The increase in frequency of mEPSCs and mIPSC (miniature inhibitory post synaptic currents) was not accompanied by an increase in presynaptic proteins, RIM1 and Munc13, nor was there an effect on the amplitude of mEPSCs or mIPSCs. Interestingly, the physiological effect of proteasome inhibition and E1 ligase inhibition was the same. This was the first study to provide evidence for dynamic ubiquitination in rapidly regulating synaptic protein turnover and synaptic transmission (Rinetti and Schweizer 2010). Presynaptic proteins are rapidly regulated by ubiquitination, and this may result in changes in synaptic transmission. However, it is not clear which presynaptic proteins are targets.

In this thesis, we are interested in vesicle associated membrane protein (VAMP2), because of its fundamental role in regulating and stabilizing synaptic fusion (Schoch et al. 2001). VAMP2 is a presynaptic protein that binds to syntaxin and SNAP-25 in a complex that is essential for vesicle docking and exocytosis (Deak et al. 2004; Jahn and Sudhof 1994). In *Drosophila* that are mutant in a VAMP2 homolog neuronal-synaptobrevin, evoked synaptic transmission was nearly abolished and spontaneous vesicle fusion was reduced by 75% at the NMJ (Deitcher et al. 1998). Schoch et al. (2001) found that in hippocampal cultures from VAMP2 knockout mice, there was a 10-fold decrease in spontaneous vesicle fusion and fusion induced by hypertonic sucrose. In the same study, calcium-triggered fusion was

decreased 100-fold (Schoch et al. 2001). Therefore, VAMP2 is essential for both evoked and spontaneous vesicle fusion events that result in neurotransmitter release (Deitcher et al. 1998; Schoch et al. 2001). VAMP2 and ubiquitination of presynaptic proteins are important for synaptic transmission, so we developed a probe to test whether ubiquitination of VAMP2 modulates synaptic transmission. To test this hypothesis, we used proteomics to confirm that ubiquitination of VAMP2 is sensitive to E1 ligase inhibition. Furthermore, we determined which lysine residues of the VAMP2 protein were altered by E1 ligase inhibition.

In order to determine the role of ubiquitination of VAMP2 in synaptic transmission, we knocked down endogenous VAMP2 using CRISPR/Cas9 system and confirmed knock down with immunocytochemistry and electrophysiology. We found that VAMP2 protein levels were decreased in neurons transfected with our knock down construct. We also found that evoked synaptic transmission, which was measured by recording autapses, was decreased in VAMP2 knock down neurons. Our knock down construct was designed using the CRISPR/Cas9 system to target intron/exon junctions so that we could rescue with exogenous protein. We rescued VAMP2 protein levels with exogenous mutant VAMP2 which was mutated at two lysine residues that came up in our proteomics screen as targets of ubiquitination. The two lysine residues were mutated to arginine so that VAMP2 could no longer undergo ubiquitination. Neurons rescued with mutant VAMP2 with the knock down background exhibited an increase in evoked synaptic transmission. We found that E1 inhibition altered spontaneous synaptic transmission in cortical neurons. We also measured the effect of E1 inhibition on evoked synaptic transmission and whether it was dependent on ubiquitination of VAMP2. We did this by recording autapses from control neurons and neurons rescued with mutant VAMP2 with a knock down background. We found that E1 inhibition altered synaptic transmission in control neurons, but did not alter synaptic transmission in neurons with mutant VAMP2 that could not undergo ubiquitination. Our

findings suggest that ubiquitination of VAMP2 plays a role in modulating synaptic transmission.

METHODS

Plasmid Design and Amplification

Two single-guide RNA were designed to target intron/exon junctions of the VAMP2 gene (ATUM/DNA2.0). The fluorophore used was PaprikaRFP and the selection marker was kanamycin. The target sequence for the first guide RNA, g20, was 5'cccaggtggtggacatcatg3' which targeted the intron/exon junction at the start of exon 3. The target sequence for the second guide RNA, g8, was 5'taggtcggctaccgctgcca3', which targeted the intron/exon junction at the start of exon 2. Both plasmids were used in knock down experiments and both targeted intron/exon junctions so that rescue experiments could be performed. A wild type VAMP2-pHlourin plasmid and mutant VAMP2-pHlourin plasmid was used in rescue experiments with a knock down background. Mutant VAMP2-pHlourin plasmids were designed to target two lysine residues which were discovered to be sites of ubiquitination in the VAMP2 gene by our proteomics screen and each lysine residue was mutated to an arginine. Plasmid DNA was amplified using DH5-Alpha *E.coli* cells and was purified using an endo-free maxi prep kit (Qiagen 12362).

Neuronal Cell Culture

Rat brain prefrontal cortical tissue (Embryonic day 18) was purchased from BrainBits, LLC. The tissue was incubated with 1 μg/mL papain (Sigma Aldrich, catalogue number 76220) dissolved in Hibernate E media (BrainBits, LLC.) for 15 min at 37 ºC in order to enzymatically dissociate the tissue. The tissue was then washed by removing Hibernate E media with papain and adding fresh culture media twice. Culture media consisted of Minimum Essential Media free from L-glutamine and phenol red (Corning CellGro, catalogue number 17-305-CV) containing 5% fetal bovine serum **(**Denville, catalogue number FB5001**)**, 2% B27 supplement (Gibco, catalogue number 17504001), 5 mg/mL glucose (Sigma, catalogue number 68270)**,** glutamax (Gibco, catalogue number 35050061) and 0.1mg/mL

transferrin (Sigma, catalogue number T8158). The brain tissue was further dissociated mechanically by very gentle use of a p1000 pipette in culture media followed by light centrifugation (speed "1" for 4 minutes) and another mechanical dissociation using the same method. Cells were plated at a density of approximately 60,000 cells/cm^2 on 12-mm glass coverslips (Carolina Biological Supply, catalogue number 633029). Coverslips were first treated with 1 mM of HCl and then coated with 50 μg/mL Poly D Lysine (Sigma, catalogue number P6407) at 37º C for a minimum of 2 hours and a maximum of 24 hours. Cortical neurons were kept in culture in a humidity controlled incubator with carbon dioxide (5%) at 37º C. Recordings were performed on neurons maintained in culture for two to four weeks.

Transfections

Primary cortical neurons were transfected with either VAMP2 knock down constructs, wild type VAMP2-pHlourin or mutant VAMP2-pHlourin using the calcium phosphate method (Graham and van der Eb 1973) in primary cortical neurons 5-7 DIV. Approximately 0.5 to 1 μg of DNA was mixed with 0.2 M calcium chloride and added dropwise to an equal volume of 2X HEPES-Buffered Saline (HBS). The precipitate solution was allowed to form in the dark at room temperature for 20 minutes. DNA precipitate solution was added dropwise to cells in serum-free media and allowed to incubate for 20 minutes at 37º C. After incubation, cells were washed twice with serum free media and serum free media was replaced with original culture media.

Immunocytochemistry

Cortical neurons were stained after 16 days in vitro. Neurons were washed two times with phosphate buffer saline (PBS) containing calcium and magnesium. Neurons were fixed with 4% Paraformaldehyde in PBS with 4% sucrose added for 20 minutes at room temperature. Cells were washed three times with PBS for 10 minutes followed by incubation with blocking solution consisting of 10% normal goat serum (Thermo Fisher Scientific,

catalogue number 50197Z) with 0.1% Triton X-100 (Acros Organics, catalogue number 9002931) in PBS for one hour in order to permeabilize the membrane and to prevent non-specific binding. Cells were incubated with primary antibody overnight in incubation solution (5% normal goat serum with 0.1% Triton X-100 in PBS). The primary antibody used was mouse monoclonal anti-VAMP2 (1:1000); (Synaptic Systems, catalogue number 104-211) After washing three times with PBS, cells were incubated with 1:250 Alexa Fluoro 488 goat anti-mouse (Invitrogen, catalogue number A-21121) for two hours at room temperature. After washing, cells were incubated with DAPI mounting media and imaged using a Zeiss confocal microscope (LSM 880).

Electrophysiology

For mini recordings, coverslips were mounted on a Warner perfusion chamber and were constantly perfused with external solution. External solution was made up of 134 mM NaCl, 1 mM $MgCl_2$, 20 mM glucose, 3 mM $CaCl_2$, 0.34 mM NaH_2PO_4, 2.5 mM KCl, 1 mM $NaHCO_3$, 10 mM HEPES and 0.1% DMSO (pH 7.3 with NaOH, 310 mOsm with sucrose). Thick-walled borosilicate glass capillaries (OD: 1.5 mm, ID: 0.86 mm; Sutter Instruments) were used to pull electrodes to a final resistance of 3-6 MΩ. Cells were perfused in external solution at a rate of 2 mL/min containing 0.5 μM tetrodotoxin (TTX) in order to inhibit action potentials. The internal solution consisted of 0.6 mM EGTA, 5 mM Na_2ATP, 10 mM Na_2-phosphocreatine, 100 mM Cs-methanesulfonate, 0.3 mM Na_3GTP, 5 mM $MgCl_2$ and 30 mM HEPES (pH 7.2, 295 mOsm). After five minutes of recording, the solution was either switched to another control external solution or to one containing 10 μM Ziram (Chem Service Inc., catalogue number 137304). Ziram stock solution (10 mM in DMSO) was prepared fresh weekly and stored at 4º C. Spontaneous synaptic transmission was recorded by measuring mini excitatory post synaptic currents (mEPSCs). Mini EPSCs were recorded from rat cortical neurons using the whole-cell voltage patch clamp technique using an Optopatch

Patch-Clamp (Cairn Research LTD.). Cells were clamped at -75 mV and currents were filtered at 3 kHz before being sampled at 20 kHz using custom LabView programs (National Instruments, Austin, TX). Recordings were analysed offline after further filtering and processing via an event detection algorithm based on threshold crossings of the first derivative. The frequency and amplitude of mEPSCs were normalized by dividing each value by the mean of the baseline frequency and amplitude, respectively. Autaptic transmission was measured by recording the autaptic response in response to a brief depolarization of the neuron to elicit an escape action potential. For autapse recordings, neurons were voltage clamped at -75 mV and were depolarized for 0.5 ms to +50 mV every 10 seconds. TTX was not added to the external solution during autapse recordings. When an autaptic response was observed, the amplitude of the response was measured and was averaged for each recording. Unless otherwise stated, all reagents were purchased from Sigma-Aldrich.

RESULTS

Ziram increased the frequency but not the amplitude of spontaneous vesicle fusion events measured by recording mEPSCs

Cortical neurons were voltage clamped at -75 mV and mEPSCs were recorded to measure spontaneous synaptic transmission. Under control conditions there was no change in the amplitude or frequency of mEPSCs when control solution was washed in after a five-minute baseline recording. When ziram (10 μM) was applied after a five-minute baseline recording, there was a rapid increase in the frequency but not the amplitude of mEPSCs. The rapid and nearly four-fold increase in the frequency of mEPSCs peaked ten minutes after addition of ziram (Figure 2). The data were normalized by dividing all data points by the mean of the frequency or amplitude during the first five minutes (Figure 2).

A one-way ANOVA using the unequal variance bootstrap approach for the frequency and amplitude of mEPSCs was performed. Since ziram's effect on the frequency peaked ten minutes after application, we compared the mean of the frequency or amplitude during the first five minutes (t= 0-5 min) with the mean of the frequency or amplitude five minutes around the maximum effect (t= 12.5-17.5 min). We found that there was a statistically significant main effect of ziram treatment on mEPSC frequency ($F= 1.69$; $p < 0.05$) but not on mEPSC amplitude ($F= 0.57$; $p > 0.05$). Post-hoc tests were performed to determine the statistical significance of the difference between the means and in order to obtain a resampled distribution. The null hypothesis that we tested was that the mean ratio of the control condition was the same as the mean ratio of the ziram condition. We also resampled the data in order to determine the uncertainty of our estimate of the difference between the two groups. For both the resampled distribution and the null distribution 10,000 simulations were used and a 95% confidence interval (CI) was obtained (Figure 3). The actual difference between the mean frequency of mEPSCs for the control and ziram condition was 3.22 (95% CI = 1.64,

4.7). We found that there was a statistically significant difference in mEPSC frequency between control and ziram recordings ($p < 10^{-5}$).

VAMP2 protein levels are decreased in neurons transfected with our knock down construct.

In order to determine whether ubiquitination of VAMP2 is involved in synaptic transmission, we transfected neurons with a VAMP2 knock down construct. Neurons were transfected with our CRISPR/Cas9 construct targeting intron/exon junctions of VAMP2 in order to allow for rescue with exogenous VAMP2. Our knock down construct expressed ParpikaRFP which allowed us to visualize transfected neurons. Cortical neurons were fixed and immunocytochemistry was performed in order to estimate VAMP2 protein levels. The perinuclear zone is continuous with the endoplasmic reticulum (ER) and during translation, a protein is translocated into the lumen of the ER for further processing. Translocation into the ER during translation is a critical step for proper trafficking through cellular compartments to the right location in a highly regulated and specific manner. In control non-transfected neurons, we can see perinuclear localization of VAMP2, which implies that the VAMP2 is translated, translocated to the ER and trafficked properly. In neurons transfected with our VAMP2 knock down construct, there is no perinuclear localization of VAMP2, suggesting that the protein is not translated (Figure 4).

Perinuclear localization of VAMP2 is abolished in neurons transfected with our knock down construct compared to control neurons in the same culture (Figure 4). We used the calcium phosphate method which resulted in sparsely transfected neurons. Low transfection rates are disadvantageous, because one cannot evaluate non-autonomous presynaptic phenotypes of the knock down. Since mEPSCs are recorded from the postsynaptic cell, it is necessary to have all cells transfected in order to determine defects in spontaneous synaptic transmission as a result of knocking down a presynaptic protein. Although we are unable to

evaluate defects in spontaneous synaptic transmission, we are able to record from transfected and non-transfected neurons in the same culture. This is advantageous because there is often culture to culture variation so this is an ideal control. Another advantage of sparse transfection in neurons is that one is able to evaluate autonomous postsynaptic phenotypes as a result of the knock down. One way to evaluate defects in postsynaptic phenotypes is to measure evoked synaptic transmission, which we did by recording from autapses.

Knocking down VAMP2 in cortical neurons decreased the amplitude of the autaptic response and was rescued with wild-type or mutant VAMP2.

In order to measure defects in evoked synaptic transmission in cortical neurons transfected with VAMP2 knock down construct, we measured the autaptic response after a brief (<1 ms) depolarization. If a neuron formed an autapse, which is a synapse onto itself, the resulting autaptic response was measured and averaged for each recording and plotted in a box and whisker plot. Measuring the autaptic response, we found that there was nearly a three-fold change in the autaptic response of VAMP2 knock down neurons compared to control neurons (Figure 5). We observed a 63% decrease in evoked synaptic transmission in VAMP2 knock down neurons compared to control neurons. Fold change was determined by dividing the amplitude of the autaptic response in control neurons by that of VAMP2 knock down neurons. A percent decrease was determined by calculating the percent change of the autaptic response in control neurons compared to VAMP2 knock down neurons.

In cortical neurons transfected with wild type or mutant VAMP2 with a knock down background, the autaptic response was measured in order to determine whether defects in synaptic transmission were rescued. The fold change was calculated by dividing the amplitude of the autaptic response in rescue neurons by that of VAMP2 knock down neurons. Percent increase was calculated by determining the percent change of wild type or mutant

VAMP2 compared to VAMP2 knock down alone. There was a two-fold change in the autaptic response of neurons transfected with exogenous wild type VAMP2 which corresponds to a 100% increase. There was nearly a three and a half-fold change in neurons transfected with mutant VAMP2 with the knock down background which corresponds to nearly a 250% increase (Figure 5). There was a 126% rescue with mutant VAMP and a 77% rescue with wild-type VAMP2. Percent rescue was determined by comparing the amplitude of the autaptic response for control neurons with that of either mutant VAMP2 or wild-type VAMP2 neurons. The amplitude of the autaptic response is larger in mutant VAMP2 neurons than in wild-type VAMP2 neurons. Two separate constructs were used for mutant rescue, while only one construct was used for wild-type rescue suggesting that alterations in synaptic transmission are sensitive to the amount of VAMP2 present in the cell.

A one-way unequal variance ANOVA was performed using bootstrapping (10,000 simulations). We found that there was a statistically significant main effect of the transfection condition on the amplitude of the autaptic response (F statistic = 1, $p < 10^{-5}$). Post-hoc tests were performed to analyze the difference between groups means. We generated a resampled and a null distribution for each comparison (Figure 6). Control and VAMP2 knock down neurons statistically differed from each other with an actual difference of 0.92 nA; 95% CI (0.63, 1.21); $p<10^{-5}$. The difference between the means of knock down and mutant VAMP2 rescue neurons was 1.3 nA; 95% CI (0.95, 1.64) and this was statistically significant ($p<10^{-5}$). The difference between the means of knock down and wild-type VAMP2 rescue neurons was 0.58 nA with a 95% CI (0.24, 0.96) and $p<10^{-5}$.

Ziram increases the autaptic response in control neurons while it appears to have no effect on the autaptic response in mutant rescue neurons with VAMP2 knock down background.

As described above, we were unable to measure defects in spontaneous synaptic transmission from transfected neurons due to sparse transfection**.** Instead, we determined defects in evoked synaptic transmission by recording from autapses**.** Control cortical neurons and neurons rescued with mutant VAMP2 with knock down background were voltage clamped at -75 mV and depolarized briefly for 0.5 milliseconds. Similar to mini recordings we obtained a five-minute baseline recording and washed in either control or ziram (10 μM) solution. The autaptic response was measured for ten minutes after ziram was added. The maximum peak of the effect of ziram occurred ten minutes after application while the increase in the autaptic response was observed immediately after application. Ziram (10 μM) increased the autaptic response in control cortical neurons up to one and a half-fold (Figure 7). Strikingly, ziram (10 μM) did not alter the autaptic response in neurons rescued with mutant VAMP2 with knock down background (Figure 5). This finding supports our hypothesis that ubiquitination of VAMP2 is necessary in order for ziram to alter evoked synaptic transmission.

DISCUSSION

E1 ligase inhibition rapidly increases the frequency of mEPSCs but does not alter the amplitude of mEPSCs in cortical neurons (Figure 2). This finding is consistent with previous findings in our lab using hippocampal cultures with E1 inhibitor application (Rinetti and Schweizer 2010). Rinetti and Schweizer (2010) found that in hippocampal neurons, there was a rapid increase in mEPSC frequency that reached a maximum effect 10 to 15 minutes after ziram application. We found that with a slightly longer duration of application (>15 minutes), ziram causes an initial increase in the frequency of mEPSCs in cortical neurons and a subsequent decrease back to baseline level (Figure 2). This observation is consistent with previous studies in cortical neurons in our lab (Katherine Myers Gschweng, personal communication). One explanation for the initial increase and resulting decline in mEPSC frequency is that ziram's effect reaches a maximum peak at ten minutes after application and after the peak effect is reached, neurons are no longer sensitive to the effect. It is also possible that a homeostatic mechanism is responsible for the rapid increase and subsequent decrease in mEPSC frequency. The presynaptic and postsynaptic response may change with increased exposure time to ziram and the change may occur after the peak maximum effect is observed. Ziram could also have several targets in neurons contributing to the initial increase and subsequent decrease in spontaneous vesicle fusion, where in one process there is an increase and in another process, there is a decrease.

Ziram is known to have multiple sites of action that could all be through different molecular mechanisms; ziram inhibits aldehyde dehydrogenase, impairs mitochondrial function and depletes cellular sulfyhryls (Fitzmaurice et al. 2014; Yamano and Morita 1995). Ziram was also found to regulate calcium homeostasis, in addition to acting as an E1 ligase inhibitor (Chou et al. 2008; Jin et al. 2014; Rinetti and Schweizer 2010). In order to attribute the observed effect on protein ubiquitination, it would be important in future experiments to

test another E1 ligase inhibitor such as NSC624206 (Ungermannova et al. 2012). It is also essential to test a deubiquitinating enzyme inhibitor, such as G5 (Fontanini et al. 2009), and measure the effect of synaptic transmission in cortical neurons. Studies in our lab suggest that dynamic ubiquitination regulates synaptic transmission. DUBs are believed to counter the role of ubiquitination, but in our studies DUB inhibition appears to have the same effect on synaptic transmission as E1 inhibition (Katherine Myers Gschweng, personal communication). Follow up studies are important for determining the role of dynamic ubiquitination on presynaptic protein ubiquitination in synaptic transmission.

To determine the role of ubiquitination of presynaptic proteins in synaptic transmission, we developed a probe to investigate whether ubiquitination of VAMP2 was involved. First, we wanted to knock down VAMP2 using the CRISPR/Cas9 system. In this study, we used the CRISPR/ Cas9 system instead of RNAi because CRISPR/Cas9 targets the genome leading to reduction in expression of the gene that is likely to be permanent. Another advantage of using CRISPR/Cas9 is that it is not found endogenously in the mammalian system, unlike RNAi, so it does not alter endogenous machinery and is less prone to off target effects (Barrangou et al. 2015; Jackson and Linsley 2010). Perinuclear localization of VAMP2 was used as a proxy for estimating VAMP2 protein levels in neurons transfected with our knock down construct. Detecting perinuclear localization of VAMP2 allows us to detect whether the protein is translated and translocated to the ER during translation, which is a crucial first step for proper protein sorting and trafficking. In control non-transfected neurons, there is perinuclear localization of VAMP2 suggesting that VAMP2 is translated and trafficked properly. Perinuclear localization of VAMP2 in cells transfected with our knock down construct is abolished compared to non-transfected control neurons in the same culture, suggesting that VAMP2 is not translated (Figure 4). Our findings indicate that our

CRISPR/Cas9 construct successively prevented VAMP2 from being produced and this was accomplished in post-mitotic neurons.

CRISPR/Cas9 has been widely used in dividing cells because one is able to select a transfected cell and create a cell line that is homogenous for the mutation. Only a small number of studies have successfully used the CRISPR/Cas9 system to knock down proteins in post-mitotic neurons in order to evaluate synaptic function. Most of these studies used calcium phosphate to sparsely transfect neurons and record autonomous postsynaptic phenotypes from transfected neurons and un-transfected controls in the same culture by recording from pairs (Incontro et al. 2014; Straub et al. 2014; Wei et al. 2016). In this study, we used calcium phosphate to sparsely transfect cortical neurons and we determined defects in evoked synaptic transmission as a result of VAMP2 knock down by recording autapses. We found that there was a decrease in the autaptic response of VAMP2 knock down neurons compared to controls (Figure 5). Previous studies at the NMJ of *Drosophila* and in hippocampal cultures from VAMP2 knock out mice found that spontaneous and evoked synaptic transmission was nearly abolished (Deitcher et al. 1998; Schoch et al. 2001). The reduction in evoked synaptic transmission in our cultures was not as dramatic, but we still see an almost 63% decrease. We found that knocking down VAMP2 using CRISPR/Cas9 was sufficient to impair evoked synaptic transmission, but it was not completely abolished. The fact that VAMP2 knock down did not completely abolish synaptic transmission suggests that the knockout is not complete. Synaptic vesicles contain approximately 70 copies of VAMP2, and only a small number are needed in order for vesicle fusion to occur (Sinha et al. 2011). Our observation is consistent with this hypothesis, and with previous findings that evoked synaptic transmission is not completely abolished with VAMP2 deletion due to incomplete knockout and redundancy (Deak et al. 2004; Schoch et al. 2001).

Since our knock down constructs targeted intron/exon junctions, we were able to rescue with exogenous VAMP2 protein. Incontro et al. (2014) pioneered this simple rescue experiment design and used it to knock down the GluN1 subunit of the ionotropic glutamate receptor (NMDAR) using the CRISPR/Cas9 system and to rescue with exogenous GluN1. We found that expression of either exogenous wild type VAMP2 or mutant VAMP2 rescued defects in synaptic transmission after VAMP2 knock down (Figure 5). It is surprising that mutant VAMP2 rescues synaptic transmission because previous studies in our lab found that mutant VAMP2 and wild-type VAMP2 have different sorting properties in synaptic vesicles. In this study, we also found that the rescue with mutant VAMP2 was different than rescue with wild-type VAMP2. This may be because more cDNA was added with mutant rescue experiments, but it is also possible that mutant and wild-type VAMP have different effects on synaptic transmission. Further investigation into the different properties of mutant and wild-type VAMP2 rescue is important. Overall, our findings indicate that knocking down endogenous VAMP2 with the CRISPR/Cas9 system decreases synaptic transmission and we are able to rescue it with exogenous wild type and mutant VAMP2 expression (Figure 4).

To determine whether ubiquitination of VAMP2 is involved in evoked synaptic transmission, we measured the effect of ziram on the autaptic response in control neurons and neurons rescued with mutant VAMP2 with the knock down background. We found that ziram increases the autaptic response in control neurons, while it did not alter the autaptic response in neurons transfected with mutant VAMP2 that cannot undergo ubiquitination (Figure 7). The fact that ziram only alters evoked synaptic transmission in control neurons and not in neurons with mutant VAMP2 supports our hypothesis that ubiquitination of VAMP2 plays a role in synaptic transmission. Ubiquitination of VAMP2 is an essential molecular mechanism and target by which ziram alters evoked synaptic transmission. Our results are very exciting but have some limitations so follow up experiments are crucial. A result that is surprising is

that ziram increases the autaptic response in cortical neurons, while previous studies in our lab found that ziram decreases evoked synaptic transmission which was measured by recording from pairs. An explanation for the discrepancy in our observations is that there is a difference in experimental setup. Our finding is also limited by the small sample size, which needs to be increased in order to ensure reproducibility of the result and reliability of the observation. In order to ensure reliability of the observation, we also need to conduct experiments in order to determine the effect of ziram on neurons rescued with wild type VAMP2 with the knock down background. These experiments are important because it is necessary to ensure that the act of transfecting, knocking down VAMP2 and rescuing is not causing the observed difference between control and mutant VAMP2 rescue neurons.

Although there are some limitations, it is clear that E1inhibition alters evoked synaptic transmission and that there is a role for ubiquitination of VAMP2 in the response. It would be interesting to follow up with NSC624206 and G5 to see if there is dynamic regulation of ubiquitination in autapses and whether it is different in neurons where VAMP2 cannot undergo ubiquitination. Another exciting follow-up experiment would be to package our constructs into a virus and measure spontaneous synaptic transmission. The fact that our VAMP2 knock down construct recapitulates the evoked synaptic transmission phenotype from previous studies (Deak et al. 2004; Deitcher et al. 1998; Schoch et al. 2001) and that we are able to rescue with exogenous VAMP2 is promising. Packaging our knock down and rescue constructs into a virus would allow us to transfect all neurons and determine whether ubiquitination of VAMP2 is involved in spontaneous synaptic transmission.

Similar knock down and rescue studies could be conducted in the future to determine the role of ubiquitination of presynaptic proteins on synaptic transmission. Future studies could use other presynaptic proteins that came up in our proteomic search, such as synaptotagmin and SNAP-25. Synaptotagmin and SNAP-25, like VAMP2, are crucial for

vesicle docking and exocytosis (Jahn and Sudhof 1994). Future studies could also be done with RIM1 and Munc13, which are active zone proteins believed to be regulated by ubiquitination (Speese et al. 2003; Yao et al. 2007). RIM1 and Munc13 are essential for vesicle docking and exocytosis, and their interaction plays an important role in synaptic plasticity (Yang and Calakos 2011). These future studies would provide insights into understanding how presynaptic proteins interact and how they are regulated, which is important for understanding synaptic transmission and plasticity.

FIGURES

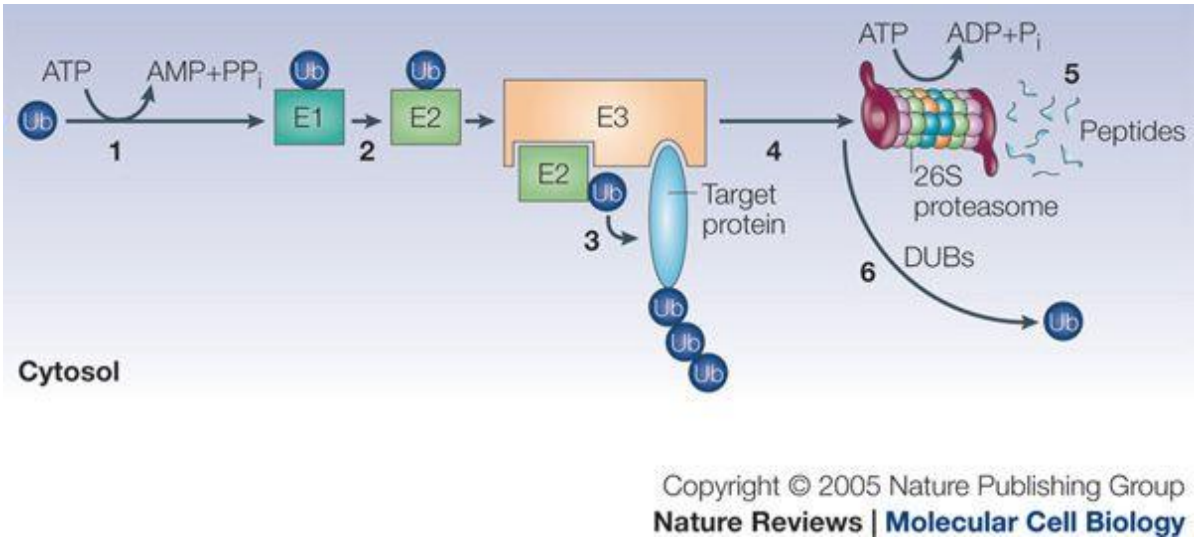


Figure 1. **Ubiquitination of a Target Protein Adapted from Welchman, Gordon, and Mayer (2005) .** Ubiquitination of a target protein begins with ubiquitin binding to E1 which activates (1), transferring ubiquitin to E2 (2) and the E2 ubiquitin complex interacting with E3 and the target protein. The E3 ligase assists or directly transfers ubiquitin from E2 to the target protein. After transfer of ubiquitin to a target protein (3), the process can stop at monoubiquination, or the ubiquitin cascade can be repeated adding a chain of ubiquitin to a target protein, resulting in polyubiquination. Ubiquitination can act as a signal to target a ubiquitin conjugated protein to the proteasome for degradation (4, 5) or the protein can be modified by a DUB which removes ubiquitin and reverses the process of ubiquitination.

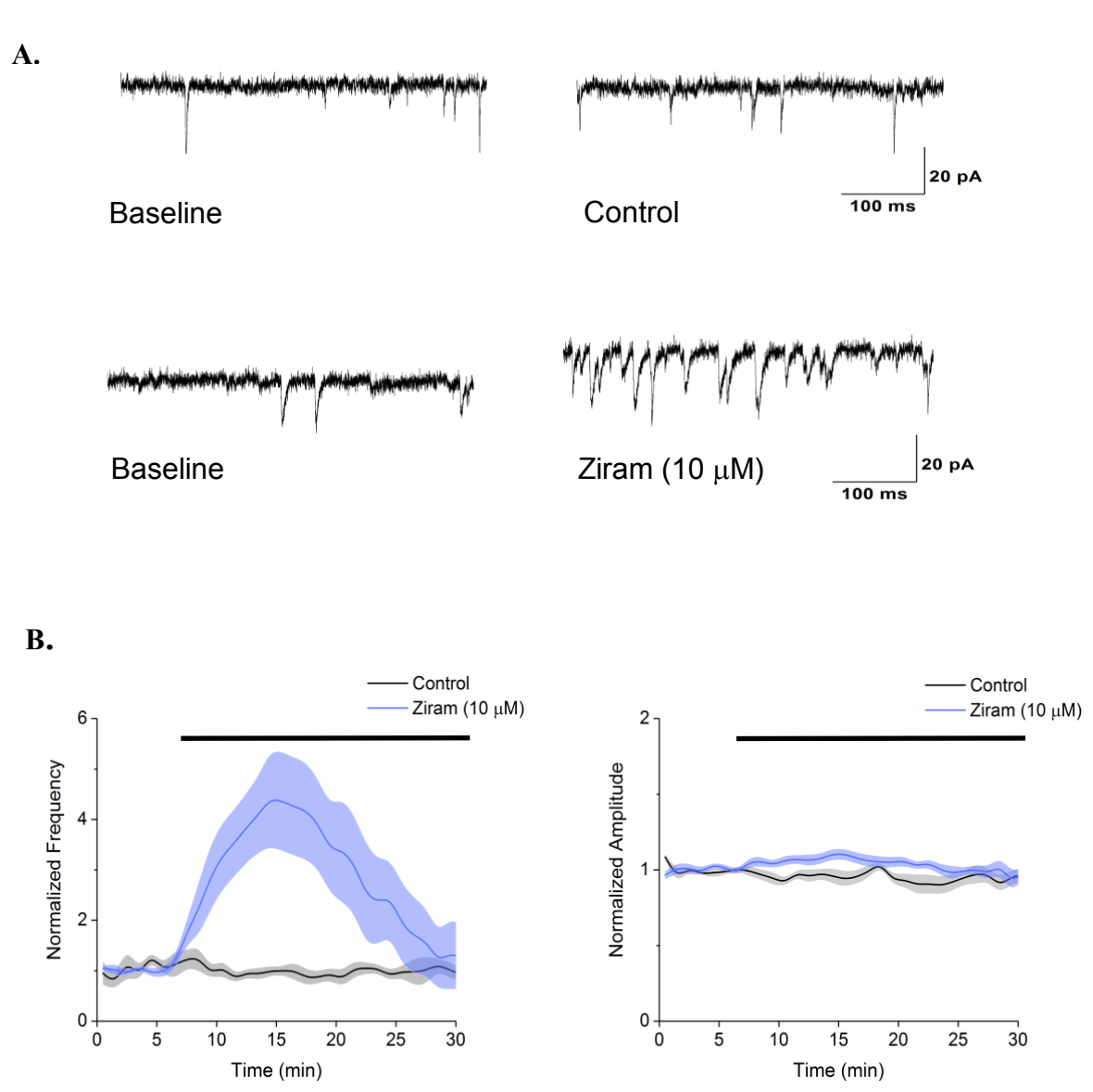


Figure 2. Ziram increases the frequency of mini excitatory post synaptic currents (mEPSCs) in primary cortical neurons. Neurons were voltage clamped at -75 mV and a baseline recording was obtained for 5 minutes in control solution, followed by perfusion with 10 μM of Ziram (N=5) or control solution (N=5). (A) Example trace of mEPSCs during voltage clamp experiments. (B) Normalized frequency (left) and amplitude (right) of mEPSCs. The black line indicates that ziram (10 μM) was added to external solution. Each line represents the mean ± SEM (lighter shaded region).

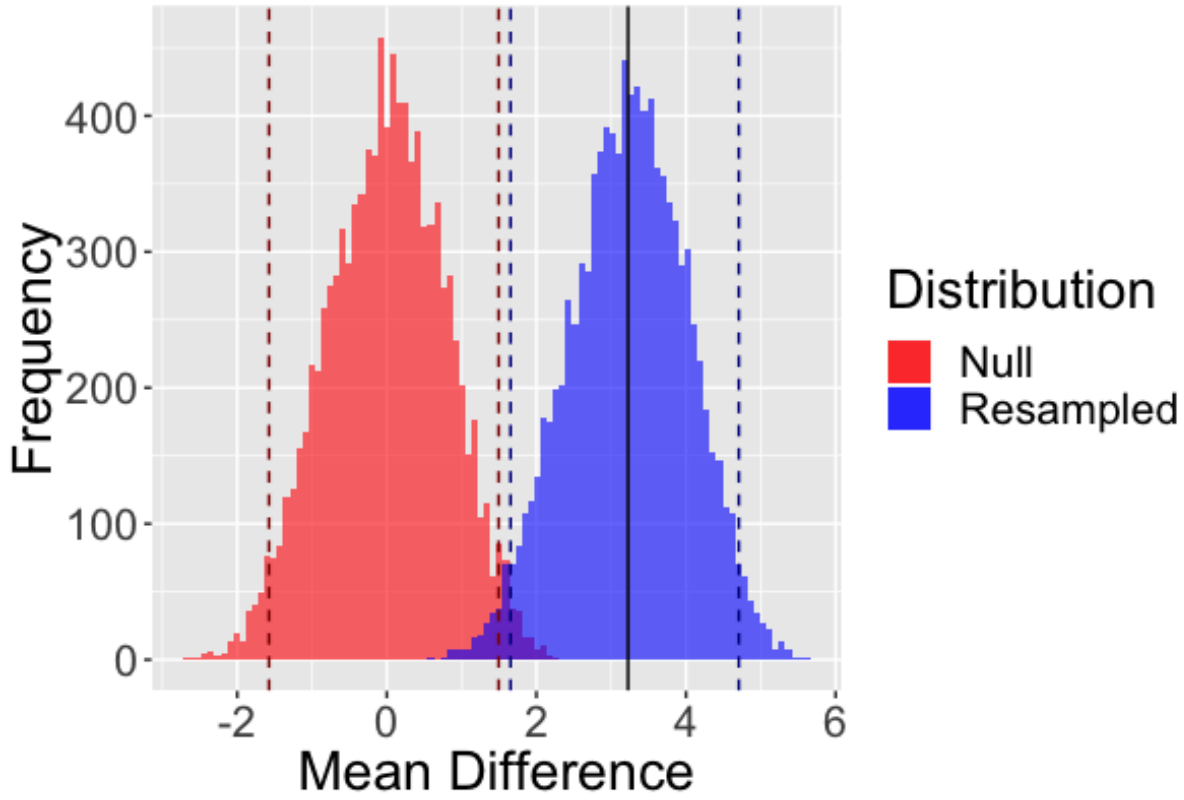


Figure 3. Resampled and null distributions of the frequency of mEPSCs comparing the difference between the control (N=5) and ziram (N=5) condition. Bootstrapping (10,000 simulations) was used to obtain a resampling distribution of the data with a 95% CI (dashed blue lines). A null distribution was also run with a 95% CI (dashed red lines). The black line represents the actual difference between the mean of the two groups.

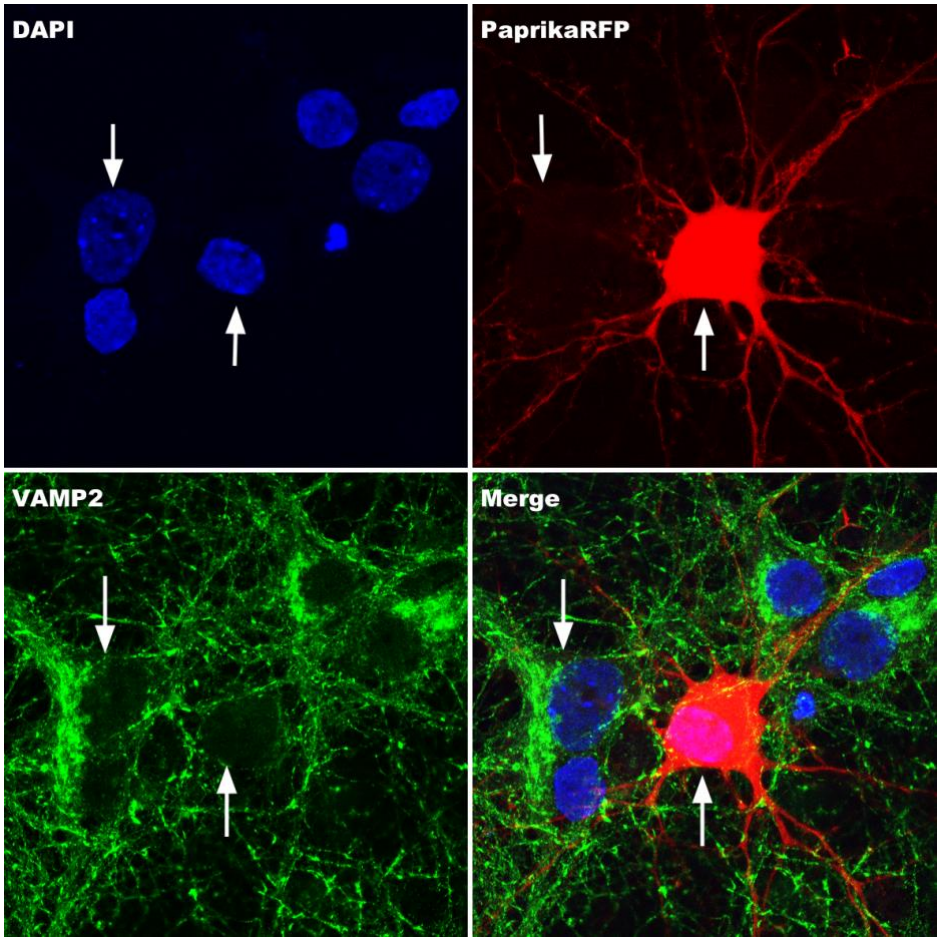
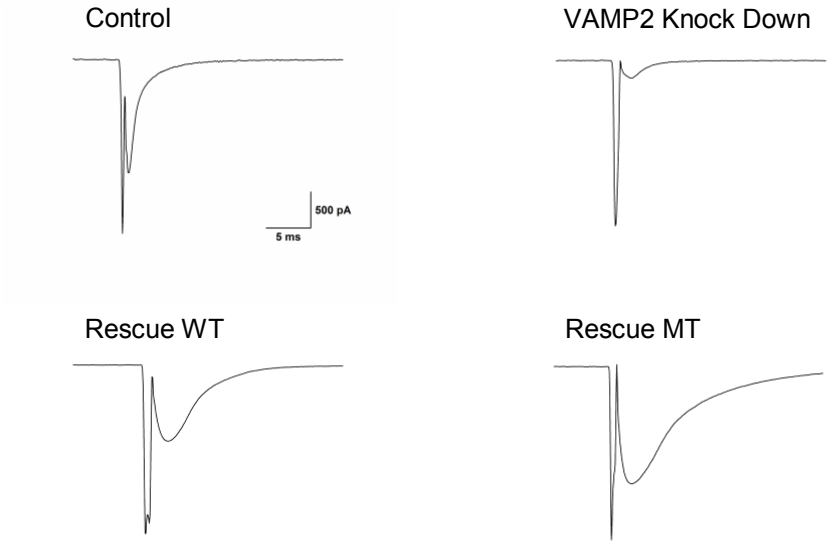


Figure 4. Perinuclear localization of VAMP2. Perinuclear localization was used to determine VAMP2 protein levels in non-transfected control neurons and VAMP2 knock down neurons in the same culture. Cortical neurons were stained with DAPI and a VAMP2 antibody. PaprikaRFP was the selection marker used to express whether neurons were transfected with the VAMP2 knock down construct. Arrows indicate an un-transfected control neuron or a neuron transfected with the CRISPR/Cas9 knock down construct. Perinuclear localization of VAMP2 is present in control neurons while it is non-existent in VAMP2 knock down neurons.

A.



B.

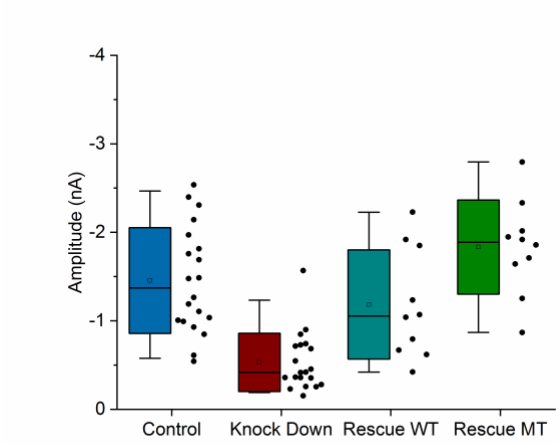
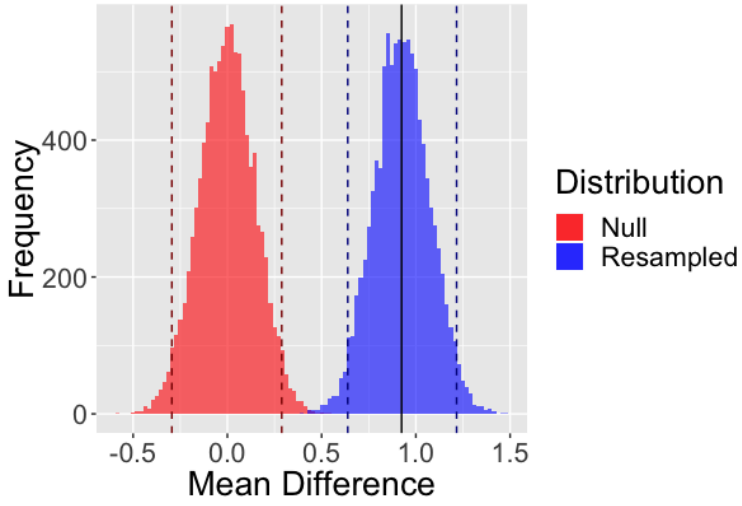
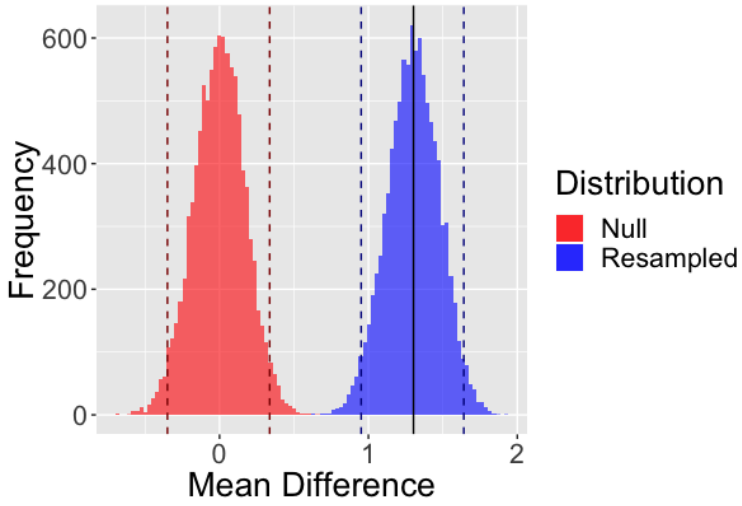


Figure 5. The amplitude of the autaptic response in cortical neurons. (A) Example traces of the autaptic response in control and transfected neurons. Autaptic response was measured in control cortical neurons, neurons transfected with the VAMP2 knock down constructs alone and neurons transfected with either the wild type or mutant VAMP2 constructs with a knock down background. (B) Box-and-whisker plot of the amplitude of the autaptic response in control (N=20), VAMP2 knock down (N=20), wild type VAMP2 (N=10) and mutant VAMP2 (N=10) cells. The box represents the standard deviation (SD), the whiskers represent the confidence interval, the middle of the box represents the median and the square inside the box represents the mean value.

A.



B.



C.

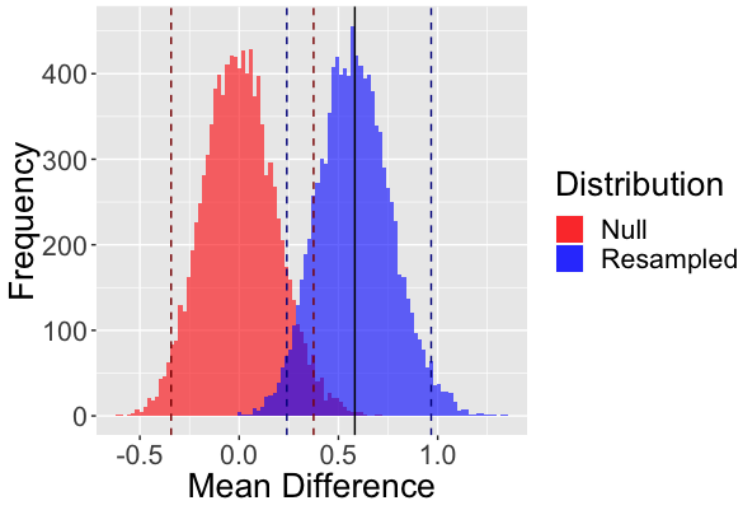


Figure 6. Resampled and null distributions comparing the mean of the amplitude of the autaptic response of Control with VAMP2 Knock Down (A), Mutant VAMP2 Rescue with Knock Down (B), or Wild-Type VAMP2 Rescue with Knock Down (C).

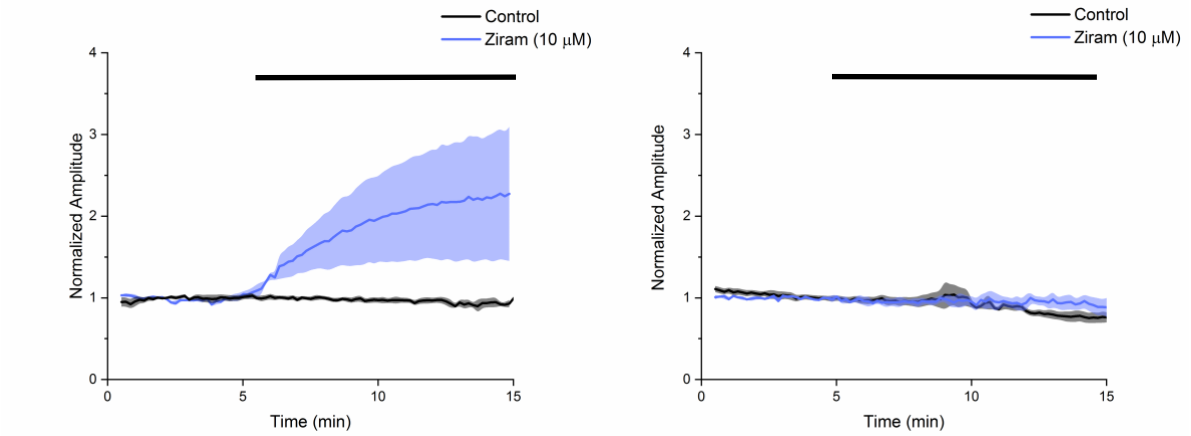


Figure 7. Ziram increases the autaptic response in control cortical neurons but does not alter the autaptic response in mutant VAMP2 neurons with knock down background.

Autaptic response was measured in control cortical neurons (N=3) and mutant VAMP2 knock down neurons (N=2). A five-minute baseline recording was first obtained and ziram (10 μM) was added to external solution. The black line indicates addition of ziram.

REFERENCES

Barrangou, R., A. Birmingham, S. Wiemann, R. L. Beijersbergen, V. Hornung, and Av Smith. 2015. 'Advances in CRISPR-Cas9 genome engineering: lessons learned from RNA interference', *Nucleic Acids Res*, 43: 3407-19.

Betz, A., M. Okamoto, F. Benseler, and N. Brose. 1997. 'Direct interaction of the rat unc-13 homologue Munc13-1 with the N terminus of syntaxin', *J Biol Chem*, 272: 2520-6.

Braten, O., I. Livneh, T. Ziv, A. Admon, I. Kehat, L. H. Caspi, H. Gonen, B. Bercovich, A. Godzik, S. Jahandideh, L. Jaroszewski, T. Sommer, Y. T. Kwon, M. Guharoy, P. Tompa, and A. Ciechanover. 2016. 'Numerous proteins with unique characteristics are degraded by the 26S proteasome following monoubiquitination', *Proc Natl Acad Sci U S A*, 113: E4639-47.

Chen, Hong, Simona Polo, Pier Paolo Di Fiore, and Pietro De Camilli. 2003. 'Rapid Ca2+-dependent decrease of protein ubiquitination at synapses', *Proceedings of the National Academy of Sciences of the United States of America*, 100: 14908-13.

Chou, A. P., N. Maidment, R. Klintenberg, J. E. Casida, S. Li, A. G. Fitzmaurice, P. O. Fernagut, F. Mortazavi, M. F. Chesselet, and J. M. Bronstein. 2008. 'Ziram causes dopaminergic cell damage by inhibiting E1 ligase of the proteasome', *J Biol Chem*, 283: 34696-703.

Deak, F., S. Schoch, X. Liu, T. C. Sudhof, and E. T. Kavalali. 2004. 'Synaptobrevin is essential for fast synaptic-vesicle endocytosis', *Nat Cell Biol*, 6: 1102-8.

Deitcher, D. L., A. Ueda, B. A. Stewart, R. W. Burgess, Y. Kidokoro, and T. L. Schwarz. 1998. 'Distinct requirements for evoked and spontaneous release of neurotransmitter are revealed by mutations in the Drosophila gene neuronal-synaptobrevin', *J Neurosci*, 18: 2028-39.

Dikic, I., S. Wakatsuki, and K. J. Walters. 2009. 'Ubiquitin-binding domains - from structures to functions', *Nat Rev Mol Cell Biol*, 10: 659-71.

Dobie, F., and A. M. Craig. 2007. 'A fight for neurotransmission: SCRAPPER trashes RIM', *Cell*, 130: 775-7.

Fitzmaurice, A. G., S. L. Rhodes, M. Cockburn, B. Ritz, and J. M. Bronstein. 2014. 'Aldehyde dehydrogenase variation enhances effect of pesticides associated with Parkinson disease', *Neurology*, 82: 419-26.

Fontanini, A., C. Foti, H. Potu, E. Crivellato, R. Maestro, P. Bernardi, F. Demarchi, and C. Brancolini. 2009. 'The Isopeptidase Inhibitor G5 Triggers a Caspase-independent Necrotic Death in Cells Resistant to Apoptosis: A COMPARATIVE STUDY WITH THE PROTEASOME INHIBITOR BORTEZOMIB', *J Biol Chem*, 284: 8369-81.

Glickman, M. H., and A. Ciechanover. 2002. 'The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction', *Physiol Rev*, 82: 373-428.

Graham, F. L., and A. J. van der Eb. 1973. 'A new technique for the assay of infectivity of human adenovirus 5 DNA', *Virology*, 52: 456-67.

Hershko, A., and A. Ciechanover. 1992. 'The ubiquitin system for protein degradation', *Annu Rev Biochem*, 61: 761-807.

Hochstrasser, M. 2009. 'Origin and function of ubiquitin-like proteins', *Nature*, 458: 422-9.

Hurley, J. H., S. Lee, and G. Prag. 2006. 'Ubiquitin-binding domains', *Biochem J*, 399: 361-72.

Incontro, S., C. Asensio, R. Edwards, and R. Nicoll. 2014. 'Efficient, Complete Deletion of Synaptic Proteins using CRISPR', *Neuron*, 83.

Jackson, A. L., and P. S. Linsley. 2010. 'Recognizing and avoiding siRNA off-target effects for target identification and therapeutic application', *Nat Rev Drug Discov*, 9: 57-67.

Jahn, R., and T. C. Sudhof. 1994. 'Synaptic vesicles and exocytosis', *Annu Rev Neurosci*, 17: 219-46.

Jin, J., A. J. Lao, M. Katsura, A. Caputo, F. E. Schweizer, and S. Sokolow. 2014. 'Involvement of the sodium-calcium exchanger 3 (NCX3) in ziram-induced calcium dysregulation and toxicity', *Neurotoxicology*, 45: 56-66.

Kaeser, P. S., L. Deng, M. Fan, and T. C. Sudhof. 2012. 'RIM genes differentially contribute to organizing presynaptic release sites', *Proc Natl Acad Sci U S A*, 109: 11830-5.

Kaeser, P. S., L. Deng, Y. Wang, I. Dulubova, X. Liu, J. Rizo, and T. C. Sudhof. 2011. 'RIM proteins tether Ca2+ channels to presynaptic active zones via a direct PDZ-domain interaction', *Cell*, 144: 282-95.

Richmond, Janet, Warren Davis, and Erik Jorgensen. 1999. 'UNC-13 is required for synaptic vesicle fusion in C. elegans', *Nature Neuroscience*, 2: 959-64.

Rinetti, G. V., and F. E. Schweizer. 2010. 'Ubiquitination acutely regulates presynaptic neurotransmitter release in mammalian neurons', *J Neurosci*, 30: 3157-66.

Ronai, Z. A. 2016. 'Monoubiquitination in proteasomal degradation', *Proc Natl Acad Sci U S A*, 113: 8894-6.

Schoch, S., F. Deak, A. Konigstorfer, M. Mozhayeva, Y. Sara, T. C. Sudhof, and E. T. Kavalali. 2001. 'SNARE function analyzed in synaptobrevin/VAMP knockout mice', *Science*, 294: 1117-22.

Sinha, R., S. Ahmed, R. Jahn, and J. Klingauf. 2011. 'Two synaptobrevin molecules are sufficient for vesicle fusion in central nervous system synapses', *Proc Natl Acad Sci U S A*, 108: 14318-23.

Speese, S. D., N. Trotta, C. K. Rodesch, B. Aravamudan, and K. Broadie. 2003. 'The ubiquitin proteasome system acutely regulates presynaptic protein turnover and synaptic efficacy', *Curr Biol*, 13: 899-910.

Straub, C., A. J. Granger, J. L. Saulnier, and B. L. Sabatini. 2014. 'CRISPR/Cas9-mediated gene knock-down in post-mitotic neurons', *PLoS One*, 9: e105584.

Ungermannova, D., S. J. Parker, C. G. Nasveschuk, D. A. Chapnick, A. J. Phillips, R. D. Kuchta, and X. Liu. 2012. 'Identification and mechanistic studies of a novel ubiquitin E1 inhibitor', *J Biomol Screen*, 17: 421-34.

Wei, M., J. Zhang, M. Jia, C. Yang, Y. Pan, S. Li, Y. Luo, J. Zheng, J. Ji, J. Chen, X. Hu, J. Xiong, Y. Shi, and C. Zhang. 2016. 'alpha/beta-Hydrolase domain-containing 6 (ABHD6) negatively regulates the surface delivery and synaptic function of AMPA receptors', *Proc Natl Acad Sci U S A*, 113: E2695-704.

Weissman, A. M. 2001. 'Themes and variations on ubiquitylation', *Nat Rev Mol Cell Biol*, 2: 169-78.

Welchman, R. L., C. Gordon, and R. J. Mayer. 2005. 'Ubiquitin and ubiquitin-like proteins as multifunctional signals', *Nat Rev Mol Cell Biol*, 6: 599-609.

Welchman RL, Gordon C, Mayer RJ. 2005. 'Ubiquitin and ubiquitin-like proteins as multifunctional signals.', *Nat Rev Mol Cell Biol*, 6: 599-609.

Winget, J. M., and T. Mayor. 2010. 'The diversity of ubiquitin recognition: hot spots and varied specificity', *Mol Cell*, 38: 627-35.

Xu, Guoqiang, and Samie Jaffrey. 2011. 'The new landscape of protein ubiquitination', *Nature Biotechnology*, 29: 1098-100.

Yamano, T., and S. Morita. 1995. 'Effects of pesticides on isolated rat hepatocytes, mitochondria, and microsomes II', *Arch Environ Contam Toxicol*, 28: 1-7.

Yang, Ying, and Nicole Calakos. 2011. 'Munc13-1 is required for presynaptic long-term potentiation', *Journal of Neuroscience*, 31.

Yao, I., H. Takagi, H. Ageta, T. Kahyo, S. Sato, K. Hatanaka, Y. Fukuda, T. Chiba, N. Morone, S. Yuasa, K. Inokuchi, T. Ohtsuka, G. R. Macgregor, K. Tanaka, and M.

Setou. 2007. 'SCRAPPER-dependent ubiquitination of active zone protein RIM1 regulates synaptic vesicle release', *Cell*, 130: 943-57.