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14. ABSTRACT

Fragile X syndrome (FXS) is the most common single gene cause of autism and intellectual dysfunction. It is marked by devastating alterations in cognition and behavior that originate in infancy. Approximately 1 in 4000 live births are affected by the disorder; therefore it represents a major health problem that also profoundly impacts a sizeable number of military families. A core symptom of the disorder is hypersensitivity of the senses, including hypersensitivity to touch, such that normal sensory stimuli are perceived as aversive. This contributes directly to many of the challenges faced by FXS individuals, including hyperarousal, social withdrawal and anxiety. The two partnering laboratories have collaborated on understanding this disruption for a number of years by working on an experimental mouse model of FXS. Studies from our laboratories have begun to define how the development of synapses and circuits in the sensory cortex are altered in FXS. We have found that there is abnormal activity in parts of the brain that process sensory inputs that could be due to changes in the neurotransmitter GABA, which normally dampens brain activity. In this proposal we will determine the extent of the alteration in synapses, neurons, circuits and behavior in the FXS model and ask the following three questions: 1) how do changes in the activity of neurons in the brain of FXS mice lead to an altered response to touch? 2) what are the alterations in GABA and brain connectivity that lead to a difference in the response of neurons in the circuit? 3) can we fix the problems in the aberrant response to touch in mice by improving GABA signaling during early brain development? These studies are designed to understand a critical problem in the FXS field, address important knowledge gaps, and ultimately to determine whether we can find ways to rectify the development of brain circuits that contribute to altered touch sensation. Our experimental design will employ cutting-edge techniques to record from neurons in the sensory cortex and is designed to incorporate the complementary expertise of the partnering laboratories. The ultimate outcome will be in identifying the network basis for hyperarousal to sensory stimuli, a hallmark symptom in FXS, and will inform the future development of novel treatments for children with FXS.

15. SUBJECT TERMS
None listed.

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TABLE OF CONTENTS

	Page
1. Introduction	4
2. Keywords	4
3. Accomplishments	4
4. Impact	9
5. Changes/Problems	9
6. Products	9
7. Participants & Other Collaborating Organizations	10

1. Introduction:

Fragile X syndrome (FXS) is the most common inherited form of mental impairment, affecting roughly 1 in 2,500 males and 1 in 7,000. The disease is caused by a CGG trinucleotide repeat expansion in the 5' untranslated region of the *FMR1* gene, which leads to its transcriptional silencing and loss of the Fragile X Mental Retardation Protein (FMRP) which is important for translational control of a large number of messenger RNAs, many of which encode for synaptic proteins. The resultant dysregulated expression of these proteins leads to disruptions in synaptic and circuit formation during development and ultimately underlies the severe cognitive and behavioral symptoms of the disorder. One of the core symptoms that is particularly prominent in FXS individuals, is the problem of hypersensitivity to sensory stimuli, which results in hyperarousal, anxiety and seizures. In the somatosensory system, hypersensitivity to touch is a sensory gating defect that manifests behaviorally as tactile defensiveness and avoidance of (or negative response to) otherwise neutral tactile stimuli.

Sensory hypersensitivity in FXS likely results from excessive neuronal or circuit hyperexcitability in sensory regions of the cortex. But whether such hyperexcitability causes a simple exaggerated response to sensory stimuli in sensory cortices, versus subtle changes in sensory processing (e.g. disruptions in adaptation to repetitive sensory stimulation, changes in receptive field size, etc.) has not been established. Our goal is to gain further insight into the synaptic and circuit alterations underlying the sensory hypersensitivity and then target those mechanisms to correct this debilitating problem for FXS individuals. Work in both the partnering PI's laboratories through this collaboration has focused attention on the involvement of GABAergic dysfunction both during early cortical critical periods as well as in the adult mouse cortex. Ongoing studies are directed at determining the mechanism underlying these alterations and determining whether targeting GABAergic signaling shows promise in preclinical models of FXS.

2. Keywords:

Fragile X, Autism, GABA, Interneuron, Sensory hypersensitivity, Synapse

3. Accomplishments:

Major Goals of the Project:

The Project was split into 3 Aims each with a Major Goal

1. To test in vivo whether Fmr1 KO mice have dysfunctional inhibitory circuitry that causes a lack of neuronal adaptation and avoidance behaviors (tactile defensiveness).

2. Use in vitro electrophysiological recording in brain slices to determine whether the connectivity of the neurons in the sensory microcircuit is disrupted in Fmr1 KO mice.

3. Use pharmacological intervention to determine whether administration of TrkB agonist can rescue the behavioral deficits as well as the synaptic deficits in sensory cortex of Fmr1 KO mice.
What was accomplished in each Aim:

****Please note that this is the final progress report after the NCE period. Therefore the progress reported is materially the same as the report submitted in September ****

Aim 1: <u>To investigate the role of cortical inhibitory circuitry underlying the lack of neuronal and behavioral adaptation to repetitive whisker stimulation in *Fmr1* KO mice:</u>

This Aim was performed in the Portera-Cailliau laboratory at UCLA

The major goal of Aim 1 was to test three separate but related hypotheses:

1. That the behavioral phenotype of *Fmr1* KO mice to repetitive sensory stimulation is an avoidance response to an aversive sensory stimulus.
2. That a defect in interneuron circuitry in the cortex is responsible for the lack of sensory adaptation in *Fmr1* KO mice.
3. That this major sensory processing defect in *Fmr1* KO mice depends on loss of FMRP prior to the critical period but persists into adulthood.

Major Task 1: To test whether dysfunctional inhibitory circuitry in barrel cortex causes the lack of neuronal adaptation and avoidance behaviors (tactile defensiveness) in Fmr1 KO mice:

<u>Subtask 1: Does the behavioral manifestation extend to repetitive visual stimuli</u>? (Months 1-12)
We proposed to investigate whether the neuronal adaptation to chronic sensory stimulation was also absent in primary visual cortex (V1) in Fmr1 KO mice. However, as indicated in the 2019 annual report, we were not be able to determine

whether adaptation to visual stimuli is also reduced in Fmr1 KO mice (like we had for neurons in somatosensory cortex, S1, to tactile whisker stimulation), because of differences between V1 and S1 that make it harder for us to look for adaptation in V1. These include the small numbers of orientation selective neurons in V1, and the slower time course over which we can observe adaptation (tens of seconds in V1 compared to a few seconds in S1). Although we had proposed to continue these experiments in Year 3 of the project, delays caused by the Pandemic have forced us to abandon those experiments in favor of others that would be more fruitful (see below). It is worth noting that deficits in adaptation/habituation are seen for tactile stimulation in human subjects with FXS and also for auditory stimuli in the Fmr1 KO mice {Castren, 2003 #1864;Van der Molen, 2012 #1330;Sinclair, 2017 #1865}{Lovelace, 2018 #2201;Ethridge, 2016 #2175}.

<u>Subtask 2: Do adult *Fmr1* KO mice exhibit neuronal and behavioral adaptation to repetitive whisker stimulation and do network alterations (loss of neuronal adaptation) require loss of FMRP before and up to the critical period</u>? (Months 1-6)

The first part of this sub-task was already completed, as we already published a study demonstrating that adult Fmr1 KO mice do manifest both neuronal and behavioral adaptation {He, 2017 #2133}. The two are related to one another, as show below (Fig. 1). As stated in previous annual reports, we abandoned the experiments to investigate conditional Fmr1 knockout mice (in which Fmr1 is deleted in cortical neurons after the 2nd postnatal week) because some of the CamKII-Cre x Fmr1$^{fl/fl}$ showed evidence of germline recombination (an issue related to CamKII-Cre mice). We are still very interested in pursuing these studies in the future, but we were not able to complete them during Year 3 or the NCE period.

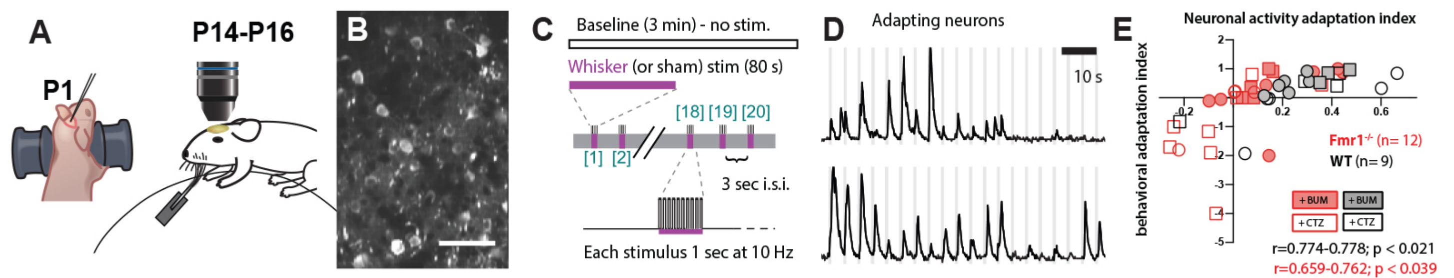

Figure 1: The degree of behavioral adaptation correlates with neuronal adaptation in L2/3 in both WT and Fmr1 KO mice. A) Cartoon of the injection of rAAV vector to express GCaMP6s in somatosensory cortex at P1 (left) and in vivo imaging with whisker stimulation at P14-16 (right). **B)** Example field of view of neurons expressing GCaMP6s in barrel cortex (confirmed by intrinsic signal imaging) at P15 (xyt SUM projection of 100 frames at 7.8 Hz). **C)** Protocol for whisker stimulation and for recording spontaneous and whisker-evoked activity (20 epochs of 10 Hz stimulation for 1 s, with a 3 s i.s.i. **D)** Example GCaMP6s fluorescence traces from L2/3 neurons showing time-locked responses, and progressive adaptation to repetitive bouts of whisker stimulation (vertical grey bars). **E)** We found a significant correlation between the neuronal adaptation index (x-axis) and the behavioral adaptation index (y-axis). Positive values for neuronal adaptation indicate that firing of L2/3 neurons (as calculated by median z-score of the ΔF/F calcium signal) was smaller for the last 5 whisker stimulations than for the first 5. Similarly, positive values for behavioral adaptation indicate that the avoidance/defensive behaviors that mice exhibited were less prominent in the last 5 stimulations than with in first 5. Note that, on average, *Fmr1*$^{-/-}$ mice showed less neuronal adaptation and less behavioral adaptation

<u>Subtask 3: Is the increase in locomotion/activity an avoidance response</u>? (Months 12-36)

We already completed this subtask. The goal was to conduct simultaneous in vivo calcium imaging recordings in awake, head-restrained mice that are allowed to run on a floating polystyrene ball, so that both measures of sensory adaptation (i.e., avoidance responses and network activity) could be assessed and correlated in individual animals. We previously reported that Fmr1 KO mice perceive repetitive whisker stimulation as aversive, because they run preferentially away from the side of stimulation (He et al., 2017). This was the first demonstration, to our knowledge, of an avoidance response in fragile X mice that is akin to tactile defensiveness in humans with FXS. In more recent and still unpublished studies (carried out in Years 2 and 3 of funding), we have now demonstrated that the degree of behavioral adaptation correlates very well with the degree of neuronal adaptation, both in wild type and in Fmr1 KO mice, such that mice with facilitation of responses tend to show more avoidance, and mice with significant adaptation appear to tolerate much better the repetitive tactile stimulation (**Fig. 1**).

We have also been conducting experiments to assess whether Fmr1 KO mice exhibit maladaptive behaviors in a novel social touch assay. This was not one of the original experiments we proposed but is a natural extension of these studies, as atypical tactile sensory processing in children with autism could negatively impact their social interactions. Using this social touch assay we have been able to demonstrate that Fmr1 KO mice show signs of avoidance when presented with other mice on a moving platform, as well as hyperarousal (presumably a sign of stress or anxiety), as manifested by persistently dilated pupils in response to repetitive bouts of forced interactions with a stranger mouse (**Fig. 2**). On the other hand, other studies we have recently performed in Fmr1 KO mice using high speed cameras to track pupil size in animals performing a visual discrimination task have shown that, even under baseline conditions, Fragile X mice have significantly larger pupils than WT mice. This is consistent with the notion that Fmr1 KO mice are in a state of

hyperarousal and this may explain why they show sensory hypersensitivity (tactile defensiveness) in response to repetitive tactile stimulation. We are excited by these novel results because they signify that hyperarousal to sensory stimuli, caused by lack of adaptation at the circuit level, is not just something that affects fragile X mice during the somewhat artificial whisker stimulation paradigm, but also during more behaviorally relevant social interactions. We believe these studies have highly significant translation potential for humans.

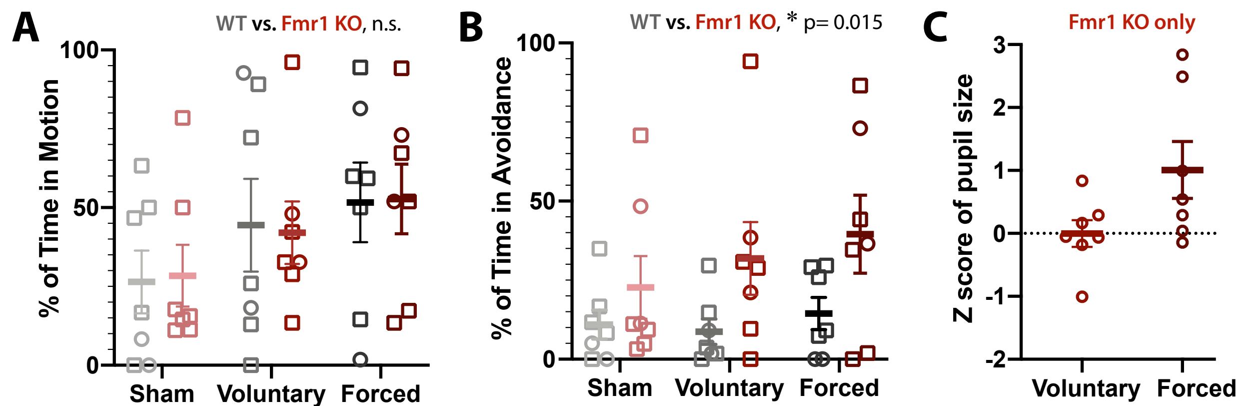

Figure 2: Fmr1 KO mice exhibit maladaptive avoidance response in social touch assay. A) The proportion of time that mice spent in motion was similar between WT and KO mice in the social touch assay, although mice of both genotypes ran more during both voluntary interactions (whisker-whisker) than during forced interactions (snout-snout) than at baseline (with no stranger mouse). **B)** In contrast, Fmr1 KO mice spent significantly more time displaying avoidance behaviors during both voluntary and forced interactions than WT mice (but no difference at baseline). **C)** Pupils were analyzed in KO mice during the social touch and we found so far that Fmr1 KO mice have more dilated pupils (assign of hyperarousal) during forced interactions than with voluntary interactions.

Subtask 4: Is the deficit in adaptation due to decreased inhibition? (Months 1-24)

The goal of this subtask was to test whether hypoactivity in subtypes of inhibitory interneurons in barrel cortex was associated with the reduced adaptation in the firing of pyramidal neurons to repetitive whisker stimulation. This was based on a hypothesis that reduced activity of parvalbumin (PV) interneurons in barrel cortex might explain why L2/3 pyramidal neurons exhibit persistently elevated firing in response to ongoing whisker stimulation. Indeed, in separate and now published studies we performed in visual cortex, we demonstrated that PV cells are abnormally hypoactive in V1 of Fmr1 KO mice, a defect that correlates with poor behavioral performance on a perceptual learning task {Goel, 2018 #2195}. We completed a detailed series of experiments using in vivo two-photon calcium imaging in PV interneurons in WT mice with the same whisker stimulation paradigm (**Fig. 3**).

Specifically, we find that PV neurons are also hypoactive in S1 cortex at postnatal day 15, and this could indeed contribute to the ack of adaptation of pyramidal neurons that we previously reported in Fmr1 KO mice {He, 2017 #2133}. On the other hand, the activity of PV neurons to repetitive whisker stimulation does not reveal that they are preferentially recruited with ongoing bouts of stimulation. Hence, it is unlikely that the progressive adaptation of L2/3 pyramidal neurons is due to a direct inhibition from PV neurons. Instead, it is likely due to a more nuanced and complex network phenomenon. In the final months of the award, we are testing whether artificially raising the activity of PV neurons in Fmr1 KO mice with activating DREADDs can restore adaptation in L2/3 pyramidal neurons and, importantly, rescue tactile defensiveness in KO mice. Although these experiments were not part of what we originally proposed, we pursued these as am important ay to address the question we originally asked: is the deficit in adaptation due to reduced inhibition?

We hope to complete those experiments in the coming months.

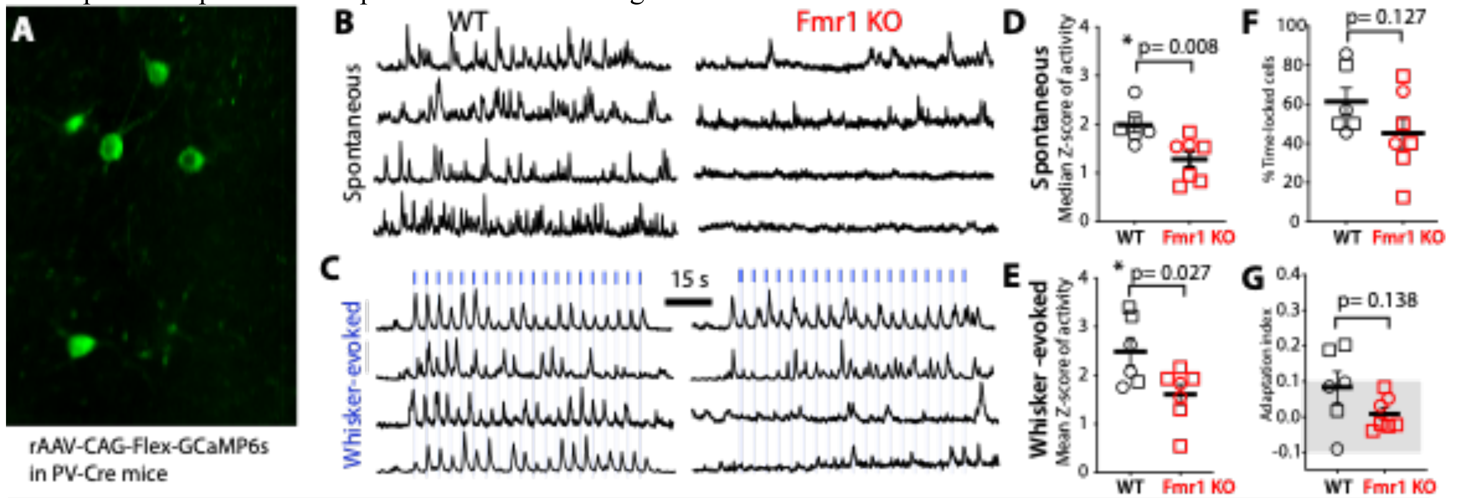

Figure 3: Calcium imaging of PV neuron activity in juvenile mice. PV-Cre mice were injected at P10 with rAAV-CAG-FLEX-GCaMP6s into barrel cortex at the time of the cranial window surgery and then PV neurons in layer 2/3 were imaged with 2P microscopy (8 Hz) at P15. **(A, B)** Representative calcium traces of spontaneous (top) and whisker evoked activity (bottom) (under chlorprothixene). **(C, D)** On average, PV neurons of Fmr1 KO mice (n= 6) had significantly lower spontaneous (C) and whisker-evoked activity (D) than those in WT mice (n= 7). Mann-Whitney tests. There were no differences in the % of PV neurons that are time-locked to epochs of whisker stimulation or in the adaption index to repetitive whisker stimulation (not shown). O: females; : males.

Aim 2 To determine the synaptic, cellular and local circuit basis for adaptation deficit in acute slices of somatosensory cortex

This Aim was performed in the Contractor laboratory at Northwestern University

Major Goals of Aim 2:

1. Determine if there are major disruptions in connectivity between neurons and interneurons in L4 of cortex of Fmr1 KO mice

2. Determine whether there are disruptions in L2/3 connectivity in Fmr1 KO mice

3. Determine whether connections from thalamus are altered in Fmr1 KO mice

4. Determine whether the dynamic properties of synapses are altered in Fmr1 KO mice

The focus over the past year has been on mapping connectivity in the different cortical layers which were the Major goals 1 and 2. To do this we used both a glutamate uncaging strategy to map the connections to neurons in L4 and L2/3 as well as using an optogenetic strategy. In the first set of experiments we made whole-cell patch clamp recordings from neurons in these layers. The extracellular solution contained caged glutamate (MNI-glutamate) which could be focally activated by photolysis by 405 nm wavelength light. Using a grid centered on the recorded neuron to uncage glutamate we are able to map synaptic responses to individual neurons and create a connectivity density map. The constructed a heat maps will be compared between WT and Fmr1 Ko animals to determine whether there are any gross alterations in

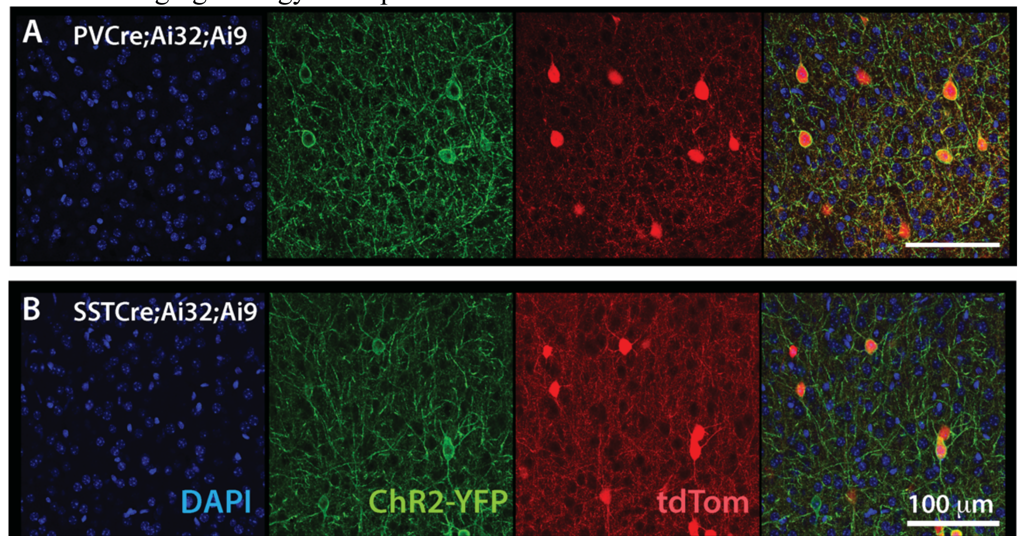

Figure 4: ChR2 and tdTom in L2/3 interneurons. (A) Image of L2/3 in PV^{Cre} mice crossed to both Ai32 (ChR2-YFP) and Ai9 (tdTom) and (B) SST^{Cre} mice crossed to Ai32 and Ai9 alleles .

the connectivity map. These experiments are ongoing and as they are performed blind to genotype we do not have the completed and compiled data set from which we will be able to establish the extent and type of disruptions in connectivity between neurons and interneurons in the cortical layers.

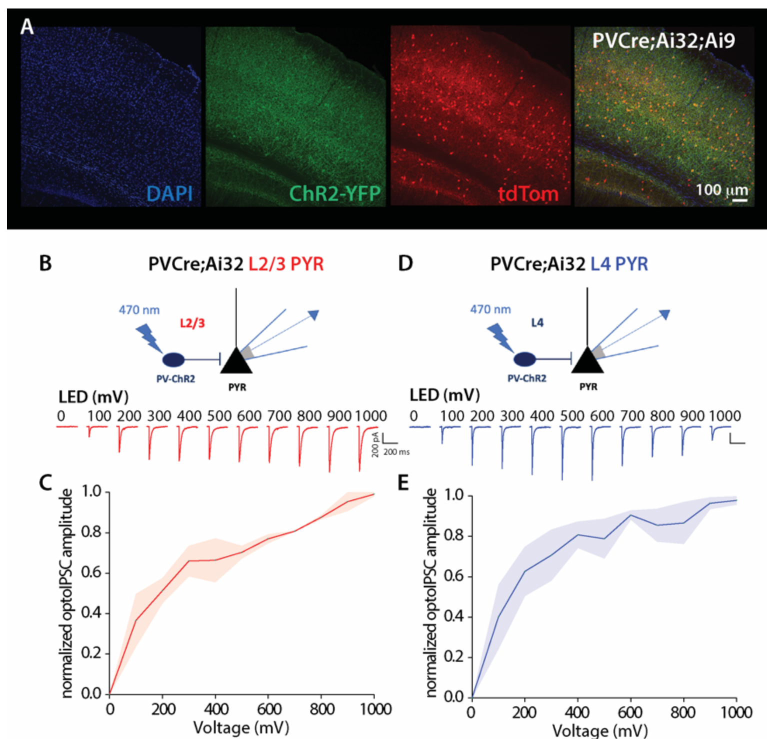

Figure 5: Photoactivation of PV interneurons recorded in excitatory neurons. (A) Expression of tdTom and ChR2 in the cortex in PVCre;Ai32;Ai9 mice (B) & (C) Recording from L2/3 PYR measuring the optoIPSCs stimulated by full field illumination of slice in PV^{Cre};Ai32 mice. D & E: Recordings from L4 PYR; example traces and grouped data. Calibration 200pA, 200ms

As a second approach to mapping connectivity (to address Major Goals 1-3) we are using an optogenetic approach. For this we have developed mice in which channelrhodopsin (ChR2) expression is limited to the two dominant neurochemically defined interneuron sub-populations parvalbumin (PV) fast spiking interneurons and somatostatin (SST) interneurons (PV^{Cre};Ai32,PV-ChR2 and SST^{Cre};Ai32,SST-ChR2). Figure 4 shows images of labeled interneurons (both tdTom and ChR2) in the cortex. As is evident selective expression of Cre in these interneurons subtypes can drive expression of fluorescent markers as well as optogenetic actuator ChR2. These mice are further crossed to Fmr1 KO mice so that a functional comparison of cortical connectivity can be made.

It has not been established precisely how connections between excitatory and inhibitory neurons are disrupted in the cortex of Fmr1 KO mice and therefore this study is the first to directly address this question. As examples of these experiments we include control recordings from these optogenetic mapping experiments. In the first we have recorded from L2/3 (Figure 5 B and C) or L4 neurons (Figure 5 D and E) and photostimulated monosynaptic GABA inputs to these neurons from connected PV neurons in the slice using full field illumination with 470nm wavelength light. Increasing the LED power causes a graded increase in the GABA response until a plateau is reached when all inputs to the recorded cell are synchronously activated (max optoIPSC). Comparison of the max optoIPSC in WT and Fmr1 KO mice is being done to establish if there are differences in the PV input to the excitatory neurons in the Fmr1 KO mouse cortex. We have also performed similar experiments in SST-ChR2 mice (Figure 6). In this case we have crossed SST^{Cre} mice to Ai9 (for tdTom expression) and Ai32 (for ChR2 expression). We again include examples of recordings from L2/3 (Figure 6 B and C) and L4 (Figure 6 D and E) neurons that demonstrate the ability to activate monosynaptic input from SST neurons in the cortical circuit. The first comparison we are making is between the amplitude of these macro current in WT and Fmr1 KO mice. This will enable us to conclude whether there are gross differences in the connectivity of the two major GABA interneurons in the cortex.

In the next set of experiments, we are asking whether individual synaptic connections between PV

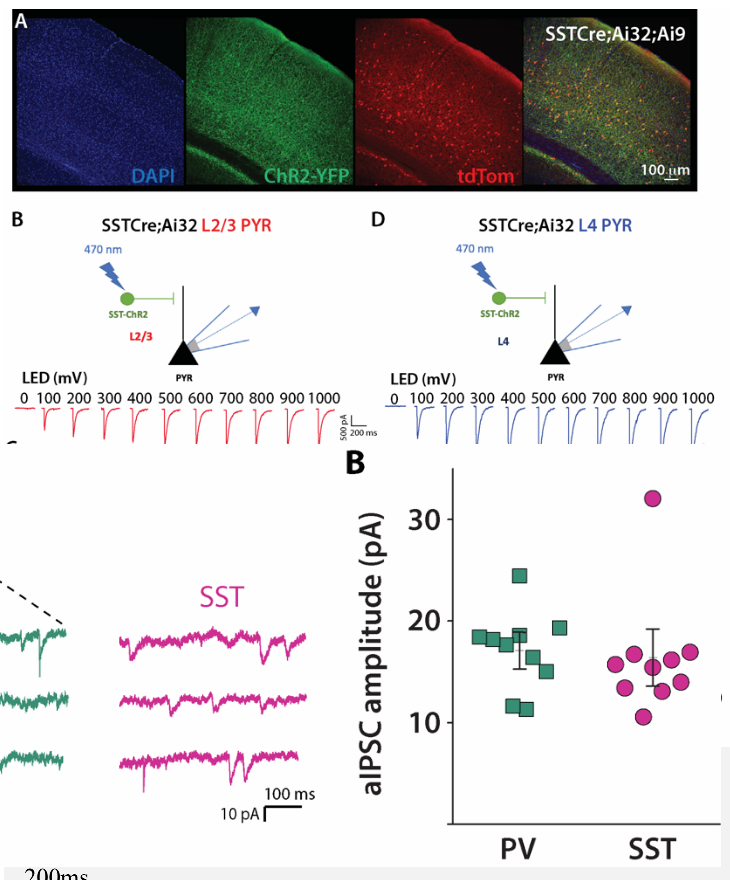

Figure 7: Desynchronizing release from PV and SST to measure quantal size. A: Macro current (optoIPSC) recorded after photoactivation of PV interneuron. Inset shows desynchronized quantal events from PV and SST interneurons. B: Average amplitude of quantal asynchronous events (aIPSCs).

and SST interneurons and cortical excitatory neurons are altered in the Fmr1 KO animals. Again we have taken an optogenetic approach using mice in which Cre is expressed in the PV or SST neurons to specifically express ChR2. By desynchronizing release from the optically stimulated interneurons using Sr^{2+} replacement of extracellular Ca^{2+} (example recordings shown in Figure 7), we are measuring the average quantal size of synapses made by PV and SST interneurons onto PYR (aIPSC), which is a measure of the synaptic potency of individual inputs. The amplitude of aIPSCs can be measured after Sr^{2+} replacement in a 500ms window after the residual synchronized current (Figure 7A). By systematically measuring the max optoIPSC and the quantal aIPSC we can calculate the number of functional inhibitory inputs from both PV and SST neurons onto individual PYR neurons in cortex of Fmr1 KO mice (number of synapses = **max optoIPSC/aIPSC**) and are comparing this to WT littermate comtrols. We are currently assessing these measures in continued experiments which will be completed during the NCE period.

The experiments outlined above are ongoing and require complicated breeding schemes with multiple crosses. As with many other labs across the world our productivity in the last year was curtailed however we are currently increasing breedings of mice to be able to complete these experiments.

What opportunities for training and professional development has the project provided?

Nothing to report

How were the results disseminated to communities of interest?

Nothing to report

What do you plan to do during the next reporting period to accomplish the goals?

In the next period of the award which is in NCE additional data acquisition will occur to complete the Aims

4. Impact:

What was the impact on the development of the principal discipline(s) of the project?

Nothing to report

What was the impact on other disciplines?

Nothing to report

What was the impact on technology transfer?

Nothing to report

What was the impact on society beyond science and technology?

Nothing to report

5. Changes/Problems:

Nothing to report

6. Products:

Publications, conference papers, and presentations:

Nomura T, Musial TF, Marshall JJ, Zhu Y, Remmers CL, Xu J, Nicholson DA, Contractor A (2017) Delayed Maturation of Fast-Spiking Interneurons Is Rectified by Activation of the TrkB Receptor in the Mouse Model of Fragile X Syndrome. J Neurosci 37:11298-11310.

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Karpf S, CT Riche, D di Carlo, A Goel, WA Zeiger, A Suresh, C Portera-Cailliau and B Jalali (2020). Spectro-temporal encoded multiphoton microscopy and fluorescence lifetime imaging at kilohertz frame rates. Nature Communications, in press

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7. Participants:

Name: Anis Contractor (Northwestern)
Project Role: PI
Researcher Identifier (e.g. ORCID ID) :
Nearest person month worked: 2.4
Contribution to Project: Overall lead for the project, provides scientific direction, mentors students and postdocs, analyses data and performs administrative duties
Funding Support: None

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Funding Support: None

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Contribution to Project: Performed experiments and analyzed data
Funding Support: None

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Researcher Identifier (e.g. ORCID ID) :
Nearest person month worked: 2.5
Contribution to Project: Performed experiments and analyzed data
Funding Support: None

Name: Yiwen Zhu (Northwestern)
Project Role: Graduate Student
Researcher Identifier (e.g. ORCID ID) :
Nearest person month worked: 12
Contribution to Project: Performed experiments and analyzed data
Funding Support: None (Complete only if the funding support is provided from other than this award.)

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Contribution to Project: Performed experiments and analyzed data
Funding Support: None

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Contribution to Project: Performed experiments and analyzed data
Funding Support: None

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Contribution to Project: Performed experiments and analyzed data
Funding Support: None

Name: Anand Suresh (UCLA)
Project Role: Postdoctoral Fellow
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Nearest person month worked: 12
Contribution to Project: Performed experiments and analyzed data
Funding Support: None

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Project Role: Postdoctoral Fellow
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 6
Contribution to Project: Performed experiments and analyzed data
Funding Support: None