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The Role of mGlu1/5 Signaling and Fragile X Mental Retardation Protein for Cell Surface Expression of the potassium channel Kv4.2

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Abstract

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Patients with fragile X syndrome (FXS), the most common inherited form of intellectual disability, frequently suffer from epileptic seizures. The higher susceptibility to seizure development is recapitulated in *Fmr1* Knockout (KO) mice, a mouse model for FXS, but the underlying molecular mechanisms are unknown. The Bassell lab has shown previously that total and cell surface levels of the potassium channel Kv4.2, a major regulator of neuronal excitability, are decreased in the brains of *Fmr1* KO mice. Of note, a mutation in Kv4.2 leads to temporal lobe epilepsy in human patients. Therefore, we hypothesize that the decreased levels of total and cell surface Kv4.2 in hippocampal neurons of *Fmr1* KO mice may contribute to the epileptic phenotype. Previous studies have shown that signaling through metabotropic glutamate receptors 1/5 (mGlu1/5) is dysregulated in *Fmr1* KO mice, and that antagonists of mGlu5 rescue the seizure phenotype in *Fmr1* KO mice and partially restore Kv4.2 cell surface levels. The purpose of this research was to determine how mGlu1/5 signaling regulates the potassium channel Kv4.2 by analyzing endogenous Kv4.2 cell surface expression in mouse hippocampus, and by using a pH-sensitive fluorescence reporter protein to quantify Kv4.2 cell surface levels in cultured cell lines. Our study suggests that signaling through mGlu1/5 negatively regulates Kv4.2 cell surface levels. Furthermore, we show that transient siRNA-mediated reduction of FMRP is sufficient to decrease Kv4.2 cell surface expression. This thesis

motivates further research using the pH-sensitive reporter protein in fixed or live cells to study the efficiency of mGlu1/5 downstream signaling antagonists to restore Kv4.2 cell surface levels in the absence of FMRP. In the future, this may help to design therapies to treat seizures in FXS.

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Introduction

Fragile X Syndrome (FXS) is the most commonly inherited form of intellectual disability and is the most common monogenic cause of autism. The disease affects approximately 1 in 2500-4000 males and 1 in 7000-8000 females (Bassell and Warren, 2008). While in the past years, considerable progress has been made towards therapeutic strategies to ameliorate certain aspects of the syndrome, the exact underlying molecular mechanisms still remain poorly understood. A better understanding of these pathological mechanisms will be useful to develop more targeted, and thus more efficient therapeutic strategies.

Inheritance of the Fragile X Syndrome

FXS is an X-linked disease and is caused by a CGG trinucleotide repeat expansion in the 5'UTR of the fragile X mental retardation gene (*FMR1*). Unaffected individuals have an average of 5-55 repeats in the UTR region. 55-200 repeats denote a permutation, while more than 200 repeats result in a full mutation of the gene. At this range, hypermethylation of the CpG island embedded within the *FMR1* promoter occurs. This leads to the transcriptional silencing of *FMR1* (McConkie-Rosell et al., 1993; Stoger et al., 1997). Only a full mutation (>200 repeats) causes hypermethylation of the *FMR1* gene, transcriptional silencing, and therefore loss of expression of the Fragile X Mental Retardation Protein (FMRP), resulting in FXS.

An individual who has the premutation in the *FMR1* gene is a carrier of the disease. While carriers do not develop FXS, they can suffer from other disorders, such as FXTAS (fragile X associated tremor/ataxia syndrome) (Berry-Kravis et al., 2007)or FXPOI (Fragile X associated primary ovarian insufficiency) (Sherman, 2000). In addition, general population studies of both adults and infants have found that approximately 1/250 females and 1/800 males are carriers

of the *FMR1* premutation worldwide (Fragile X Foundation.org). Because of the prevalence of this disease, it is important to continue to conduct research that will alleviate symptoms and ultimately help cure the disease.

Fragile X Symptoms

Approximately 1 in 4000 males and 1 in 4000-6000 females are born with the full mutation. However, only half of the females who have the full *FMR1* mutation display symptoms of FXS while all males with the full mutation suffer from symptoms. This is because the affected gene, *FMR1*, is located on the X chromosome. Females have a second X chromosome, which if normal, still produces FMRP; due to X-chromosomal inactivation, there is a 50% chance in every cell that the normal X chromosome is activated, which can ameliorate the FXS phenotype. Some physical features that characterize the FXS phenotype include: elongated face, prominent ears, flat feet, and macroorchidism, or enlarged testicles (Butler et al., 1992). Fragile X patients suffer from a variety of symptoms and complications including: intellectual disability, developmental delays, learning disabilities, hyperactivity, attention deficit, anxiety, mood disorders, and seizures. The amount and extent of these symptoms vary by the individual; however, females usually display fewer and less severe symptoms due to their extra X chromosome with an unaffected *FMR1* gene (de Vries et al., 1996).

Three common symptoms of FXS that are important to this research are hyperactivity, increased sensitivity to sensory stimuli, and epilepsy. These symptoms can be explained through neuronal hyperexcitability. In fact, hyperactivity, increased sensitivity to sensory stimuli and epilepsy are all symptoms that have been observed in *Fmr1* knockout (KO) mice. *Fmr1* KO mice, in which the *Fmr1* gene is deleted, are a useful mouse model of FXS, and have been

shown to recapitulate many of the symptoms observed in human patients. Moreover, eletrophysiological experiments have demonstrated that *Fmr1* KO neurons are hyperexcitable (Chuang et al., 2005). Because *Fmr1* KO mice show macroorchidism and cognitive and behavioral deficits that are comparable to human patients, Fmr1 KO mice are considered to be a reliable transgenic model for the study of FSX (Kooy et al., 1996).

Dysregulation of potassium channels in FXS

FMRP is a selective RNA binding protein that plays an important role in the post-transcriptional regulation of gene expression, where it binds approximately 4% of mRNA in brain, and regulates their translation, transport or stability. FMRP was shown to regulate the translation and transportation of select mRNAs at the synapse and its domain structure is representative of its function (Figure 1). FMRP contains several RNA-binding motifs, including: two KH domains (KH1 and KH2), an RGG box, and an N-terminal RNA-binding domain (Siomi et al., 1993; Zalfa et al., 2005).

The underlying causes of hyperexcitability in FXS are not fully understood. Yet, one possible cause may be the dysregulation of specific target mRNAs of FMRP. This dysregulation may ultimately lead to the neuronal hyperexcitability seen in FXS. Recent evidence has suggested that FMRP mediates regulation of specific ion channels (Brown et al., 2010; Strumbos et al., 2010; Gross et al., 2011; Lee et al., 2011). Prior research has also shown that potassium channels are key regulators of neuronal excitability. This suggests that dysregulated potassium channel function might contribute to the neuronal hyperexcitability seen in FXS, and ultimately lead to the epileptic phenotype found in FXS patients.

As stated above, recent evidence indicates that FMRP plays a role in the expression and/or function of specific potassium channels, such as Kv3.1b, slack and Kv4.2 (Brown et al., 2010; Strumbos et al., 2010; Gross et al., 2011; Lee et al., 2011). It was shown that FMRP regulates the Slack potassium ion channel by binding to the C-terminus of Slack in order to activate it (Brown et al., 2010). Unlike Slack regulation, FMRP regulates Kv3.1b by binding to mRNA encoding the protein (Strumbos et al., 2010). Moreover, two recent studies suggest that FMRP associates with the mRNA of the potassium channel Kv4.2, and regulates Kv4.2 protein expression and function. Kv4.2 is the major potassium channel regulating neuronal excitability in the hippocampus (Birnbaum et al., 2004). While Kv3.1b and slack are important for auditory processing in the brain stem, only Kv4.2 has been linked to epilepsy in humans or animal models (Singh et al., 2006; Barnwell et al., 2009).

Kv4.2 channels mediate transient A-type currents, that rapidly hyperpolarize cells in response to depolarization. Thus, Kv4.2 channels reduce the back-propagation of action potentials into dendrites, control the excitability of neurons, and regulate neuronal capability to undergo long lasting changes in its signal transmission (Birnbaum et al., 2004; Chen et al., 2006). Previous research has shown that Kv4.2 knockout (KO) mice, with a deletion in the Kv4.2 gene, have a much higher sensitivity to convulsant stimuli, and a mutation in the Kv4.2 gene leads to temporal lobe epilepsy in humans (Barnwell et al., 2009). Furthermore, seizures influenced and changed hippocampal Kv4.2 protein phosphorylation and localization levels in several different animal models (Francis et al., 1997; Lugo et al., 2008). Therefore, a possible mechanism underlying the heightened neuronal excitability and eventual epilepsy in FXS patients is the loss of direct regulation of Kv4.2 by FMRP. This hypothesis is supported by a

recent study from our lab showing that neuronal and dendritic Kv4.2 cell surface expression of hippocampal slices is reduced in Fmr1 KO mice brains (Figure 2, Gross et al., 2011). FMRP is usually a negative regulator of mRNA translation, and only a few target mRNAs have been reported to be positively regulated by FMRP (Bechara et al., 2009; Fahling et al., 2009); however, the Bassell lab has demonstrated that FMRP normally associates with Kv4.2 mRNA, and that total and cell surface levels of Kv4.2 are reduced in the absence of FMRP (Figure 2; Gross et al., 2011). Therefore, this indicates that FMRP positively regulates Kv4.2 and that Kv4.2 dysregulation in the absence of FMRP may contribute to synaptic hyperexcitability and epileptic seizures in Fmr1 KO mice and human patients. However, a study of another lab has recently suggested that Kv4.2 protein levels are upregulated in FXS (Lee et al., 2011). This apparent contradiction to our lab's results might be due to the different mouse strains, cell types and ages of neuronal cultures used. The different results obtained in the two studies will require future clarification, and it is therefore important to corroborate our hypothesis that FMRP is a positive regulator of Kv4.2 expression.

The precise molecular mechanisms and details of Kv4.2 regulation by FMRP are not clear. We hypothesize that when neurons receive excitatory stimuli, Kv4.2 is internalized and endocytosed, leading to reduced cell surface levels of Kv4.2, which promotes neuronal excitation. FMRP counteracts this mechanism by increasing Kv4.2 mRNA translation and protein synthesis leading to enhanced Kv4.2 membrane levels, which allows for the reduction of neuronal excitability (Figure 4). We speculate that in the absence of FMRP, this positive, counteractive mechanism is gone, leading to lower surface expression of Kv4.2 and increased neuronal excitability.

Dysregulated neurotransmitter receptor signaling in FXS

A second theory regarding neuronal hyperexcitability is the widely accepted "mGluR theory of FXS" which proposes that excessive signaling through metabotropic glutamate receptors 1 and 5 (mGlu1/5) in the absence of FMRP leads to neuronal hyperexcitability (Bear et al., 2004). This theory is based on research showing that in the absence of FMRP, signaling through mGlu1/5 is exaggerated and stimulus-insensitive, leading to impaired downstream signaling and impaired mGlu1/5-dependent synaptic plasticity (Figure 3). Therefore, a possible therapeutic approach for FXS is to target the excessive mGlu1/5 signaling through mGlu1/5 antagonists. In fact, clinical trials using mGlu5 antagonists in patients with FXS are underway with partially promising results (Gross et al., 2012). It has been shown previously that the mGlu5 antagonist 2-methyl-6-(phenylethynyl)-pyridine (MPEP) rescues the increased susceptibility of *Fmr1* knockout mice to audiogenic seizures (Yan et al., 2005), suggesting that reducing mGlu5 signaling ameliorates neuronal hyperexcitability in FXS. In line with this assumption, the Bassell lab has shown that MPEP partially rescues reduced Kv4.2 surface levels in *Fmr1* KO hippocampal slices (Figure 2, Gross et al., 2011). This suggests that mGlu1/5 may have a direct role in the regulation of Kv4.2.

Apart from mGlu1/5 signaling, evidence is increasing that other, intracellular signaling pathways are dysregulated in the absence of FMRP. For example, absence of FMRP results in excess activity of phosphoinositide 3-kinase (PI3K) and mTOR, downstream signaling targets of mGlu1/5 and other cell surface receptors (Gross et al., 2010; Sharma et al., 2010; Gross and Bassell, 2011; Hoeffer et al., 2012). Moreover, other labs have shown that impaired signaling takes place through other signaling molecules downstream of other receptors, such as ERK1/2

and GSK3β (reviewed in Gross et al., 2012). Therefore, another possible therapeutic approach is to target the downstream regulators of mGlu1/5, such as PI3K.

Hypothesis and Specific Aims

The precise molecular mechanisms underlying dysregulated signaling through mGlu1/5 causes hyperexcitability in FMRP-deficient neurons have not been addressed so far. The overall goal of this research is to give more insight into the molecular mechanisms leading to neuronal hyperexcitability in FXS by studying the role of FMRP and mGlu1/5-medicated signaling for the regulation of Kv4.2, a potassium channel that is important in the control of neuronal excitability. We hypothesize that both, FMRP and signaling through mGlu1/5 affect the cell surface levels of Kv4.2 in hippocampal neurons. To test our hypothesis, we analyzed the effect of inhibition or activation of mGlu1/5 signaling in the presence and absence of FMRP on Kv4.2 cell surface expression using two different approaches: In the first approach, we analyzed endogenous Kv4.2 cell surface levels in hippocampal slices from wild type and *Fmr1* knockout mice using cell surface biotinylation. In the second approach, we used a pH-sensitive fluorescent Kv4.2 recombinant protein, pHluorin. PHluorin is a derivative of the green fluorescent protein (GFP), which fluoresces green at physiological pH (7.4), but loses fluorescence at acidic pH (Miesenbock et al., 1998). Thus, the Kv4.2 recombinant protein will fluoresce green when it is expressed on the cell surface, but will lose its fluorescence when it is endocytosed and is in the acidic environment of a vesicle. We used Neuro 2A cells, a mouse neuroblastoma cell line to analyze the effect of siRNA-mediated knockdown of *Fmr1* as well as mGlu1/5 stimulation and inhibition on Kv4.2 surface expression, using the pH sensitive fluorescent Kv4.2 reporter in Neuro2A cells.

The first aim of this research was to investigate the role of mGlu1/5 activity and downstream signaling on Kv4.2 surface expression and to better understand the function of FMRP for this regulation (Figure 5). A major part of this aim was to establish the use of pHluorin to quantify cell surface levels of proteins as a novel technique in the lab, and to optimize quantification and analysis methods of pHluorin expression for future use in primary neurons and for live cell imaging.

As mentioned above, previous studies suggest that excessive PI3K activity underlies increased mGlu1/5 signaling, and that PI3K antagonists can rescue certain phenotypes in *Fmr1* knockout mice (Gross et al., 2010). The second aim of this research was to determine if antagonizing PI3K signaling is an effective novel therapeutic strategy to restore Kv4.2 surface levels to WT levels, and therefore, to ameliorate the epilepsy phenotype in FXS patients (Figure 5). To achieve this goal, we have analyzed whether the PI3K antagonist Wortmannin restores Kv4.2 cell surface levels to WT levels in hippocampal slices.

The results of our research reconfirmed previous findings of the Bassell lab that loss of FMRP leads to reduced Kv4.2 cell surface levels, as shown by surface biotinylation experiments in mice hippocampal slices and experiments with a pHluroin Kv4.2 fusion protein in Neuro 2A cells. Our research also suggests that excessive mGlu1/5 signaling may decrease cell surface levels of Kv4.2 in wild type neurons; thus, mGlu1/5 may directly regulate surface levels of Kv4.2. Lastly, our results provide initial data motivating further analyses of PI3K antagonists, such as Wortmannin, for their efficiency to rescue surface levels of Kv4.2 in hippocampal slices of *Fmr1* KO mice.

Methods

Plating of N2A Cells

Glass cover slips in a 12 well-plate were coated with poly-lysine (1mg/mL) at 37°C for 2 hours. The cover slips were washed 3 times with autoclaved water. N2A cells were then plated on the cover slips.

Transfection of N2A Cells

The plated N2A cells were transfected with control siRNA and *FMR1* siRNA (sifmr1: NM_008031_stealth_298, sense, UGG CGC UUU CUA CAA GGC AUU UGU A; sictr: NM_008031_stealth_control_298, sense, UGG UUU CCA UCG GAA UUA CUG CGU A, from *Invitrogen*) using Lipofectamine 2000 (Invitrogen) according to the user's manual (as in Gross et al., 2010). The N2A cells were left to incubate overnight at 37°C. After the incubation period, we transfected the cells with a cDNA plasmid expressing pH-sensitive green fluorescent protein (pHluorin) fused to the Kv4.2 protein and allowed the cells to incubate overnight (pHluorin-Kv4.2 cDNA was a kind gift from Andreas Jeromin). The pHluorin protein is attached to the N-terminus of the Kv4.2 protein, resulting in a recombinant Kv4.2 protein that fluoresces at physiological pH or pH 7.4 (Figure 6). This fusion protein will not fluoresce at pH 6.0. pH 6.0 is the pH of the environment inside mature vesicles which carry Kv4.2 to and from the membrane (Figure 6). Thus, this recombinant protein allows for the quantification of surface levels of Kv4.2.

Cell Treatment

One day after transfection with pHluorin-Kv4.2, we stimulated some cells with the mGlu1/5 agonist DHPG (100µM, 15 min, *Tocris*), and treated others with the mGlu5 antagonist

MPEP (10μM, 15min, *FRAXA*). We then fixed the cells with 4% paraformaldehyde (PFA, pH7.4). We then washed the cover slips 3 times with PBS (phosphate-buffered saline, *VWR*) for 5 minutes per wash. In some experiments, we then rinsed the coverslips with water, mounted them onto slides using mounting media and let them dry over night before imaging. In other experiments, cells were further processed for fluorescence immunostaining (see below).

Fluorescence Immunostaining

After fixation and washing, the cells were placed in TBS50 buffer (50mM Tris/HCl (pH 7.4), 150mM NaCl) for about 5 minutes. Afterwards, we permeabelized cells in 0.3% Triton X-100/TBS50 for 5 minutes. We then washed the cells in Tris-Glycine (200mM Tris/HCl (pH 7.4), 100mM Glycine) buffer for 5 minutes. The cells are placed in IF buffer (2g BSA, 0.1% Triton X-100/TBS50) for another 5 minutes. After a brief rinsing of the cells in 0.1% Triton X-100/TBS50, we blocked the cells by inverting each coverslip onto a 40μl drop of blocking buffer (2% BSA, 2% Fetal Bovine Serum, 0.1% Triton X-100) for 1-2 hours. After blocking, we rinsed cells in IF buffer and then blotted each coverslip on a kimwipe and incubate cell-side-down on 35-40μl drop of primary antibody (mouse anti-Kv4.2 antibody (1:2000), *NeuroMAB*). We incubated for 1 hour in a humid chamber at room temperature and then washed the cells in IF buffer 3 times for 10 minutes each. After the washing, we blotted the coverslips and incubate cell-side-down on a 35-40μl drop of secondary antibody (donkey anti-mouse Cy3-conjugated, *Jackson Immunoresearch*) for 30 minutes at room temperature. We once again washed the cells in IF buffer 3 times for 10 minutes each. After washing the cells in TBS50 one time for 5 minutes, we rinsed the coverslips with water and mounted them on slides.

Image acquisition and analysis

We imaged the cells using a widefield fluorescence Nikon Ti microscope. We acquired 9 z-stacks at 0.6µm intervals and deconvolved the images using AutoQuant 2.0 software (Cybernetics). We quantified the protein expression by using the Image J software program (NIH). Kv4.2 cell surface and total levels were quantified by measuring the average total fluorescence intensity of the 5 most in focus z-stacks of pHluorin (green channel, surface Kv4.2), and Cy3 (red channel, total Kv4.2). For each image, regions of interest where chosen to include only transfected cells (identified through red fluorescence), and background subtracted. In the experiments, in which an immunostaining was not performed, only cell surface levels of Kv4.2 were measured (pHluorin, green fluorescence). Two different methods were used to quantify protein expression in these experiments using the Image J software. Both methods only used a single, most in focus z-stack. After background subtraction, the region of interest was chosen by tracing around the whole cell in order to measure the fluorescence of the entire cell (method 1). In method 2, we confined the region of interest to the membrane by drawing a small box around 4 separate segments of the cell membrane of each cell analyzed (as shown in Figure 9).

Biotinylation Assays of Hippocampal Mice Slices

We prepared ACSF (*artificial cerebrospinal fluid,* 20mM HEPES (pH7.4), 1.2mM $CaCl_2$, 147mM NaCl, 2.7mM KCl, 1mM $MgCl_2$, 10mM Glucose) and equilibrated it at 37°C/ 5% CO_2 overnight. At the day of the experiment, we dissected hippocampi from WT and *Fmr1* KO mice (P19-P23) on ice and cut the hippocampi into 400-µm thick slices using a tissue chopper. Hippocampal slices were transferred immediately into 1 mL of ice-cold ACSF. The slices were distributed evenly into 6 wells of a 12well plate, which contained 500µL of the pre-equilibrated ACSF. After distribution, the slices were incubated at 37°C, 5% CO_2 for 20 minutes. Some

slices were treated with 200nM Wortmannin (0.5µL of a 200µM Wortmannin stock solution, *Tocris*) for 40 min while other slices were treated with 100µM DHPG (1µL of a 50mM DHPG stock solution) for 20 min. This was followed by immediate biotinylation on ice with 50µL of 50mM EZ-link sulfo-NHS-Biotin (*ThermoScientific*) added to each well. This reaction was quenched for 10min with 100mM glycine added to each well. The slices were transferred into tubes with 1mL of ice-cold TBS (65mM Tris (pH 7.4), 150mM NaCl). The tubes were spun down at 10,000g at 4°C for 2 minutes. The supernatant was entirely removed and 250µL of RIPA buffer was added into each tube. The slices in the RIPA buffer (65mM Tris, 150mM NaCl, 1% Triton X-100, 0.1% SDS, 1mM EDTA, 0.5% deoxycholic salt, 50mM NaH_2PO_4, 10mM $Na_4P_2O_7$, 1mM Na_3VO_4, 50mM NaF, pH7.4) were sonicated until the hippocampal tissue was solubilized. Following a 15 minute centrifugation (20,000g) at 4°C, the supernatant was transferred into fresh tubes and the protein concentrations were measured through Bradford Assays (see below). Neutravidin-beads were prepared by transferring 150µL of the beads per reaction to fresh tubes and washing the tubes 3 times with 1mL RIPA buffer. After the washes were complete, the beads were re-suspended in 200µL RIPA buffer. 100µg of protein lysate was added to the beads, and the solution was incubated 2 hours while rotating at 4°C. The beads were washed four times with 1 mL RIPA buffer, and then resuspended in 40µL 2x SDS-loading buffer and incubated at 90-95°C for 20 minutes. Following incubation, the tubes were spun down for 2 minutes at max speed at room temperature and the supernatant was transferred to fresh tubes without the beads. The tubes were kept at -20°C until the proteins were run on a polyacrylamid protein gel.

Bradford Assay to measure protein concentrations

The Bradford protein assay was used to determine the protein concentration of the sample fractions. A standard curve was prepared with known concentrations of BSA. The Bradford reagent was added to all samples after each protein sample was diluted 1:100 µL in PBS. The absorbance at 595nm was measured and quantified by spectrophotometry. Protein concentrations of the samples were calculated based on the standard curve.

SDS-PAGE and Western Blotting

4µg samples of the lysates (total protein) and 15µg of the supernatant from the beads (cell surface protein) were run on an 8% SDS polyacrylamide gel, and then transferred to PVDF membranes. The membranes were probed with antibodies specific for Kv4.2 (*Upstate,* 1:2000), NR1 (*BD Pharmingen*, 1:1000) and α-tubulin (Sigma, 1:200,000). NR1, which is not a target of FMRP, was used as a negative control in order to compare Kv4.2 total and cell surface protein expression in the different genotypes and following treatments. Specific signals were detected using enhanced chemiluminescence followed by autoradiography to visualize the specific bands. The 'gel analysis tool' from Image J (NIH) was then used to perform densitometric analysis of the detected bands. As a control for the protein loading, α-tubulin, a housekeeping protein that is not changed in the absence of FMRP, was also detected on the membranes to determine variances in the amount of protein in the lanes.

Results

The overall goal of this research was to investigate the role of mGlu1/5 activity and downstream signaling on Kv4.2 surface expression, and to better understand the function of FMRP for this regulation. To achieve this goal, we used two different experimental approaches. One approach employed a method that already has been established in the lab (Gross et al., 2011), namely surface biotinylation of acute hippocampal slices. The other approach involved a method that has not been previously used in our laboratory, namely quantitative imaging of a Kv4.2 fusion protein with a pH-sensitive green fluorescent reporter protein, pHluorin, in cultured Neuro2A cells (Miesenböck et al., 1998; also see Fig 6). One part of my thesis was therefore to begin to establish pHluorin as a tool to analyze cell surface expression of specific proteins in the lab.

This research had two aims and we used two experimental methods to explore those aims. The first aim was to analyze the effect of mGlu1/5 activity on Kv4.2 cell surface expression in the presence and absence of FMRP by (a) treating acute hippocampal slices from wild type (WT) and *Fmr1* knockout (KO) mice with the mGlu1/5 agonist DHPG followed by cell surface biotinylation, and (b) treating Neuro 2A cells expressing a Kv4.2 pHluorin reporter construct with DHPG following siRNA-mediated *Fmr1* knockdown. The second aim was to test if antagonizing an important downstream signaling molecule of the mGlu1/5 pathway, phosphoinositide-3 kinase (PI3K), which was shown to be over-active in the absence of FMRP (Gross et al., 2010; Sharma et al., 2010; Gross and Bassell, 2011; Hoeffer et al., 2012), might be an effective therapeutic strategy to restore reduced Kv4.2 cell surface expression in the absence of FMRP to normal levels. In addition, we attempted confirm the finding of the Bassell

lab that Kv4.2 surface levels are decreased when FMRP is absent or reduced (Gross et al., 2011), because a recently published study reported partially contradictory findings (Lee et al., 2011).

Surface Levels of Kv4.2 in Hippocampal Slices of Fmr1 KO Mice may be reduced compared to wild type

Surface biotinylation of hippocampal slices revealed a trend towards reduced levels of surface Kv4.2 in hippocampal slices of KO Fmr1 mice as compared to hippocampal slices of WT mice (Figure 7, n=3, p(genotype)=0.67; p(treatment)=0.67; p(interaction)=0.26;Two-Way ANOVA). Interestingly, total levels of Kv4.2 were reduced, however; the reduction was very small compared to surface levels, which suggests that surface levels of Kv4.2 are more affected than total levels in *Fmr1* KO mice (Figure 7, n=3, p(genotype)=0.67; p(treatment)=0.60; p(interaction)=0.96; Two-Way ANOVA). These results corroborate previous findings of the Bassell lab (Gross et al., 2011).

Because of the preliminary nature of these results, and to be able to better evaluate the effects of *Fmr1* KO in these experiments, we also conducted a paired t-test. The t-test showed no significance and no statistical trend towards reduced Kv4.2 surface levels in *Fmr1* KO hippocampal slices as compared to Kv4.2 surface levels in hippocampal slices of WT mice (p=0.281).

Review of procedure for experiments with pHluorin-Kv4.2 fusion protein to analyze Kv4.2 cell surface expression in Neuro 2A cells

Neuro 2A cells were either transfected with control siRNA or *Fmr1*-specific siRNA (*Fmr1* siRNA) followed by transfection with plasmid DNA coding for a Kv4.2-pHluorin fusion protein. The cells were then treated with either a media change (untreated condition) or with the

mGlu1/5 agonist DHPG. The untreated condition included a media change to control for the media change when the DHPG treatment was administered. DHPG was added to fresh media (end concentration 100µM) and treatment consisted of replacing the old media with fresh DHPG-containing media. After treatment for 20 min and fixation with paraformaldehyde, Kv4.2-specific immunocytochemistry, using a Cy3-coupled secondary antibody, was performed in two independent experiments. The immunocytochemistry protocol allowed for the simultaneous quantification of cell surface Kv4.2 (pHluorin, green fluorescence) and total Kv4.2 protein levels (Cy3, red fluorescence). This is because permeabilization of the cells during immunocytochemistry, allowed for quantification of total Kv4.2 levels. In two additional independent experiments, immunocytochemistry was not performed. This precaution was taken because there was concern that permeabilization with Triton X-100, during the immunocytochemistry protocol, could potentially lead to a loss of the pH-gradient inside the cell, resulting in the green fluorescence of endocytosed Kv4.2 pHluorin protein.

Decreased Surface levels of Kv4.2 in Fmr1 knockdown Neuro 2A cells

In the *Fmr1* knockdown experiments that were followed by immunocytochemistry, Kv4.2 total and cell surface levels were quantified by measuring GFP-specific (cell surface) and Cy3-specific (total) fluorescence intensity. Ratios of Kv4.2 cell surface to total levels were calculated to quantify relative cell surface levels. This method allowed for quantification of the effect of *Fmr1* knockdown and mGlu1/5 activation on Kv4.2 cell surface levels, thereby eliminating the possibility that an observed effect might be due to changes in total Kv4.2 expression levels. As compared to control siRNA-transfeted cells, the *Fmr1* siRNA-transfected

cells had significantly reduced Kv4.2 cell surface levels (Figure 8, n=30, *p(siRNA)=0.014; p(treatment)=0.32; p(interaction)=0.39; two-way ANOVA).

A similar result was attained when comparing results from control and *Fmr1*-knockdown Neuro 2A cells that were imaged after fixation without immunocytochemistry. We used two different methods to quantify Kv4.2 cell surface levels in this experimental approach. We first measured fluorescence intensity of the entire cell. The results showed a trend towards reduced Kv4.2 cell surface levels in *Fmr1* siRNA-transfected cells as compared to cells transfected with control siRNA (Figure 9, n=11, p(siRNA)=0.06; p(treatment)=0.69; p(interaction)=0.40; Two-Way ANOVA). The second method used to quantify Kv4.2 surface levels in Neuro 2A cells transfected with control and *Fmr1*-specific siRNA was to quantify fluorescence of separate membrane segments (see methods and Figure 9). Using this method, which ensured quantification of just the membrane excluding any unspecific autofluorescence within the cell, we could show that Kv4.2 cell surface levels were significantly reduced in *Fmr1*-siRNA Neuro 2A cells as compared to the control (Figure 9, n=11, two independent experiments, *p(siRNA)<0.0001; p(treatment)=0.28; p(interaction)=0.31; Two-Way ANOVA).

Lowering the pH after fixation does not quench fluorescence of pHluorin

As previously stated, Kv4.2 total and surface levels were quantified in control and *Fmr1*-knockdown Neuro 2A cells using two techniques. One technique was to transfect the Neuro 2A cells and then perform immunocytochemistry in order to quantify both total and surface levels of Kv4.2 (Figure 8). The second technique mimicked the first except that immunocytochemistry was not performed, thus, allowing only for the quantification of cell surface levels of Kv4.2 (Figure 9). Two different techniques were used due to the concern that the

immunocytochemistry procedure would lead to green fluorescence of the endocytosed pHluorin. The immunocytochemistry included cell permeabilization with the detergent Triton X-100 at pH 7.4, which is expected to destroy the pH differences inside cell, leading to loss of the acidic milieu inside the vesicles. Changes in the pH lead to changes in the confirmation of pHluorin, leading to loss or gain of fluorescence, respectively (see Figure 6 and introduction). Although it is expected that paraformaldehyde fixation stalls the pHluorin in its previous confirmation, we were concerned that the prolonged treatment with Triton X-100 and buffers at pH7.4 during immunocytochemistry could induce conformational changes in the endocytosed Kv4.2 pHluorin and lead to green fluorescence of both endocytosed and cell surface Kv4.2. A preliminary analysis showed that lowering the pH to 5.8 following fixation did not eliminate fluorescence of surface pHluorin, suggesting that once fixed with paraformaldehyde, the pHluorin maintains its previous conformation (Figure 10). In the future, more experiments are needed to further ensure the usability of pHluorin in combination with immunocytochemistry on fixed cells.

Signaling through mGlu1/5 may lead to a decrease in Kv4.2 cell surface expression in WT hippocampal slices and Neuro 2A cells

We next investigated whether activation of mGlu1/5 receptor signaling affects Kv4.2 cell surface expression. Treatment of WT hippocampal slices with DHPG, an mGlu1/5 agonist, led to a trend towards reduced Kv4.2 cell surface levels as compared to the WT untreated condition, although results were not significant (Figure 7, n=3, p(genotype)=0.67; p(treatment)=0.67; p(interaction)=0.26;Two-Way ANOVA). Our results suggest that Kv4.2 surface levels may be reduced in WT hippocampal slices treated with DHPG as compared to the WT untreated

condition, however a t-test showed no significant difference or trend (p=0.18). Similarly, using the pH-sensitive reporter method, treatment with DHPG of control Neuro 2A cells (with immunocytochemistry) also may reduce Kv4.2 cell surface protein levels relative to total protein levels as compared to the untreated control condition (Figure 8, n=26, p(siRNA)=0.014; p(treatment)=0.32; p(interaction)=0.39;Two-Way ANOVA; paired t-test (untreated compared to DHPG treatment) p=0.315). Similar results were observed after treatment with DHPG of control Neuro 2A cells, for which no immunocytochemistry was performed using both the whole cell analysis (Figure 9, n=11, p(siRNA)=0.06; p(treatment)=0.69; p(interaction)=0.40; Two-Way ANOVA, paired t-test (untreated compared to DHPG) p=0.318) and the sample membrane analysis (Figure 9, n=11,p(siRNA)<0.0001; p(treatment)=0.28; p(interaction)=0.31; Two-Way ANOVA). In the sample membrane analysis, we detected a statistical trend towards reduced Kv4.2 membrane levels in Neuro 2A cells transfected with scrambled siRNA after DHPG treatment (p=0.078, paired t-test). Furthermore, treatment with the mGlu5 antagonist, 2-methyl-6-(phenylethynyl)-pyridine (MPEP), led to a trend towards increased Kv4.2 cell surface expression in control cells using both analysis methods, whereas *Fmr1* knockdown cells were unaffected.

Interestingly, treatment with DHPG of hippocampal slices from *Fmr1* KO mice led to a trend towards increased surface Kv4.2 levels as compared to hippocampal slices of the untreated KO condition, although variability in the results was high (Figure 7, n=3, p(genotype)=0.67; p(treatment)=0.67; p(interaction)=0.26;Two-Way ANOVA). In contrast, none of the treatments, neither MPEP nor DHPG, had an obvious effect on Kv4.2 cell surface levels in the *Fmr1* knockdown cells.

Levels of surface Kv4.2 may increase with Wortmannin treatment of hippocampal slices of Fmr1 KO mice

It has been shown previously that the mGlu5 antagonist MPEP partially rescues reduced Kv4.2 surface levels in *Fmr1* KO hippocampal slices (Gross *et al*., 2011). Here, we analyzed whether antagonizing a downstream signal molecule of mGlu1/5, namely the phosphoinositide-3 kinase (PI3K), likewise regulates Kv4.2 surface expression in *Fmr1* KO hippocampal slices. PI3K is an important downstream signal molecule of mGlu1/5. Previous studies have shown that PI3K signaling is exaggerated and dysregulated in *Fmr1* KO mice, suggesting that antagonizing PI3K activity might be therapeutic for FXS (Gross et al., 2010; Sharma et al., 2010; Gross and Bassell, 2011; Hoeffer et al., 2012). Thus, here we used Wortmannin, a PI3K antagonist, to treat hippocampal slices of *Fmr1* KO mice. The results showed a trend towards slightly increased surface Kv4.2 levels in hippocampal slices of *Fmr1* KO mice as compared to untreated *Fmr1* KO hippocampal slices (Figure 7, n=3, p(genotype)=0.67; p(treatment)=0.67; p(interaction)=0.26;Two-Way ANOVA).

NR1 Surface Protein Levels Remain Constant in Hippocampal Slices from WT and Fmr1 KO mice

Previous studies have shown that the NR1 protein is not regulated by FMRP (Gross et al., 2010; Gross et al., 2011). Thus, we used NR1 as a negative control for the surface biotinylation experiments in hippocampal slices. NR1 cell surface and total protein levels remained relatively constant in hippocampal slices of most conditions (cell surface: Figure 7, n=3, p(genotype)=0.38; p(treatment)=0.48; p(interaction)=0.39;Two-Way ANOVA; total levels: Figure 7, n=3, p(genotype)=0.21; p(treatment)=0.45; p(interaction)=0.90;Two-Way ANOVA).

However, there was a rather large increase in NR1 total protein and cell surface levels in DHPG-treated hippocampal slices of *Fmr1* KO mice as compared to all the other conditions (Figure 7).

Discussion

The goal of this thesis was to investigate the role of signaling through mGlu1/5 and FMRP for cell surface expression of the potassium channel Kv4.2. There were three major outcomes of this thesis work:

(1) This thesis could corroborate previous findings of the Bassell lab that the absence of FMRP, which causes the intellectual disability fragile X syndrome, leads to reduced Kv4.2 cell surface levels.

(2) This thesis provides initial support of the hypothesis that mGlu1/5 signaling regulates Kv4.2 cell surface expression.

(3) This thesis began to establish a novel technique in the lab, namely the use of pH-sensitive fluorescent reporter proteins to study protein cell surface expression.

Absence or reduced levels of FMRP lead to reduced cell surface levels of Kv4.2

An important finding of this research is that cell surface levels of Kv4.2 in Neuro 2A cells, in which FMRP expression was reduced by *Fmr1*-specific siRNA, are significantly reduced as compared to Neuro 2A cells transfected with control siRNA. Previous research of the Bassell lab has shown that in *Fmr1* knockout mice, Kv4.2 cell surface levels are reduced; however, the results presented here provide the first evidence that transient and acute reduction of FMRP expression levels likewise reduces Kv4.2 cell surface expression. The observed reduction of Kv4.2 surface expression in the *Fmr1* KO mice could be due to a compensatory, and thus indirect effect during development; however, the results of the transient knockdown of FMRP expression presented here suggest a direct role of FMRP in controlling Kv4.2 cell surface levels.

Thus, the reduction in Kv4.2 surface levels is likely not due to potential indirect compensatory effects.

It is important to note that these results were obtained using two different techniques to quantify the fluorescence of the Kv4.2 pHluorin reporter. Both the results from Neuro 2A cell experiments that included immunocytochemical staining for total Kv4.2 levels and the subsequent calculation of surface to total level ratios, and the results from Neuro 2A cell experiments that were analyzed by a technique only measuring Kv4.2 pHluorin fluorescence in membrane segments without quantifying total levels of Kv4.2 protein showed statistically significant reductions of Kv4.2 surface levels after *Fmr1* knockdown. However, quantification of Kv4.2 pHluorin fluorescence in the entire cell, instead of just membrane segments, showed a trend toward Kv4.2 surface levels being reduced after *Fmr1* knockdown, which was not significant. This difference in the results may be due to the inclusion of unspecific autofluorescence within the cells when fluorescence in the entire cell was quantified. In general, results of these experiments may also be highly variable due to differences in the efficiency of the siRNA-mediated *Fmr1* knockdown. All the Neuro 2A cells that were imaged and quantified for Kv4.2 pHluorin fluorescence may not have been transfected equally with *Fmr1*-specific siRNA. While the *Fmr1*-specific siRNA has been shown to efficiently knockdown FMRP protein levels to about 50% (Gross et al., 2010), and is frequently used in the Bassell lab, variations in siRNA-transfection efficiency might create differences in the observed effects of Kv4.2 cell surface expression. In the future, simultaneous quantification of FMRP levels in such experiments might help to monitor this variability.

In a different experimental approach, we also detected a trend towards reduced cell surface levels of Kv4.2 protein in hippocampal slices of *Fmr1* KO mice as compared to WT. This corroborates previous findings of the Bassell lab (Gross et al., 2011), and reaffirms that FMRP is a positive regulator of Kv4.2 mRNA translation and cell surface expression. This observation is important since a recent publication suggested that FMRP suppresses Kv4.2 mRNA translation, and that Kv4.2 protein and cell surface levels were elevated in *Fmr1* knockout mice (Lee et al., 2011), which is in contrast to the Bassell lab study (Gross et al., 2011). These contradictory findings might be due to different genetic backgrounds of the *Fmr1* mouse strains that were analyzed in the two studies (B6/C57 in Gross et al., 2011 versus FVB in Lee et al., 2011), and different experimental conditions, e.g. neuronal culture systems and reporter constructs employed, and will have to be further investigated in the future. However, the findings of this thesis that transient siRNA-mediated reduction of FMRP expression in a mouse neuroblastoma cell line significantly reduces Kv4.2 cell surface levels is an important corroboration of the Bassell lab's hypothesis that reduced levels of Kv4.2 might contribute to the occurrence of epilepsy observed in FXS (Berry-Kravis et al., 2010; Gauthey et al., 2010).

Kv4.2 is the major potassium channel regulating neuronal excitability in the hippocampus (Birnbaum et al., 2004). Kv4.2 mediates A-type currents, which downregulates backpropagating action potentials and dendritic excitability. Reduced levels or function of Kv4.2 have been associated with heightened neuronal excitability and epileptic seizures in animal models and humans (Singh et al., 2006; Barnwell et al., 2009); thus, this thesis provides further evidence that a possible mechanism underlying the heightened neuronal excitability and eventual epilepsy in FXS patients may be the downregulation of Kv4.2 cell surface levels when

FMRP is absent or at insufficient levels. In the future, it will be interesting to study Kv4.2 channel properties and Kv4.2 function in the *Fmr1* KO mouse model, e.g. by studying backpropagating action potentials and dendritic excitability in hippocampal CA1 neurons, as done previously for other mouse models (Sun et al., 2011).

A potential novel function of metabotropic glutamate receptors 1/5 in regulating Kv4.2 cell surface expression

One aim of this thesis was to analyze the effect of mGlu1/5 activity on Kv4.2 cell surface expression in the presence and absence of FMRP. A previous study by the Bassell lab has shown that the mGlu5 antagonist MPEP could partially rescue reduced Kv4.2 cell surface levels in hippocampal slices from *Fmr1* KO mice (Gross et al., 2011), suggesting that mGlu5 is involved in regulating Kv4.2 membrane expression. However, so far no study has investigated the role of metabotropic glutamate receptor signaling on Kv4.2 membrane trafficking and localization. My experiments showed that treatment of WT hippocampal slices with DHPG, an mGlu1/5 agonist, led to a trend towards reduced Kv4.2 cell surface levels as compared to the WT untreated condition. A similar reduction in Kv4.2 cell surface levels also occurred when DHPG treatment was administered to Neuro 2A cells transfected with control siRNA. Thus, this research is the first to show results that suggest mGlu1/5 regulates Kv4.2 surface expression. Several studies have found that exaggerated mGlu1/5 signaling does occur in *Fmr1* KO mice and other animal models of FXS, and might underlie many symptoms in patients with FXS (Bear et al., 2004). If these results showing that mGlu1/5 receptor signaling is involved in controlling Kv4.2 surface expression are confirmed, treatment with mGlu1/5 antagonists such as MPEP should be effective in controlling hyperexcitability and reducing epilepsy in Fragile X patients. This is

because Kv4.2 functions at the membrane and previous studies have shown that the function of Kv4.2 is to control neuronal hyperexcitability. Furthermore, this finding may help to understand the mechanisms by which mGlu1/5 antagonists mediate anticonvulsant and anxiolytic effects observed in animal models (Moldrich et al., 2003; Pietraszek et al., 2005; Inta et al., 2012). Interestingly, our preliminary studies have shown that the effect of mGlu1/5 signaling on Kv4.2 surface levels observed in WT hippocampal slices and control N2A cells was absent in *Fmr1* KO hippocampal slices and in N2A cells following *Fmr1* knockdown. This suggests that FMRP plays a role for the regulation of cell surface expression of Kv4.2 by mGlu1/5.

In order to be able to confirm and substantiate the results showing that mGlu1/5 regulates Kv4.2 membrane expression, and that this may involve FMRP, the surface biotinylation experiments using hippocampal slices of WT and *Fmr1* KO mice must be repeated several times in order to attain significant results. Previous studies of the Bassell lab using this technique suggest that six independent experiments are required to account for the variations within this type of experiments (Gross et al., 2011). In addition, experiments to analyze the effect of mGlu1/5 activity on control and *Fmr1* siRNA transfected Neuro 2A cells using the Kv4.2 pHluorin reporter protein also need to be repeated. The results shown in this thesis indicate mGlu1/5 signaling may regulate Kv4.2 membrane expression. In the future, we also hope to replicate these preliminary results by conducting live cell imaging in Neuro2A cells or cultured primary neurons. The analysis of Kv4.2 membrane expression in response to mGlu1/5 activity using pHluorin reporter proteins in live cell experiments could reveal a role for mGlu1/5 signaling in Kv4.2 membrane trafficking.

Another goal of our research was to test the effectiveness of antagonizing PI3K, an important downstream signaling molecule of mGlu1/5, to restore surface levels of Kv4.2 back to WT levels in the absence of FMRP. Previous studies have shown that PI3K signaling is exaggerated and dysregulated in *Fmr1* KO mice, thus, antagonizing PI3K activity may be therapeutic in FXS (Gross et al., 2010; Sharma et al., 2010; Gross and Bassell, 2011; Jeon et al., 2011; Hoeffer et al., 2012). Here, we show that the PI3K inhibitor produced a trend towards slightly increased surface Kv4.2 levels in hippocampal slices of *Fmr1* KO mice as compared to untreated *Fmr1* KO hippocampal slices. However, these effects were only marginal. In the future, more potent and subunit-selective PI3K antagonists may be used, which might be more efficient. Previous research of the Bassell lab has shown that FMRP regulates the expression and activity of a specific PI3K catalytic subunit, p110β. p110β-selective antagonists can rescue excessive protein synthesis in synaptic fractions from *Fmr1* KO mice, and in cell lines from patients with FXS (Gross and Bassell, 2011). While based on this research, we cannot draw the conclusion that antagonizing PI3K may be useful to restore Kv4.2 cell surface levels, and thus potentially ameliorate hyperexcitability in FXS, future experiments might show that targeting PI3K or other important downstream signals of mGlu1/5 may be a valuable therapeutic approach to rescuing surface levels of Kv4.2 when FMRP is absent.

Phluorin or other pH-sensitive reporter proteins maybe useful tools for the future analysis of the role of FMRP and mGlu1/5 signaling in Kv4.2 cell surface expression

Another aim of this research was to establish pHluorin, a pH-sensitive green fluorescent Kv4.2 recombinant protein, as an efficient and efficacious tool for measuring cell surface protein levels through changes in fluorescence intensity. Surface biotinylation is a more

established method for quantifying protein levels and has been used by the Bassell lab in previous research. Using pHluorin to quantify protein surface levels is a novel technique in the Bassell lab and has both advantages and disadvantages. Because pHluroin is pH-sensitive, pHluroin will fluoresce at physiological pH, the pH at the cell membrane, but will not fluoresce at more acidic environments such as the environment found inside transport vesicles (Miesenbock et al., 1998). Thus, the use of pHluorin is ideal for measuring levels of proteins that are trafficked to and from the membrane. One disadvantage of using pHluorin is that the fluorescent tag, which has a mass of 26kDa, may affect Kv4.2 membrane trafficking. pHluroin is a fusion protein that combines a green-fluorescent protein with Kv4.2, thus, the pHluroin protein is larger than endogenous Kv4.2. Because of its larger size, pHluroin may be trafficked to and from the membrane at a different rate as compared to the trafficking of endogenous Kv4.2. However, the experiments conducted with pHluorin in this research corroborated previous research in the Bassell lab, namely that surface levels of Kv4.2 are decreased in FMRP-deficient cells; thus, this result may indicate that pHluroin can accurately detect cell surface levels of Kv4.2.

In the past, most studies using pHluorin were conducted in live cells and did not involve fixation of cells with paraformaldehyde e.g. (Lin and Huganir, 2007). These studies have confirmed the pH-sensitivity of pHluorin, showing that a brief exposure to acidic buffer (<pH6) leads to loss of fluorescence. However, they did not analyze whether cell fixation with paraformaldehyde, as done in the experiments presented here, could preserve the conformation, and thus fluorescence status of pHluorin. Immunocytochemistry requires prolonged treatment with Triton X-100, a detergent, which is expected to destroy pH

differences inside the cell. The concern was that Triton X-100-mediated elimination of the acidic environment inside the vesicles of cells would lead to conformational changes in the endocytosed Kv4.2 pHluorin even after fixation with paraformaldehyde. This could lead to green fluorescence of both endocytosed and cell surface Kv4.2. However, a preliminary analysis showed that reducing the pH below 6 in the buffer after cell fixation did not visibly change fluorescence levels, suggesting that treatment with paraformaldehyde preserved the protein conformation of pHluorin. Thus, the analysis method in which pHluorin is detected in fixed cells may be a valid method for future studies of Kv4.2 cell surface expression in the lab. Additionally, in the future, we plan to use the Kv4.2 pHluorin reporter in live cell imaging experiments studying the dynamic membrane trafficking of Kv4.2, which will not involve cell fixation. A great advantage of using recombinant proteins such as pHluorin Kv4.2 reporters is the possibility to study the effect of deletion mutations of Kv4.2 on mGlu1/5 and FMRP dependent cell surface expression. Previous research has shown that the C-terminus of Kv4.2 is necessary for its interaction with SAP97, an important scaffold protein in the postsynaptic density (Gardoni et al., 2007). In the future, it will be interesting to analyze how C-terminal deletions of Kv4.2 may influence mGlu1/5-dependent Kv4.2 membrane trafficking in the presence or absence of FMRP.

Summary and Future Outlook

This thesis presents preliminary data suggesting that mGlu1/5 activity and downstream signaling regulate Kv4.2 cell surface expression, which may involve FMRP. We expect that continuation of the studies initiated during this thesis will provide important insight into so far unknown mechanisms of Kv4.2 control. So far, the majority of studies have analyzed the effects

of ionotropic glutamate receptors, such as NMDA receptors, on Kv4.2 function (Jung et al., 2008; Lei et al., 2008; Lei et al., 2010; Jung et al., 2011). Furthermore, the role of several protein kinases, such as ERK1/2, PKA and PKC for Kv4.2 has been elucidated (Anderson et al., 2000; Schrader et al., 2006; Hammond et al., 2008; Schrader et al., 2009), however to our knowledge no one has studied the role of metabotropic glutamate receptor signaling and/or activity of the lipid kinase PI3K on Kv4.2. In the future, this research might thus reveal a novel, so far unknown mechanism of Kv4.2 regulation.

Previous research has suggested that FMRP regulates Kv4.2 mRNA translation and protein expression (Gross et al., 2011; Lee et al., 2011). The research presented here was focused on the effect of mGlu1/5 signaling and FMRP on Kv4.2 cell surface levels, where it exerts its function as a potassium channel. However, in the future it will be interesting to further analyze the translational control of Kv4.2 by mGlu1/5 signaling, FMRP and possible other mechanisms, such as microRNAs. Here, we used a pHluorin construct that only contained the open reading frame of Kv4.2. Previous studies have shown that FMRP associates with the 3'UTR of Kv4.2 mRNA (Gross et al., 2011; Lee et al., 2011). Thus, in the future, it will be interesting to analyze the effect of mGlu1/5 signaling and FMRP on Kv4.2 pHluorin recombinant proteins containing the Kv4.2 3'UTR.

An important future experiment will be to analyze the effect of mGlu1/5 signaling and FMRP on Kv4.2 function. Previous research in another mouse model has shown that reduction in Kv4.2 protein levels lead to functional impairments of Kv4.2, an increase in back propagating action potentials and, and an increase in dendritic excitability (Sun et al., 2011). This suggests that the here observed reduction in cell surface levels of Kv4.2 in *Fmr1* KO hippocampus

following mGlu1/5 stimulation may lead to the functional impairments and hyperexcitability seen in FXS.

As mentioned before, Kv4.2 plays a predominant role in regulating dendritic excitability in the hippocampus. Interestingly, Kv4.2 was shown to be dysregulated in several neurological diseases, such as FXS, other autism spectrum disorders, epilepsy and Alzheimer's disease (Singh et al., 2006; Marshall et al., 2008; Hall et al., 2011; Sun et al., 2011). A detailed analysis of mechanisms regulating Kv4.2 expression and function will be essential to better understand Kv4.2 dysregulation in disease and may potentially help to design future therapeutic strategies targeting Kv4.2.

References

Anderson AE, Adams JP, Qian Y, Cook RG, Pfaffinger PJ, Sweatt JD (2000) Kv4.2 phosphorylation by cyclic AMP-dependent protein kinase. *J Biol Chem* **275**:5337-5346.

Barnwell LF, Lugo JN, Lee WL, Willis SE, Gertz SJ, Hrachovy RA, Anderson AE (2009) Kv4.2 knockout mice demonstrate increased susceptibility to convulsant stimulation. *Epilepsia*.

Bassell GJ, Warren ST (2008) Fragile X Syndrome: Loss of Local mRNA Regulation Alters Synaptic Development and Function. **60**:201-214.

Bear MF, Huber KM, Warren ST (2004) The mGluR theory of fragile X mental retardation. *Trends Neurosci* **27**:370-377.

Bechara EG, Didiot MC, Melko M, Davidovic L, Bensaid M, Martin P, Castets M, Pognonec P, Khandjian EW, Moine H, Bardoni B (2009) A novel function for fragile X mental retardation protein in translational activation. *PLoS Biol* **7**:e16.

Berry-Kravis E, Raspa M, Loggin-Hester L, Bishop E, Holiday D, Bailey DB (2010) Seizures in fragile X syndrome: characteristics and comorbid diagnoses. *Am J Intellect Dev Disabil* **115**:461-472.

Berry-Kravis E, Abrams L, Coffey SM, Hall DA, Greco C, Gane LW, Grigsby J, Bourgeois JA, Finucane B, Jacquemont S, Brunberg JA, Zhang L, Lin J, Tassone F, Hagerman PJ, Hagerman RJ, Leehey MA (2007) Fragile X-associated tremor/ataxia syndrome: clinical features, genetics, and testing guidelines. *Mov Disord* **22**:2018-2030, quiz 2140.

Birnbaum SG, Varga AW, Yuan LL, Anderson AE, Sweatt JD, Schrader LA (2004) Structure and function of Kv4-family transient potassium channels. *Physiol Rev* **84**:803-833.

Brown MR, Kronengold J, Gazula VR, Chen Y, Strumbos JG, Sigworth FJ, Navaratnam D, Kaczmarek LK (2010) Fragile X mental retardation protein controls gating of the sodium-activated potassium channel Slack. *Nat Neurosci* **13**:819-821.

Butler MG, Brunschwig A, Miller LK, Hagerman RJ (1992) Standards for selected anthropometric measurements in males with the fragile X syndrome. *Pediatrics* **89**:1059-1062.

Chen X, Yuan L-L, Zhao C, Birnbaum SG, Frick A, Jung WE, Schwarz TL, Sweatt JD, Johnston D (2006) Deletion of Kv4.2 Gene Eliminates Dendritic A-Type K+ Current and Enhances Induction of Long-Term Potentiation in Hippocampal CA1 Pyramidal Neurons. *J Neurosci* **26**:12143-12151.

Chuang SC, Zhao W, Bauchwitz R, Yan Q, Bianchi R, Wong RK (2005) Prolonged epileptiform discharges induced by altered group I metabotropic glutamate receptor-mediated synaptic responses in hippocampal slices of a fragile X mouse model. *J Neurosci* **25**:8048-8055.

de Vries BB, Wiegers AM, Smits AP, Mohkamsing S, Duivenvoorden HJ, Fryns JP, Curfs LM, Halley DJ, Oostra BA, van den Ouweland AM, Niermeijer MF (1996) Mental status of females with an FMR1 gene full mutation. *Am J Hum Genet* **58**:1025-1032.

Fahling M, Mrowka R, Steege A, Kirschner KM, Benko E, Forstera B, Persson PB, Thiele BJ, Meier JC, Scholz H (2009) Translational regulation of the human achaete-scute homologue-1 by fragile X mental retardation protein. *J Biol Chem* **284**:4255-4266.

Francis J, Jugloff DG, Mingo NS, Wallace MC, Jones OT, Burnham WM, Eubanks JH (1997) Kainic acid-induced generalized seizures alter the regional hippocampal expression of the rat Kv4.2 potassium channel gene. *Neurosci Lett* **232**:91-94.

Gardoni F, Mauceri D, Marcello E, Sala C, Di Luca M, Jeromin A (2007) SAP97 directs the localization of Kv4.2 to spines in hippocampal neurons: regulation by CaMKII. *J Biol Chem* **282**:28691-28699.

Gauthey M, Poloni CB, Ramelli GP, Roulet-Perez E, Korff CM (2010) Status epilepticus in fragile X syndrome. *Epilepsia* **51**:2470-2473.

Gross C, Bassell GJ (2011) Excess protein synthesis in FXS patient lymphoblastoid cells can be rescued with a p110beta-selective inhibitor. *Mol Med*.

Gross C, Berry-Kravis EM, Bassell GJ (2012) Therapeutic Strategies in Fragile X Syndrome: Dysregulated mGluR Signaling and Beyond. *Neuropsychopharmacology* **37**:178-195.

Gross C, Yao X, Pong DL, Jeromin A, Bassell GJ (2011) Fragile X mental retardation protein regulates protein expression and mRNA translation of the potassium channel Kv4.2. *J Neurosci* **31**:5693-5698.

Gross C, Nakamoto M, Yao X, Chan CB, Yim SY, Ye K, Warren ST, Bassell GJ (2010) Excess phosphoinositide 3-kinase subunit synthesis and activity as a novel therapeutic target in fragile X syndrome. *J Neurosci* **30**:10624-10638.

Hall AM, Markwardt SJ, Overstreet-Wadiche LS, Roberson ED (2011) Tau and potassium channel Kv4.2 in Alzheimer disease-related neuronal dysfunction. In, pp Neuroscience Meeting Planner. Washington, DC: Society for Neuroscience, 2011. Online.

Hammond RS, Lin L, Sidorov MS, Wikenheiser AM, Hoffman DA (2008) Protein kinase a mediates activity-dependent Kv4.2 channel trafficking. *J Neurosci* **28**:7513-7519.

Hoeffer CA, Sanchez E, Hagerman RJ, Mu Y, Nguyen DV, Wong H, Whelan AM, Zukin RS, Klann E, Tassone F (2012) Altered mTOR signaling and enhanced CYFIP2 expression levels in subjects with Fragile X syndrome. *Genes, Brain and Behavior*:no-no.

Inta D, Filipovic D, Lima-Ojeda JM, Dormann C, Pfeiffer N, Gasparini F, Gass P (2012) The mGlu5 receptor antagonist MPEP activates specific stress-related brain regions and lacks neurotoxic effects of the NMDA receptor antagonist MK-801: Significance for the use as anxiolytic/antidepressant drug. *Neuropharmacology* **62**:2034-2039.

Jeon SJ, Seo JE, Yang S-I, Choi JW, Wells D, Shin CY, Ko KH (2011) Cellular stress-induced up-regulation of FMRP promotes cell survival by modulating PI3K-Akt phosphorylation cascades. *Journal of Biomedical Science* **18**:17.

Jung SC, Kim J, Hoffman DA (2008) Rapid, bidirectional remodeling of synaptic NMDA receptor subunit composition by A-type K+ channel activity in hippocampal CA1 pyramidal neurons. *Neuron* **60**:657-671.

Jung SC, Eun SY, Kim J, Hoffman DA (2011) Kv4.2 block of long-term potentiation is partially dependent on synaptic NMDA receptor remodeling. *Brain Res Bull* **84**:17-21.

Kooy RF, D'Hooge R, Reyniers E, Bakker CE, Nagels G, De Boulle K, Storm K, Clincke G, De Deyn PP, Oostra BA, Willems PJ (1996) Transgenic mouse model for the fragile X syndrome. *Am J Med Genet* **64**:241-245.

Lee HY, Ge WP, Huang W, He Y, Wang GX, Rowson-Baldwin A, Smith SJ, Jan YN, Jan LY (2011) Bidirectional regulation of dendritic voltage-gated potassium channels by the fragile X mental retardation protein. *Neuron* **72**:630-642.

Lei Z, Deng P, Xu ZC (2008) Regulation of Kv4.2 channels by glutamate in cultured hippocampal neurons. *Journal of Neurochemistry* **106**:182-192.

Lei Z, Deng P, Li Y, Xu ZC (2010) Downregulation of Kv4.2 channels mediated by NR2B-containing NMDA receptors in cultured hippocampal neurons. *Neuroscience* **165**:350-362.

Lin D-T, Huganir RL (2007) PICK1 and Phosphorylation of the Glutamate Receptor 2 (GluR2) AMPA Receptor Subunit Regulates GluR2 Recycling after NMDA Receptor-Induced Internalization. *The Journal of Neuroscience* **27**:13903-13908.

Lugo JN, Barnwell LF, Ren Y, Lee WL, Johnston LD, Kim R, Hrachovy RA, Sweatt JD, Anderson AE (2008) Altered phosphorylation and localization of the A-type channel, Kv4.2 in status epilepticus. *J Neurochem* **106**:1929-1940.

Marshall CR et al. (2008) Structural variation of chromosomes in autism spectrum disorder. *Am J Hum Genet* **82**:477-488.

McConkie-Rosell A, Lachiewicz AM, Spiridigliozzi GA, Tarleton J, Schoenwald S, Phelan MC, Goonewardena P, Ding X, Brown WT (1993) Evidence that methylation of the FMR-I locus is responsible for variable phenotypic expression of the fragile X syndrome. *Am J Hum Genet* **53**:800-809.

Miesenbock G, De Angelis DA, Rothman JE (1998) Visualizing secretion and synaptic transmission with pH-sensitive green fluorescent proteins. *Nature* **394**:192-195.

Moldrich RX, Chapman AG, De Sarro G, Meldrum BS (2003) Glutamate metabotropic receptors as targets for drug therapy in epilepsy. *European Journal of Pharmacology* **476**:3-16.

Pietraszek M, Sukhanov I, Maciejak P, Szyndler J, Gravius A, Wisłowska A, Płaźnik A, Bespalov AY, Danysz W (2005) Anxiolytic-like effects of mGlu1 and mGlu5 receptor antagonists in rats. *European Journal of Pharmacology* **514**:25-34.

Schrader LA, Ren Y, Cheng F, Bui D, Sweatt JD, Anderson AE (2009) Kv4.2 is a locus for PKC and ERK/MAPK cross-talk. *Biochem J* **417**:705-715.

Schrader LA, Birnbaum SG, Nadin BM, Ren Y, Bui D, Anderson AE, Sweatt JD (2006) ERK/MAPK regulates the Kv4.2 potassium channel by direct phosphorylation of the pore-forming subunit. *Am J Physiol Cell Physiol* **290**:C852-861.

Sharma A, Hoeffer CA, Takayasu Y, Miyawaki T, McBride SM, Klann E, Zukin RS (2010) Dysregulation of mTOR Signaling in Fragile X Syndrome. *J Neurosci* **30**:694-702.

Sherman SL (2000) Premature ovarian failure among fragile X premutation carriers: parent-of-origin effect? *Am J Hum Genet* **67**:11-13.

Singh B, Ogiwara I, Kaneda M, Tokonami N, Mazaki E, Baba K, Matsuda K, Inoue Y, Yamakawa K (2006) A Kv4.2 truncation mutation in a patient with temporal lobe epilepsy. *Neurobiol Dis* **24**:245-253.

Siomi H, Siomi MC, Nussbaum RL, Dreyfuss G (1993) The protein product of the fragile X gene, FMR1, has characteristics of an RNA-binding protein. *Cell* **74**:291-298.

Stoger R, Kajimura TM, Brown WT, Laird CD (1997) Epigenetic variation illustrated by DNA methylation patterns of the fragile-X gene FMR1. *Hum Mol Genet* **6**:1791-1801.

Strumbos JG, Brown MR, Kronengold J, Polley DB, Kaczmarek LK (2010) Fragile X mental retardation protein is required for rapid experience-dependent regulation of the potassium channel Kv3.1b. *J Neurosci* **30**:10263-10271.

Sun W, Maffie JK, Lin L, Petralia RS, Rudy B, Hoffman DA (2011) DPP6 establishes the A-type K(+) current gradient critical for the regulation of dendritic excitability in CA1 hippocampal neurons. *Neuron* **71**:1102-1115.

Yan QJ, Rammal M, Tranfaglia M, Bauchwitz RP (2005) Suppression of two major Fragile X Syndrome mouse model phenotypes by the mGluR5 antagonist MPEP. *Neuropharmacology* **49**:1053-1066.

Zalfa F, Adinolfi S, Napoli I, Kuhn-Holsken E, Urlaub H, Achsel T, Pastore A, Bagni C (2005) Fragile X mental retardation protein (FMRP) binds specifically to the brain cytoplasmic RNAs BC1/BC200 via a novel RNA-binding motif. *J Biol Chem* **280**:33403-33410.

Yan QJ, Rammal M, Tranfaglia M, Bauchwitz RP (2005) Suppression of two major Fragile X Syndrome mouse model phenotypes by the mGluR5 antagonist MPEP. *Neuropharmacology* **49**:1053-1066.

Figures

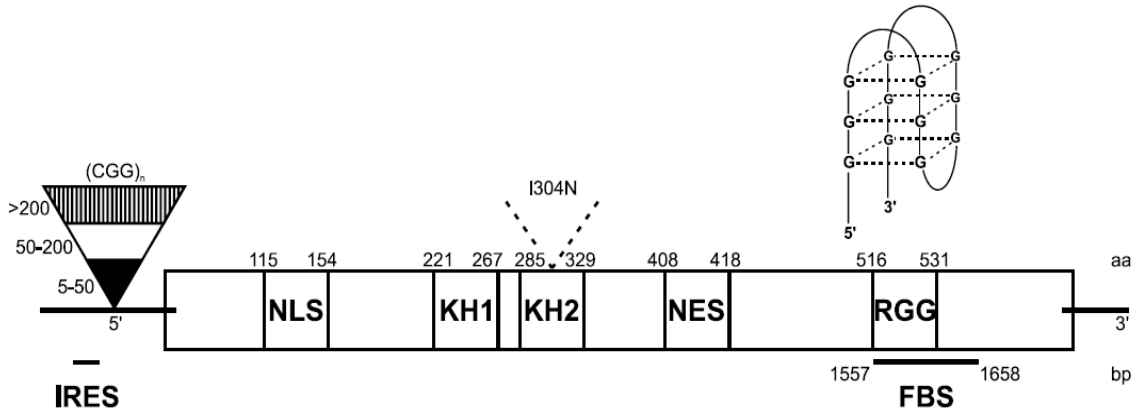

Figure 1: The FMRP domain structure: NLS = Nuclear Localization Sequence, KH = K Homology domain (RNA-binding motif). NES = Nuclear Export Sequence. RGG = RGG Box (RNA-binding motif). FMRP is a RNA binding protein, and previous work from the Bassell lab suggests that FMRP is a positive regulator of Kv4.2 mRNA translation.

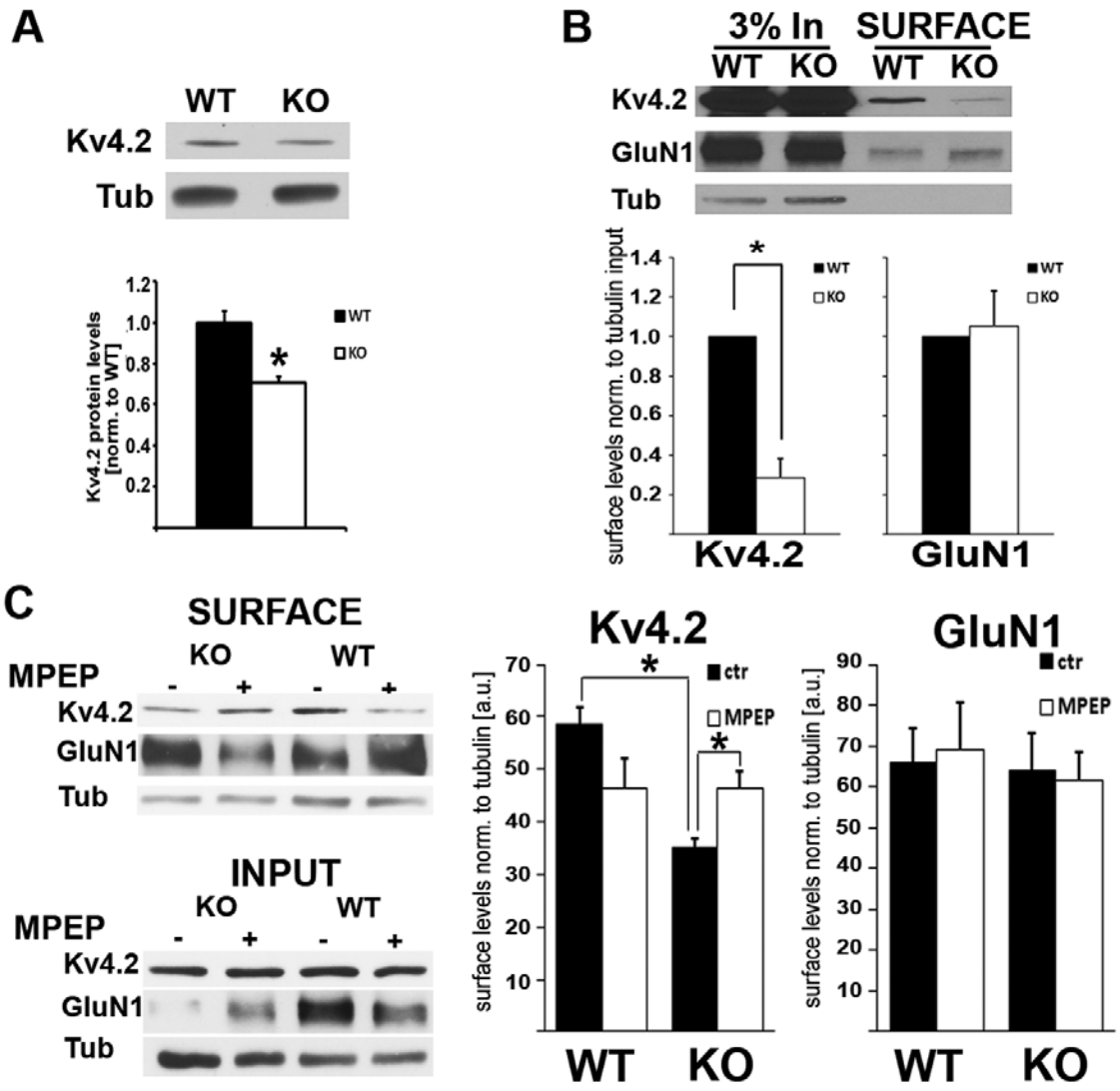

Figure 2: **(A)** Western blot analysis of total Kv4.2 protein levels shows reduced total Kv4.2 in KO cortices of mice (n=5 each WT and KO, 2 independent litters, p=.001, paired t test). **(B)** Kv4.2 surface levels in cultured cortical neurons are reduced as demonstrated in western blot analysis of surface biotinylation assays (n=4, p=0.03, paired t test). **(C)** Western blot analysis showed cell surface levels of Kv4.2, but not GluN1, a protein not regulated by FMRP, are significantly reduced in hippocampal slices from *Fmr1* KO mice. Kv4.2 surface levels can be partially rescued by pretreatment with the mGlu5 antagonist MPEP (50µm, 40min) [Kv4.2: n=6, two-way ANOVA; treatment: p=0.976, genotype: p=0.01, interaction genotype–treatment: p=0.01, Games-Howell *post hoc* analyses (unequal variances assumed): $p_{wtctr\text{-}wtMPEP}$=0.467, *$p_{wtctr\text{-}koctr}$ =0.001, *$p_{koctr\text{-}koMPEP}$= 0.036, $p_{wtctr\text{-}koMPEP}$= 0.053; GluN1: n=6, two-way ANOVA; treatment: p=0.936, genotype: p=0.500, interaction genotype–treatment: p=0.679).]. Error bars represent S.E.M. [Figure taken from Gross et al., 2011]

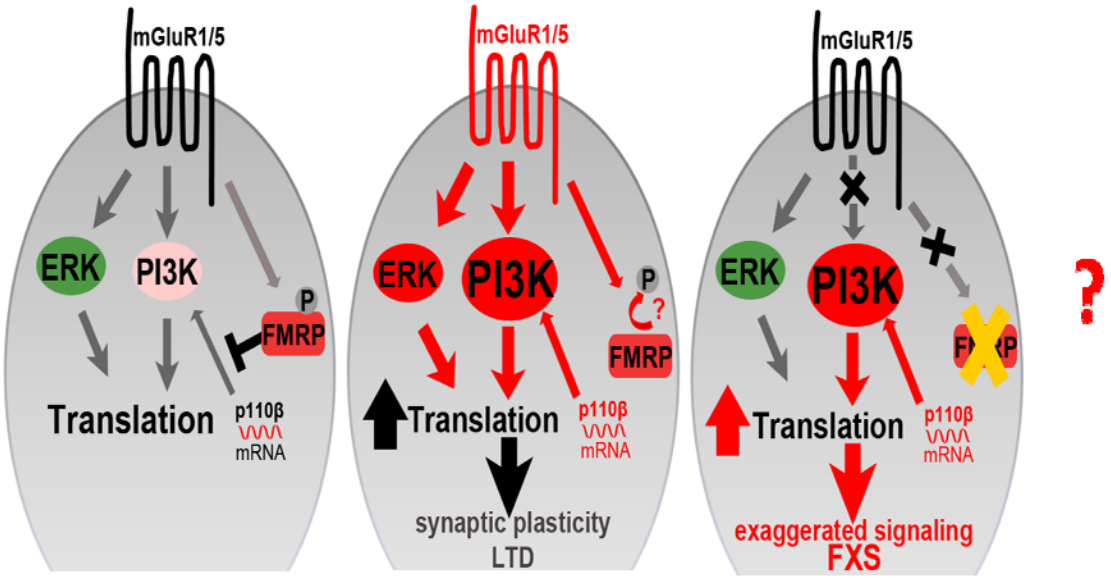

Figure 3: Proposed model for dysregulated metabotropic glutamate receptor 1/5 (mGlu1/5) signaling in FXS

The "mGluR theory of FXS" proposes that many FXS-associated phenotypes are caused by excessive and dysregulated signaling through mGlu1/5 in the absence of FMRP (Bear et al., 2004). Signaling through mGlu1/5 activates the downstream signal transduction molecules ERK1/2 (extracellular signal regulated kinase 1/2) and PI3K (phosphoinositide-3 kinase), leading to increased protein synthesis and long lasting synaptic plasticity, such as long term depression (LTD). Previous studies suggest that FMRP controls mRNA translation and protein expression of the PI3K catalytic subunit p110β, thereby allowing regulated response to plasticity-inducing stimuli. In the absence of FMRP, this regulation is lost, leading to exaggerated and stimulus-insensitive signaling through mGlu1/5. In line with the "mGluR theory", previous research has shown that the mGlu5 antagonist 2-methyl-6-phenylethynyl-pyridine (MPEP) rescues many FXS-associated symptoms in *Fmr1* knockout mice, including the increased susceptibility to audiogenic seizures (Yan et al., 2005).

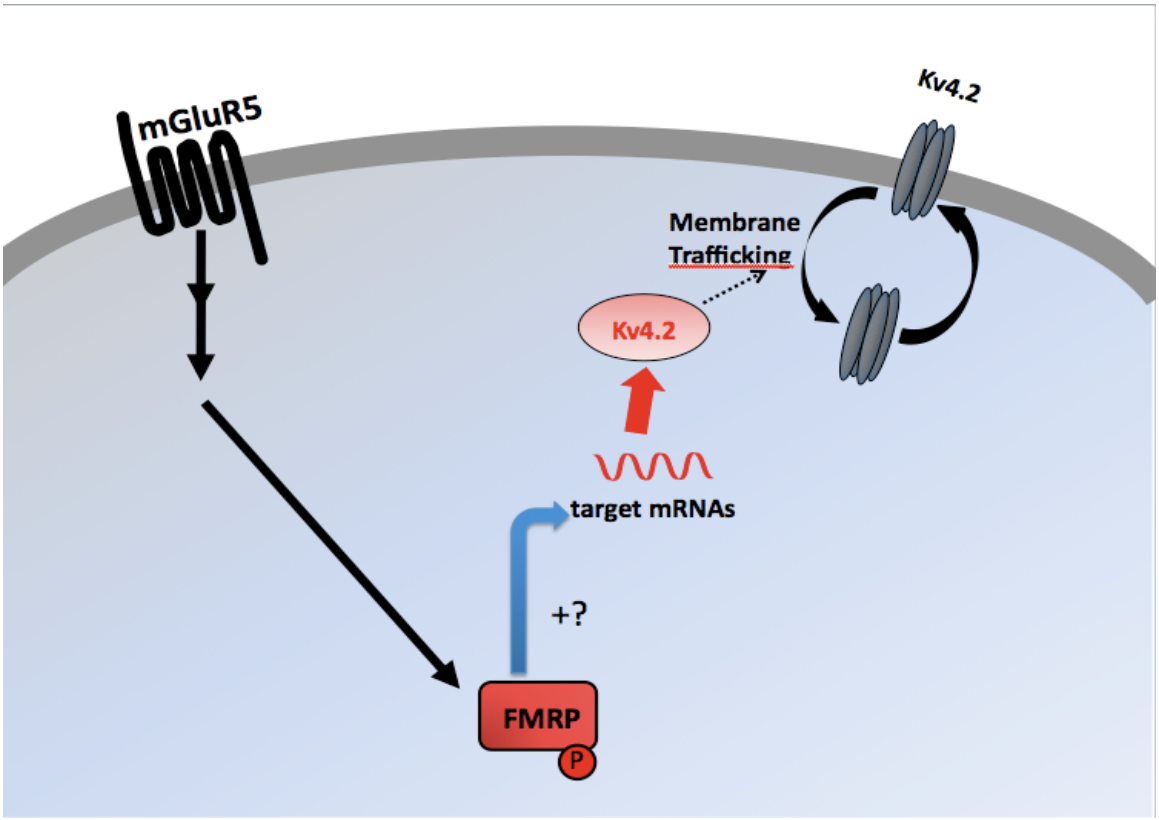

Figure 4: Proposed model for the regulation of the potassium channel Kv4.2
Kv4.2 is a major potassium channel regulating neuronal excitability in the hippocampus. Reduced plasma membrane levels of Kv4.2 lead to increased dendritic excitation. A recent study by the Bassell lab suggests that FMRP associates with the mRNA of Kv4.2 and positively regulates Kv4.2 expression and function, thereby counteracting hyperexcitability (Gross et al., 2011). This study also shows that antagonizing mGlu5 partially restores Kv4.2 cell surface expression in *Fmr1* KO mice to WT levels, suggesting that mGlu1/5 signaling might regulate Kv4.2. We hypothesize that Kv4.2 cell surface expression is negatively regulated by mGlu1/5 signaling, leading to reduced cell surface levels of Kv4.2 when the mGlu1/5 pathway is activated. FMRP may counteract the mGlu1/5-mediated effect by activating Kv4.2 mRNA translation and protein synthesis, which ultimately leads to higher levels of Kv4.2 on the cell surface. Increased Kv4.2 expression on the cell surface during mGlu1/5 signaling may serve to dampen the neuronal excitability seen in FXS.

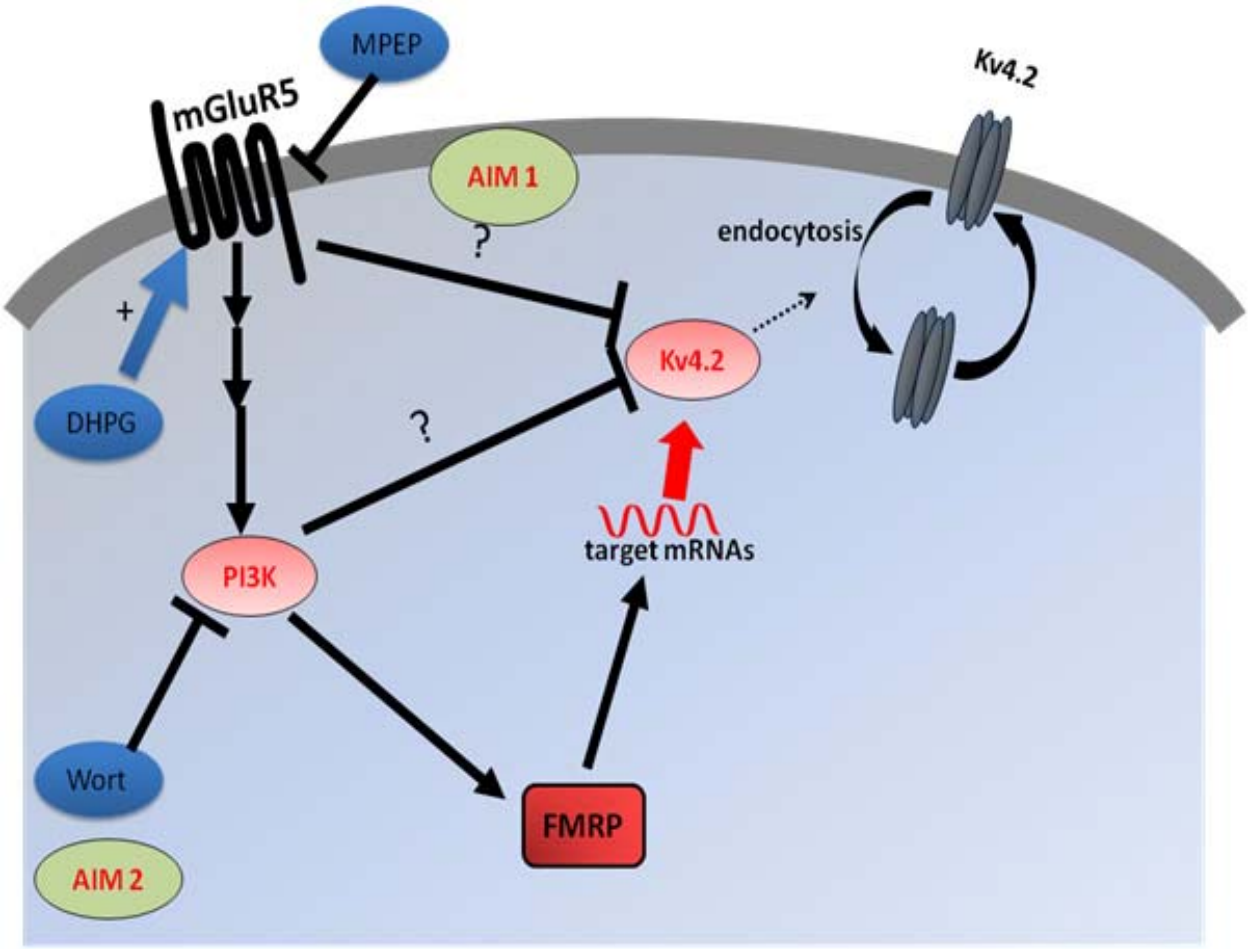

Figure 5: Overview of the hypothesis and aims of the thesis research
The overall hypothesis of this thesis is that the Fragile X Mental Retardation Protein (FMRP) and signaling through mGlu1/5 regulate Kv4.2 cell surface expression. The first aim of this research was to investigate the role of mGlu1/5 activity and downstream signaling on the regulation of Kv4.2 surface expression. We propose that activation of mGlu1/5 signaling decreases surface levels of Kv4.2. The second aim of this research is to determine if antagonizing PI3K signaling, a downstream signaling molecule of mGlu1/5 which is shown to be overactive in the absence of FMRP (Gross et al., 2010), may restore Kv4.2 surface levels in *Fmr1* knockout mice to wild type levels.

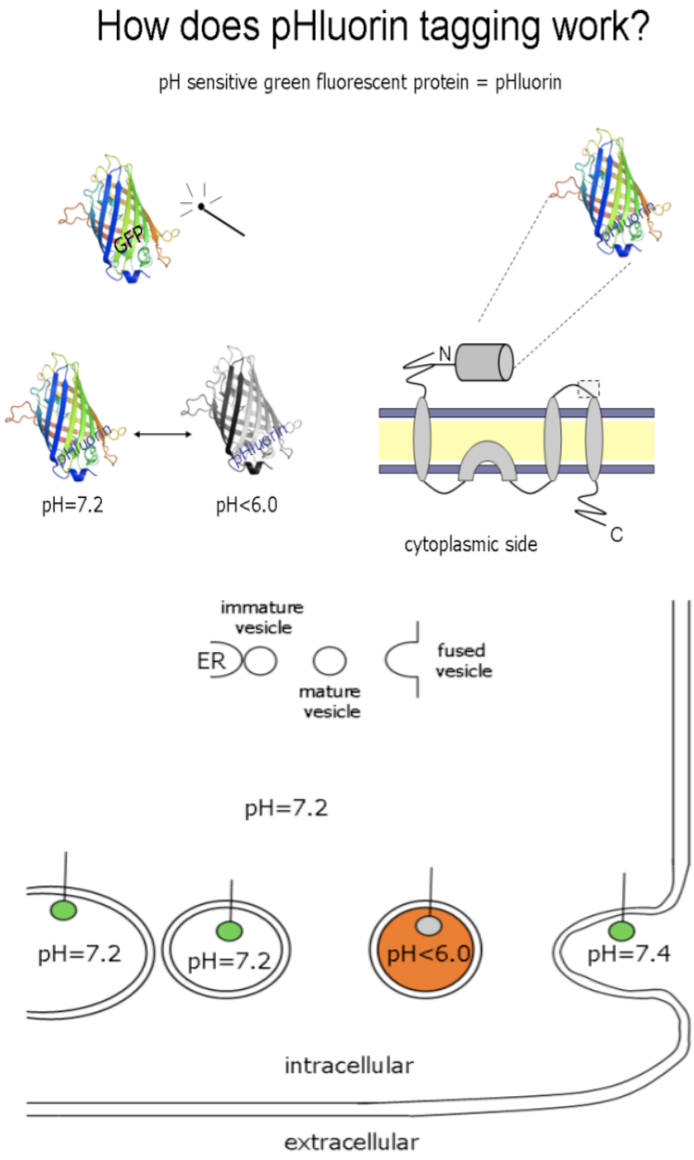

Figure 6: the pHluorin-Kv4.2 fusion protein

pHluorin is a pH-sensitive derivative of the green fluorescent protein GFP (Miesenbock et al., 1998). When pHluorin is in neutral (pH=7.2) or physiological (pH=7.4) pH environment, e.g. at the cell surface, the protein fluoresces, but loses fluorescence in acidic environment, e.g. in endocytosed vesicles. This research takes advantage of pHluorin's properties by employing a Kv4.2-pHluorin fusion protein to quantify cell surface expression of Kv4.2. The Kv4.2-pHluorin fusion protein will fluoresce green when on the cell membrane because the protein is in a neutral pH environment. However, when the protein is in a mature vesicle, the environment inside that vesicle is acidic; thus, the Kv4.2-pHluorin protein will not fluoresce. This is how we are able to distinguish between total and surface levels of Kv4.2.

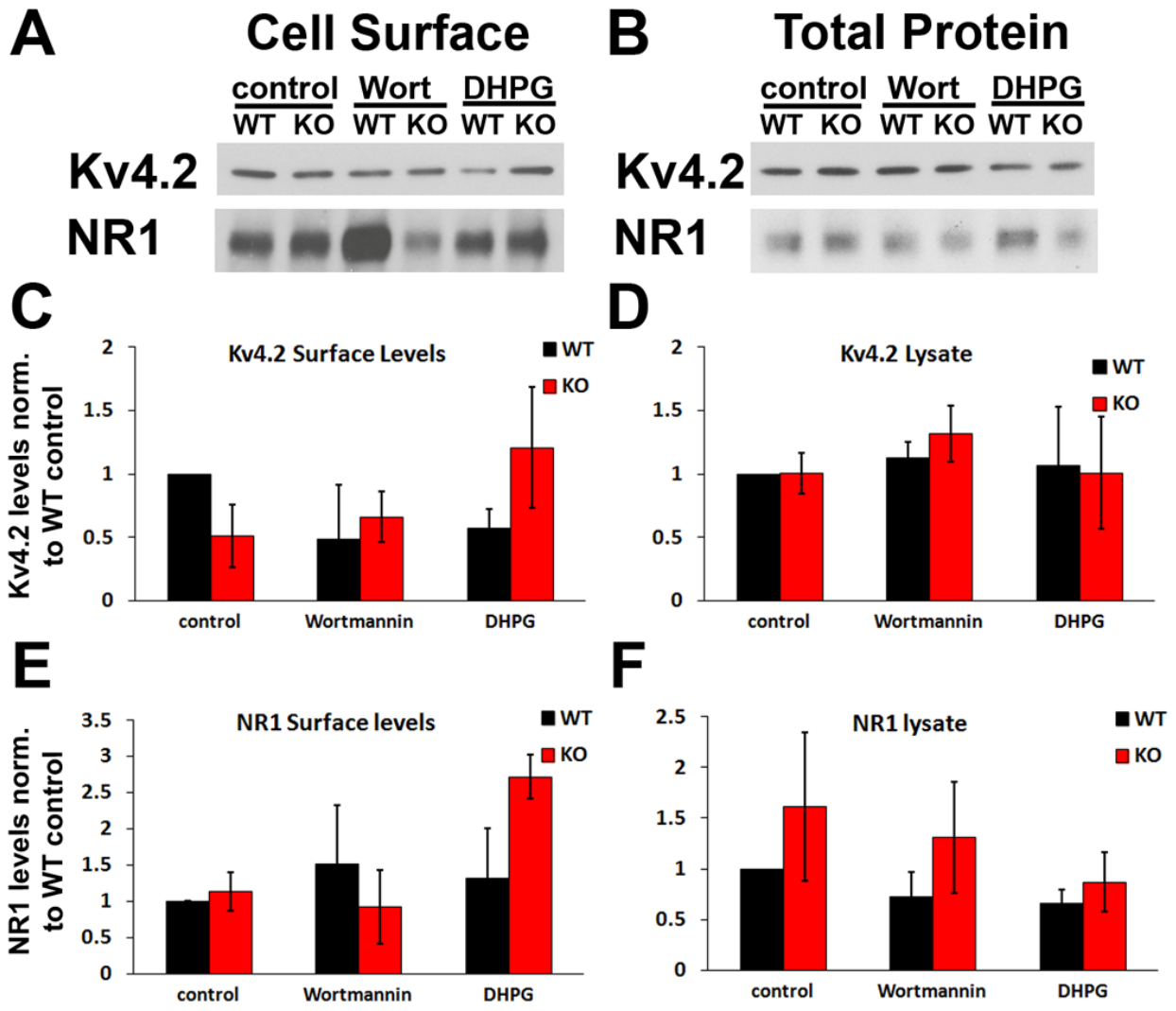

Figure 7: Surface levels of Kv4.2 in mouse hippocampal slices may be reduced in the absence of FMRP and by signaling through mGlu1/5.
(A+B) Shown are Kv4.2- and NR1-specific example western blots of cell surface proteins (**A**) and total protein fractions (**B**) from surface biotinylation experiments in hippocampal slices from WT and *Fmr1* KO mice. Some hippocampal slices were treated with either DHPG or Wortmannin (see methods, indicated in the figures).
(C) Densitometric analysis of Kv4.2-specific western blots of hippocampal cell surface protein fractions normalized to tubulin suggests that cell surface Kv4.2 levels may be reduced in hippocampal slices of *Fmr1* KO mice as compared to WT. Furthermore, there was a trend towards reduced Kv4.2 cell surface levels in WT hippocampal slices treated with DHPG, a mGlu1/5 agonist, as compared to the WT untreated condition. Interestingly, both, treatment with DHPG and treatment with the PI3K antagonist Wortmannin led to a trend towards increased cell surface levels of Kv4.2 in *Fmr1* KO hippocampal slices, although variability was

high (n=3, p(genotype) =0.67; p(treatment) =0.67; p(interaction) =0.26;Two-Way ANOVA, error bars represent S.E.M.).
(**D**) Densitometric analysis of Kv4.2-specific western blots of hippocampal lysate fractions (normalized to tubulin) suggests that total Kv4.2 levels in hippocamapal slices of KO *Fmr1* mice (normalized to tubulin) may be slightly decreased as compared to hippocampal slices of WT mice. Treatment with the PI3K antagonist furthermore leads to a trend of increased Kv4.2 total levels in *Fmr1* KO hippocampal slices (n=3, p(genotype)=0.67; p(treatment)=0.60; p(interaction)=0.96; Two-Way ANOVA, error bars represent S.E.M.).
(E) Densitormetric analysis of NR1-specific western blots of hippocampal cell surface fractions shows that NR1 cell surface levels are not reduced by the absence of FMRP or by DHPG treatment. However, in WT, Wortmannin treatment leads to a trend towards increased NR1 surface levels, whereas DHPG treatment leads to a trend towards increased surface levels in *Fmr1* KO (n=3, p(genotype)=0.38; p(treatment)=0.48; p(interaction)=0.39;Two-Way ANOVA, error bars represent S.E.M.). NR1 was chosen as a cell membrane receptor that was shown previously not to be reduced in *Fmr1* KO mice (Gross et al., 2011), which was confirmed in this study. However, future studies may follow-up on the here presented results and analyze how mGlu1/5 activity and downstream signaling affect NR1 cell surface levels in WT and *Fmr1* KO.
(F) Densitometric analysis of NR1-specific western blots of hippocampal lysates fractions (normalized to tubulin) shows a trend towards increased NR1 total levels in *Fmr1* KO in all three conditions. Both wortmannin and DHPG reduced the total levels of NR1 in *Fmr1* KO hippocampal slices (n=3, p(genotype)=0.21; p(treatment)=0.45; p(interaction)=0.90;Two-Way ANOVA, error bars represent S.E.M.).

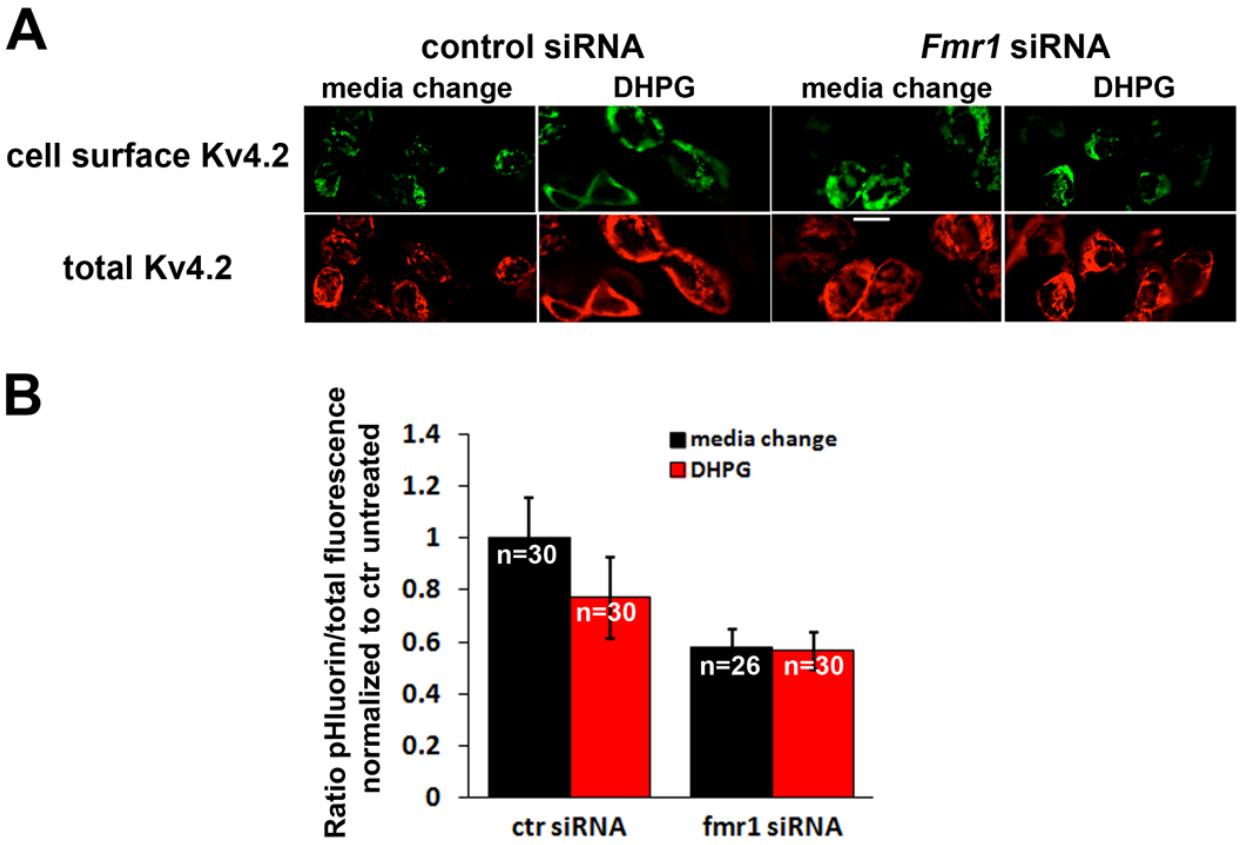

Figure 8: mGlu1/5 activity and siRNA-mediated knockdown of *Fmr1* leads to reduced Kv4.2 cell surface levels in N2A cells

Note: It is interesting that Fmr1 KO cells are insensitive to DHPG-induced reduction in Kv4.2.

N2A cells were transfected with either a scrambled control siRNA (*ctr siRNA*) or *fmr1*-specific siRNA (*fmr1 siRNA*) followed by transfection with Kv4.2-pHluorin. Cells were either treated with media change (control) or with 100µM DHPG for 20 min. After fixation, total Kv4.2 protein levels were visualized by Kv4.2-specific immunocytochemistry using a Cy3-coupled secondary antibody. Kv4.2 cell surface levels and total Kv4.2 levels were quantified by measuring GFP- or Cy3-specific fluorescence intensity, respectively.

(A) Representative images are for each analyzed condition. Scale bar is 10 µm.

(B) Shown are ratios of Kv4.2 cell surface to total levels from two independent experiments. For both experiments, ratios of all conditions were normalized to the average ratios of untreated control siRNA-transfected cells. Transfection with *Fmr1* siRNA siRNA leads to significantly reduced Kv4.2 cell surface levels as compared to control siRNA-transfected cells. Treatment with the mGlu1/5 agonist DHPG led to a trend towards reduced Kv4.2 cell surface levels in control cells (n indicated in the figure; two-way ANOVA, α=0.05; no significant effect of treatment: p=0.32, significant effect of siRNA: *p=0.014; no interaction between treatment and siRNA: p=0.39, error bars represent S.E.M.).

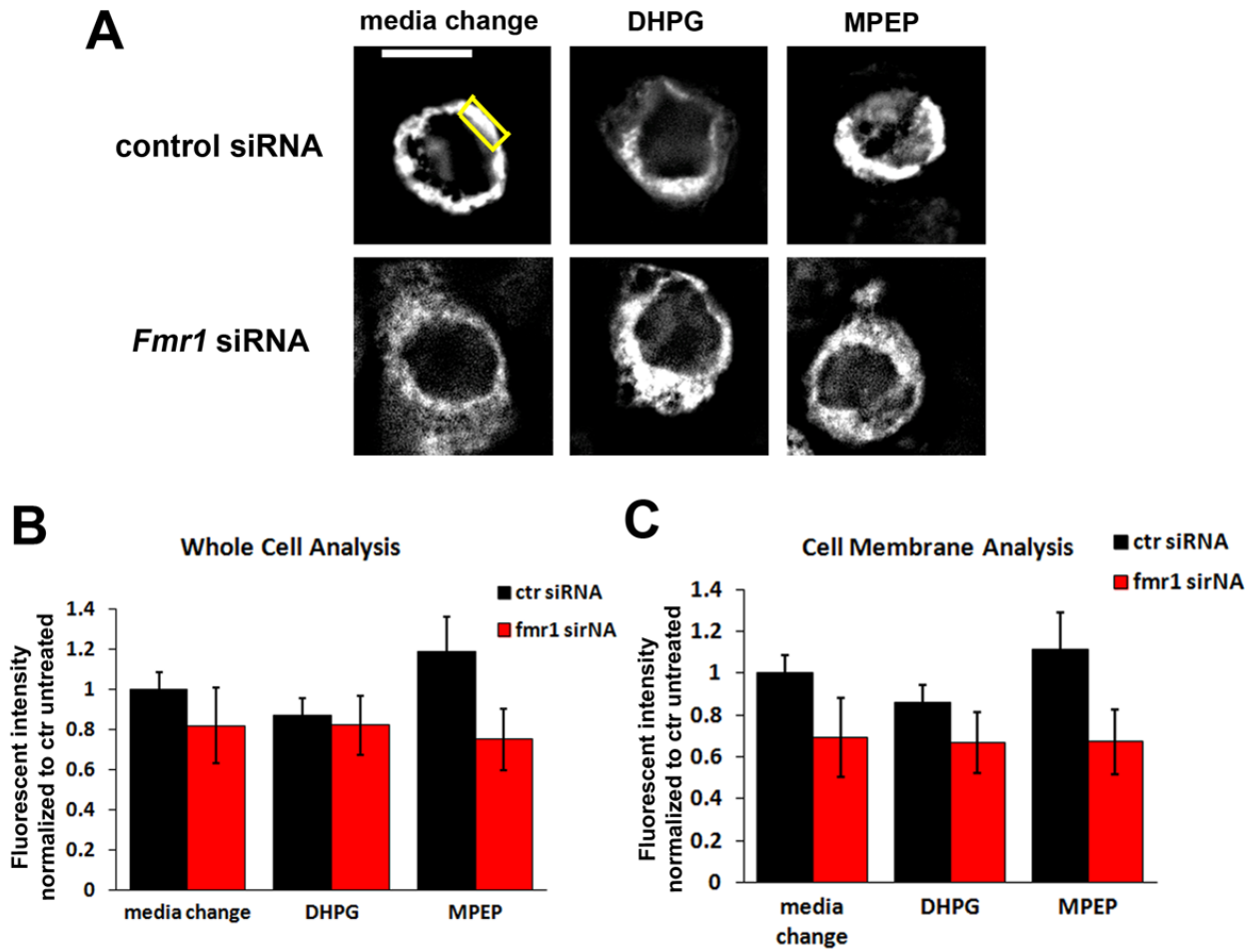

Figure 9: mGlu1/5 activity and siRNA-mediated knockdown of *Fmr1* reduce Kv4.2 cell surface levels in N2A cells:

Neuro 2A cells were transfected with either a scrambled control siRNA (*ctr siRNA*) or *Fmr1*-specific siRNA (*fmr1 siRNA*) followed by transfection with Kv4.2-pHluorin. Cells were either treated with media change as control or with 100µM DHPG for 20 min. After fixation, Kv4.2 cell surface levels were quantified by measuring GFP-specific fluorescence intensity. Fluorescence intensity was measured through two different techniques.

(A) Example images for each analyzed condition. Scale bar is 10 µm.

(B) Fluorescence intensity was quantified by measuring fluorescence of the entire cell (see methods). Shown are average Kv4.2 cell surface levels from two independent experiments, normalized to the average fluorescence intensity of the respective untreated control siRNA-transfected cells. Transfection with *Fmr1* siRNA leads to a trend towards reduced Kv4.2 cell surface levels compared to control siRNA-transfected cells. Treatment with the mGlu1/5 agonist DHPG leads to a trend towards reduced Kv4.2 cell surface levels in control cells, whereas MPEP treatment shows a trend towards increased cell surface levels in control cells. None of the treatment has an effect on *Fmr1*-siRNA-transfected cells (n=11 cells; 2 independent experiments, two-way ANOVA, α=0.05; no significant effect of treatment: p=0.70, no significant effect of siRNA: p=0.06; no interaction between treatment and siRNA: p=0.40).

(C) Fluorescence intensity was quantified by measuring fluorescence of separate membrane segments (see methods, indicated by yellow box in **A**). Shown are Kv4.2 cell surface levels from two independent experiments, normalized to the average fluorescence intensity of the respective untreated control siRNA-transfected cells. Transfection with *fmr1* siRNA leads to significantly reduced Kv4.2 cell surface levels compared to control siRNA-transfected cells. Treatment with the mGlu1/5 agonist DHPG leads to a trend towards reduced Kv4.2 cell surface levels in control cells, whereas MPEP treatment shows a trend towards increased cell surface levels in control cells. None of the treatment seems to have an effect on *Fmr1*-siRNA-transfected cells (n=11 cells, 2 independent experiments; two-way ANOVA, α=0.05; no significant effect of treatment: p=0.28, significant effect of siRNA: *p<0.0001; no interaction between treatment and siRNA: p=0.28).

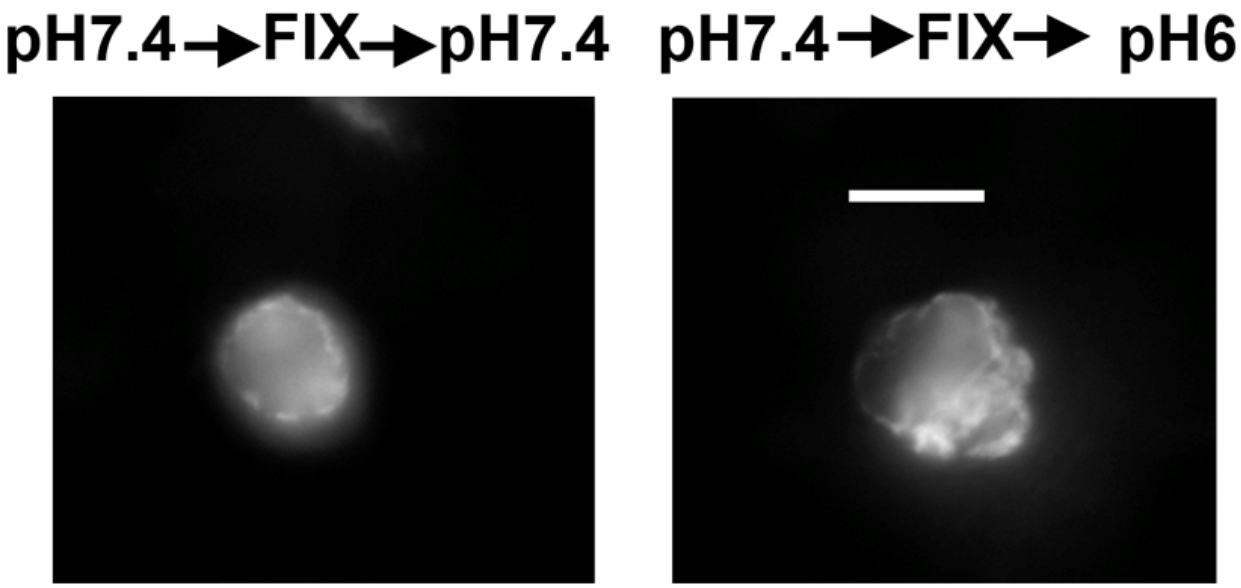

Fig 10: Lowering the pH after fixation does not quench fluorescence of pHluorin
Treatment with the detergent Triton X 100 during immunocytochemistry is expected to destroy the pH gradient within the cell, leading to neutral pH even in endocytosed, previously acidic vesicles. The analysis shown in Figure 8 is thus based on the assumption that paraformaldehyde fixation of cells stalls the protein conformation of the Kv4.2-pHluorin fusion protein and preserves its fluorescence status independently of the surrounding pH. To test this assumption, we lowered the pH to 5.8 following fixation, and could show that this treatment did not eliminate fluorescence of surface pHluorin, suggesting that once fixed with paraformaldehyde, the pHluorin maintains its previous conformation and thus fluorescence status. Above are images showing pHluorin-Kv4.2 transfected Neuro 2A cells. Cells were washed with a PBS solution of pH 7.4, and fixed with 4% paraformaldehyde, pH 7.4. After fixation, PBS solutions of either pH 7.4 (left) or pH 5.8 (right) were added to the cells. Cells that were washed with PBS solution of pH 5.8 after fixation maintained their fluorescence, suggesting that treatment of cells with 4% paraformaldehyde is sufficient to preserve the fluorescence status of pHluorin. This corroborates the validity of the experiments described in Figure 8 and 9.