Single Cell Knock-out of the Fragile X Mental Retardation 1 Gene in the Mice Cortex

SPECIFIC AIMS

Fragile X Syndrome (FXS) is the most common form of inherited mental retardation. It affects approximately 1 in 4,000 males and 1 in 8,000 females. Symptoms are both mental and physical and include facial abnormalities such as a large forehead and ears and a prominent jaw, developmental delay, hyperactive behavior, and autistic behavior. Symptoms tend to be more severe in men than in women [3,6]. Unfortunately there is still no cure for FXS, and there is little known about the disorder. FXS is a genetic disorder caused by inactivation of the Fragile X Mental Retardation 1 (*Fmr1*) gene. The inactivation is caused by the expansion of a CGG trinucleotide repeat in the 5' untranslated region of the X-linked *Fmr1* gene that leads to methylation. The *Fmr1* gene encodes the Fragile X Mental Retardation Protein (FMRP), which is expressed to high levels in neurons and is necessary for normal brain function.

The main goal of this project is to examine how the deletion of FMRP affects the structural dynamics of dendritic spines (deletions/ truncations/formations of spines) in mouse cortical neurons in vivo, and whether the effect is cell-autonomous (i.e. intrinsic to the affected neuron), or a network effect where the behavior of neurons in the circuit are disrupted, impairing input to each postsynaptic cell. Our short term goal is to use transcranial in vivo two-photon microscopy to visualize dendritic spines in the cortex of mice, and then test the CRE-DOG (Cre recombinase dependent on GFP) system for single cell knockout of FMRP. The overall objective is to make a conditional knock-out

of the *Fmr1* gene in cortical neurons to investigate the synaptic dynamics of synapses in individual neurons lacking FMRP. Our central hypothesis is that FMRP deletion affects spine dynamics. Hence, we expect to find truncated or a complete loss of dendritic spines in the cortex.

Aim 1. Delete the *Fmr1* gene in a single cell using the CRE DOG system. This *Fmr1* deletion will allow us to study the effect of FMRP deletion in the brain without the complications of whole animal knockouts. We will use the CRE-DOG system to knockout FMRP in only a few cells in cortical neurons. We expect to label a sparse subset of GFP positive neurons that will co-express tdTomato.

Aim 2. Determine whether the effect of FMRP loss on neurons is cell autonomous or produces a network effect. This will elucidate any fundamental role FMRP plays in brain development and could be helpful in determining the developmental timing requirements for FMRP. We will use transcranial in vivo two-photon microscopy to investigate the synaptic dynamics of individual neurons lacking FMRP. Imaging will be done at different time intervals (day 0, day 4, day 8) after injecting the mouse cortex with adeno associated viruses (AAVs) encoding the CRE-DOG system and FLEX-tdTomato into the mouse cortex (3 weeks of incubation of virus before imaging). We expect to find abnormalities in spines of cells lacking the *Fmr1* gene.

IMPACT

The proposed research will elucidate the specific function of FMRP in neurons, and the specific changes that dendritic spines undergo due to loss of FMRP. Furthermore, the

single cell knock out mouse models can serve as a tool to elucidate the physiological role of *Fmr1* and the mechanisms involved in mental retardation.

SIGNIFICANCE

The brain contains many mRNA-binding proteins, but only a few are required for proper neural function. The *Fmr1* gene is linked to the X- chromosome and encodes an mRNA-binding protein (FMRP) that is highly expressed in neurons. This FMRP protein functions to negatively regulate protein synthesis in the brain. Loss of function of the FMRP causes Fragile X syndrome (FXS) [11]. The absence of FMRP in the developing brain permanently alters neuronal connectivity and produces the devastating behavioral symptoms seen in FXS patients, including intellectual disability and autism.

The majority of excitatory synapses are formed by small dendritic protrusions in the cerebral cortex commonly referred to as dendritic spines. Indeed, dendritic spine formation and elimination play an important role in learning and memory. Interestingly, spine density along the apical dendrite is greater in FMRP knockout mice, which reflects and impaired developmental organization of synapse stabilization and elimination or pruning [2]. FMRP deleted mice models exhibit widespread defects in synaptic plasticity and development including an increased number of long and thin dendritic spines, instead of the mature and strong mushroom-shaped spines that healthy neurons have. Consistently, the transcripts of 40 genes identified as FMRP targets (Joshua A. Suhl) encode mostly proteins that are implicated in synaptic growth, plasticity, cell adhesion, or cytoskeletal structure and remodeling [9].

FMRP regulates proteins involved in the signaling pathways that regulate spine shape/dynamics and long-term synaptic plasticity. Abnormal spines cause dysfunctional circuits and dysfunctional circuits can cause abnormal spines [1]. The capacity of the nervous system to generate neuronal networks relies on the establishment and maintenance of synaptic contacts. When FMRP is lost, the ability of the brain to form these neuronal circuits properly are also lost. Additionally, lack of FMRP leads to defects in synaptic transmission. FMRP is a major protein regulator of the translation of many mRNAs involved in synaptic plasticity. This is why it is worthwhile examining how synaptic dynamics change when FMRP is knocked out in single neurons. Especially, it will be interesting to determine if the neuronal circuits are functioning in most of the brain.

The understanding of FXS and the implementation of meaningful treatment strategies require a thorough understanding of the temporal and spatial requirements for FMRP in establishment and maintenance of neural circuit function. The CRE-DOG system will be activated during critical periods of brain development to knock out FMRP in specific neurons. We will determine how the single knock out affects individual neurons. Previously, full KO models provided insight of the global effect when the inputs between neurons were not functioning (dysfunctional neuronal circuits). Our single neuron knock-outs will help us determine whether abnormal spine dynamics of the neuron are simply caused by absence of FMRP. It will also help determine if the inputs are not functioning properly (network effect). More importantly, we will resolve whether the postsynaptic neurons are properly functioning

INNOVATION

Learning, memory, and behavior are complicated processes that involve many regions of the brain, as well as many proteins. Targeted disruption of specific genes is a powerful tool in the journey to elucidate the specific role of proteins and brain development. Innovatively, the CRE-DOG system allows us to genetically manipulate GFP expressing cells to create single cell knock-outs. The single cell knock-out will provide insight on whether the abnormal phenotype of elongated/dense regions of dendritic spines is also observed such as in knock-out mice models. Similarly, we will be able to knock out *Fmr1* at different times, and observe different spine dynamics which will be helpful in determining the developmental time requirement of FMRP something that was previously not known. This will allow us to answer the question as to whether the abnormal FXS dendritic spine phenotype is due to the absence of FMRP during development or is the result of a lack of postnatal FMRP expression.

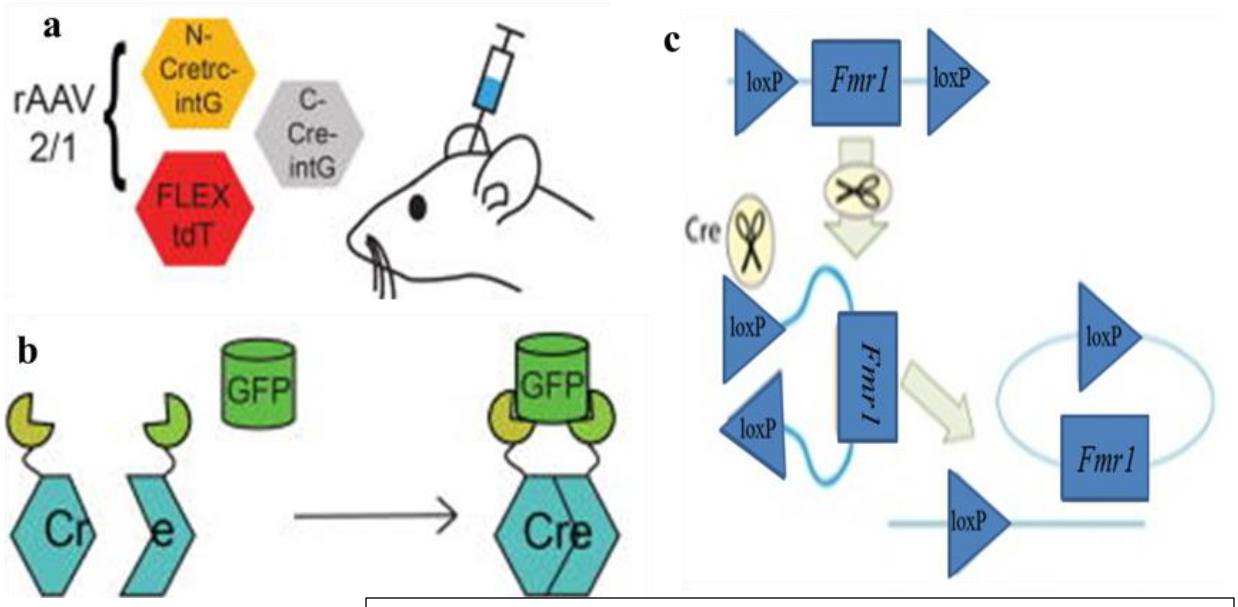
APPROACH

The model organism of choice to study FXS is the Mus musculus (mice), and this is because the expression pattern of *Fmr1* at the mRNA and protein level is similar in different tissues of humans and mice [5]. More importantly humans and mice, both being mammals share many common genetic, anatomical, and physiological features. The mouse genome was sequenced on 2002, and it is very similar to our own, which makes mouse genetic research useful for the study of human disease. Like humans, mice naturally develop diseases that affect all the systems in our bodies. Furthermore, it is easy to manipulate the mouse genome by either adding/deleting a gene to better

understand its role in the body, which is useful for modeling diseases when a mutated gene (*Fmr1)* is known to play a role in the disease (FXS) [5].

Aim 1. Sparsely knock out FMRP in mice cortical neurons using CRE-DOG

In order to conditionally knock out *Fmr1* from mice cortical neurons we must first produce mice that are both expressing GFP in cortical neurons, and that have *Fmr1* floxed between two lox p sites (recombination event will occur between lox p sites). We will first cross a Thy1 GFP-M line mice (expressing cytoplasmic GFP in a random, sparse subset of layer 5 pyramidal neurons in the cortex) with conditional knockout (CKO) mice containing a floxed *Fmr1* gene (Fmr1fl). The split Cre recombinase once reconstituted in GFP positive cells will catalyze recombination between these two lox P sites, and induce deletion of the *Fmr1* gene (Figure 1). After genotyping and confirming positive GFP mice with *Fmr1* floxed to two lox p sites we will inject AAVs encoding the CRE-DOG system and FLEX-tdTomato into the mouse barrel cortex. The CRE-DOG system targets GFP-positive neurons for full Cre reconstitution, so the *Fmr1* gene is knocked out in these Cre positive neurons. The FLEX-tdTomato will serve as the reporter for the presence of Cre (Figure 1). After 3 weeks of injection we will image, and then perform immunohistochemistry to verify the single cell knockouts. We expect to have some single cell knockouts via this CRE- DOG system. If we fail to verify a single cell knock out of *Fmr1*, we will then optimize our immunohistochemistry protocol that requires antigen retrieval in order to stain for FMRP by slightly changing the protocol (Temp., time, etc.). Furthermore, we will cut different size coronal sections (20um, 25um, 30 um, 40 um) to observe which coronal sections is optimal for immunohistochemistry.



Adapted from http://blog.addgene.org/plasmids-101-cre-lox

Figure 1: (A) Injection of AAVs encoding the CRE-DOG system and FLEX-tdTomato into the mouse cortex. N-Cretrc-intG: N-terminal part of Cre Recombinase; C-Cre-intG: C-terminal part of Cre Recombinase. (**B**) Diagram showing how Split Cre reconstitutes into full Cre upon binding GFP. (**C**) Cre catalyzes recombination between two loxP sites to induce deletion of the *Fmr1* gene [7,10].

Aim1.1: Stain for FMRP via Immunohistochemisrty

After 3 weeks of incubation with AVVs, we will transcardially perfuse the mouse with 4% paraformaldehyde (PFA), post-fix the brain in 4% PFA at 4℃ overnight, and incubate the brain in 30% sucrose solution at 4℃ overnight. We will then cut 40 µm coronal sections of the brain with a Leica Cryostat. Next, we will immunostain brain slices for FMRP (mouse anti-FMRP primary antibody; Alexa 594-conjugated goat-anti-mouse secondary antibody) and for the neuronal marker NeuN (rabbit anti-NeuN primary antibody; Alexa 488-conjugated goat-anti-rabbit secondary antibody). Finally, we will

image the stained brain slices with a Zeiss AxioImager fluorescent microscope equipped with Apotome. It is necessary to be able to stain for FMRP because this will be the way that we verify the FMRP single cell knock out (Figure 2). We expected to see all neurons expressing FMRP in WT mice since FMRP is highly expressed in all neurons (Figure 2). We expect FMRP not to be expressed in the single cell knock-outs with the CRE-DOG system, so single cell knock-out neurons shouldn't be labeled.

Aim 1.2: Test CRE-DOG system in WT mice (thy1-GFP-M mouse)

Before we can actually implement the CRE-DOG system we have to verify the efficacy of the AAVS to successfully deliver the CRE DOG system, and that the CRE DOG system can label a sparse subset of GFP positive cortical neurons in thy1-GFP-M mice. After AAV injection in a thy1-GFP-M mouse and post-incubation for three weeks we will transcardially perfuse and cut 40 um coronal sections to be observed under a fluorescence microscope to verify CRE-DOG labeling (figure 3). We expect to see co-expression of TdTomato and GFP in the neurons that the CRE-DOG system was successfully delivered to since split cre recombinase is only supposed to reconstitute in GFP positive cells which then activates FLEX-TdTomato.

Aim 2. Observe Spine Dynamics in FMRP knock outs via two photon microscopy

We plan to conditionally knock out the *Fmr1* gene in cortical neurons via the CRE-DOG system and investigate the synaptic dynamics in individual neurons lacking FMRP using transcranial in vivo two-photon microscopy. After injecting AAVs encoding the CRE-DOG system and incubating for three weeks we will begin to do in-vivo two-photon imaging. We will be collecting spine dynamics data by manually counting the dendritic

spines, and examining how the spine dynamics change over time (day 0, day 4, day 8). Specifically, we will be keeping track of cortical neurons that are co-expressing GFP and TdTomato, and counting each dendritic spine along the dendrites while keeping track if they are truncated, completely gone, or if there is formation of dendritic spines, which is what we expect to find.

PRELIMENARY FINDINGS

Using immunohistochemistry, we have confirmed FMRP expression in all cortical neurons from WT mice (Figure 2).

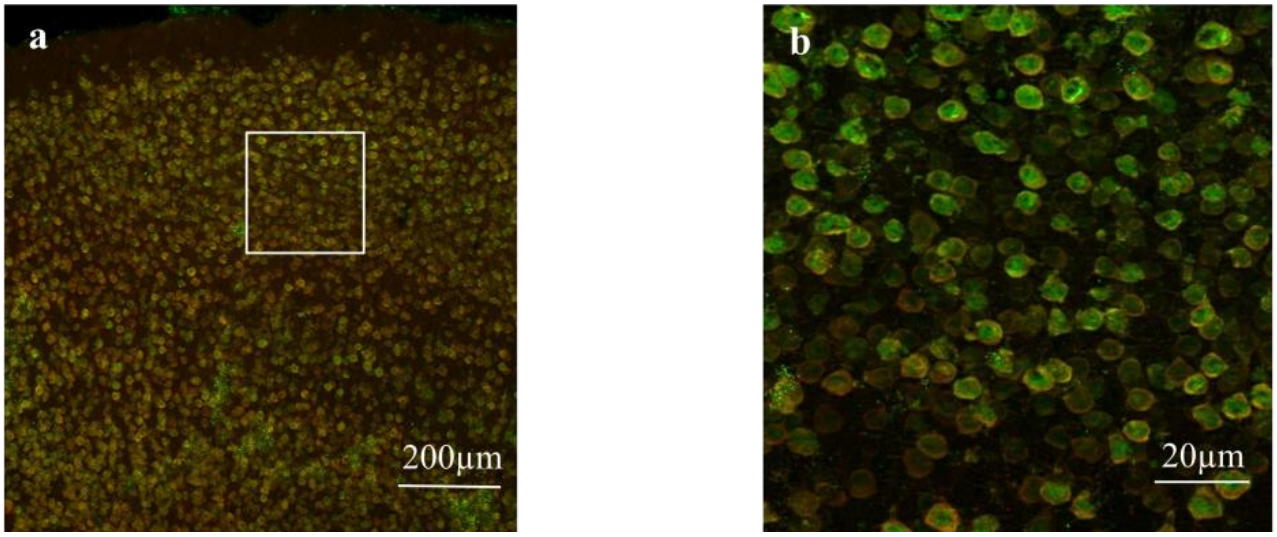


Figure 2: Low (a) and high (b) resolution images taken from the sensori-motor cortex of a wild-type mouse reveals that out of 205 NeuN positive cells counted, all of them are FMRP positive. Red channel: FMRP; Green Channel: NeuN. Scale bars: 200µm (a) 20 µm (b).

Additionally, we have confirmed the efficacy of the CRE-DOG system. We have successfully shown the CRE DOG system to label a sparse subset of GFP positive cortical neurons in a thy1-GFP-M mouse just as expected (Figure 3).

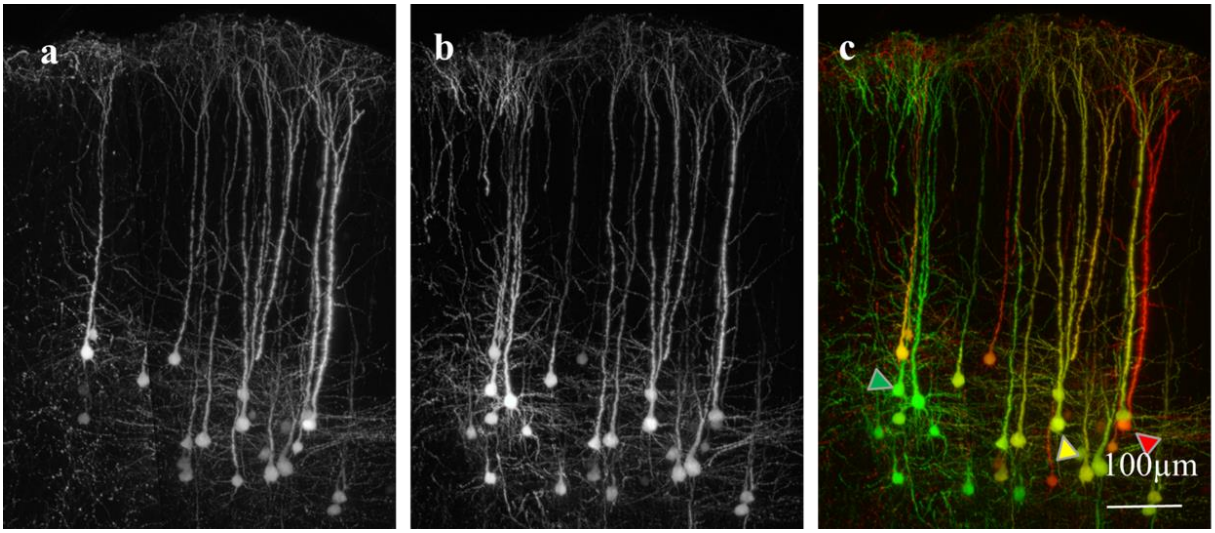


Figure 3: A subset of GFP positive pyramidal neurons in layer 5 cortex co-express tdTomato (yellow arrowhead), suggesting full Cre reconstitution. The viral infection efficiency is not 100%, as indicated by GFP positive neurons that do not co-express tdTomato (green arrowhead). Furthermore, there are some tdTomato positive cells that are GFP negative (red arrowhead). This suggests that CRE-DOG may reconstitute into full Cre in the absence of GFP with a small probability. (a) TdTomato positive Neurons. (b) GFP positive Neurons. (c) Merged image (Red & Green channels). Scale bar: 100 µm.

REFRENCES CITED:

1. Carlos Portera-Cailliau. 2011. Which Comes First in Fragile X Syndrome, Dendritic Spine Dysgenesis or Defects in Circuit Plasticity? The Neuroscientist. Vol 18, Issue 1, pp. 28 – 44.
2. Comery TA, Harris JB, Willems PJ, Oostra BA, Irwin SA, Weiler IJ, Greenough WT. 1997. Abnormal dendritic spines in fragile X knockout mice: maturation and pruning deficits. Proc Natl Acad Sci U S A 94:5401-5404.
3. E.J. Mientjesa, I. Nieuwenhuizena, L. Kirkpatrickb, 1, T. Zuc, M. Hoogeveen-Westervelda, L. Severijnena, M. Riféa, R. Willemsena, D.L. Nelsonb, B.A. Oostraa. 2006. The generation of a conditional Fmr1 knock out mouse model to study Fmrp function in vivo. Nuerobiology of Disease 21, 549 – 555.
4. Esther A. Nimchinsky, Adam M. Oberlander, Karel Svoboda. 2001. Abnormal Development of Dendritic Spines inFMR1 Knock-Out Mice. Journal of Neuroscience, 21 (14) 5139-5146.
5. Fmr1 knockout mice: a model to study fragile X mental retardation. 1994. The Dutch-Belgian Fragile X Consortium. Cell, Vol. 78, No. 1., pp. 23-33.
6. "Fragile X Syndrome." Genetics Home Reference. N.p., n.d. Web. 26 July 2016.
7. JC Tang, S Rudolph, OS Dhande, VE Abraira, S Choi, SW Lapan, IR Drew, E Drokhlyansky, AD Huberman, WG Regehr, CL Cepko. 2015. Cell type-specific manipulation with GFP-dependent Cre recombinase. Nature Neuroscience, 18, 1334 -1341.
8. Joshua A. Suhl, Pankaj Chopra, Bart R. Anderson, Gary J. Bassell, Stephen T. Warren. 2014. Analysis of FMRP mRNA target datasets reveals highly

associated mRNAs mediated by G-quadruplex structures formed via clustered WGGA sequences. Hum Mol Genet; 23 (20): 5479-5491.

9. Kashima, R., Roy, S., Ascano, M., Martinez-Cerdeno, V., Ariza-Torres, J., Kim, S., Hata, A. 2016. Augmented noncanonical BMP type II receptor signaling mediates the synaptic abnormality of fragile X syndrome. Science Signaling, 9(431), [ra58].
10. Tsien J. Z. 2016. Cre-Lox neurogenetics: 20 years of versatile applications in brain research and counting. Front. Genet. 7:19 10.
11. Verkerk AJ, et al. 1991. Identification of a gene (FMR-1) containing a CGG repeat coincident with a breakpoint cluster region exhibiting length variation in fragile X syndrome. Cell. 65:905–914.