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13. SUPPLEMENTARY NOTES

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

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1. INTRODUCTION:

Fragile X syndrome (FXS) is the most common inherited form of intellectual disability and the largest genetically identified cause of autism affecting roughly 1 in 2,500 males. One of the core deficits in autism, which is particularly prominent in FXS individuals, is the problem of hypersensitivity to a variety of sensory stimuli, which results in hyperarousal, anxiety and seizures. The underlying alterations in the development of neuronal circuits that are the basis for sensory problems in autism are not well defined. In this project the multi-PI team proposed to understand the circuit basis for altered sensory responses in the mouse model of FXS. Both in vivo imaging of neuronal activity as well as in vitro recording of individual neurons is proposed to map the connectivity and functional changes in the somatosensory cortex focusing on the role of GABAergic neurons. Furthermore, a strategy to alleviate these deficits by targeting the maturation of GABAergic interneurons will be employed.

2. KEYWORDS:

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

3. OVERALL PROJECT SUMMARY:

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.

4. KEY RESEARCH ACCOMPLISHMENTS:

What were the major goals of the project?

The Multi-PI proposal had three integrated aims. The SOW was divided so that Sp Aim 1 would be carried out in the Portera-Cailliau laboratory, Sp Aim 2 would be performed in the Contractor laboratory and Aim3 would be performed in both laboratories. For the first year of the award tasks in Aim 1 and Aim 2 were prioritized.

Aim1: To test whether dysfunctional inhibitory circuitry in barrel cortex causes the lack of neuronal adaptation and avoidance behaviors (tactile defensiveness) in Fmr1 KO mice

a. Determine whether tactile disturbances also manifest in response to visual stimuli

b. Determine whether increased locomotor activity in Fmr1 mice in response to sensory stimuli is an avoidance response

c. Determine whether adaptation deficit is due to altered inhibition

d. Determine whether the sensory alterations result from loss of FMRP during critical period development

Aim 2: Determine the alteration in connectivity and function of synapses in the sensory microcircuit

a. Determine whether there are disruption in the fine grain connectivity of interneuron subtypes and principal neurons in layer IV of the somatosensory cortex of Fmr1 KO mice

b Determine whether there are alteration in the connectivity of layer II/III neurons in Fmr1 KO mice

c. Determine whether the development of extrinsic connectivity from thalamus is altered in Fmr1 KO mice

d. Determine whether the dynamic properties of individual synaptic connections in the somatosensory cortex are altered in FXS mice

During this cycle of the award we have continued work on the objectives of these two aims.

Advances in these are outlined below.

What was accomplished under these goals?

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

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:

Subtask 1: Does the behavioral manifestation extend to repetitive visual stimuli? (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.

These experiments are ongoing, but at the present time we feel we will likely not be able to determine whether adaptation to visual stimuli is also reduced in Fmr1 KO mice. In order to make comparisons with our published study showing reduced adaptation of layer (L) 2/3 neurons in the primary somatosensory cortex of Fmr1 KO mice [1], we first used the same pattern of stimulation for primary visual cortex (V1), where mice passively view 20 visual stimuli, one after the other. Hence, each visual stimulus (a drifting sinusoidal grating) lasts 1 s and there is a 3 s long inter-stimulus interval between stimuli. However, there are important differences between V1 and S1 that make it harder for us to look for adaptation in V1. For starters, there are few orientation selective neurons in V1 (~30% with calcium imaging) and at best only 12.5% of those cells would be tuned to a particular direction of the 8 drifting gratings we present. Thus, the chances that we find neurons in V1 that respond to the visual stimulus we use for repetitive stimulation are fairly small (In contrast in S1, neurons that are whisker responsive seem to be generically tuned to whisker stimulation in general, so we could test dozens of neurons in any field of view). We could just image the same ensemble of neurons multiple times, testing all 8 different directions, but such prolonged imaging sessions would potentially harm the tissue. The other problem is that L2/3 neurons in V1 do not seem to show adaptation when presented with the same grating (say at 0 degrees) for 1 s, repeatedly every 3 s (not shown). On occasion, we do encounter neurons that seem to adapt over a different time scale (minutes instead of seconds), such that adaptation occurs after subsequent rounds of drifting gratings in 8 directions are presented (Fig. 1, left). And still, we find other orientation selective neurons that do not adapt (Fig, 1, right). We will continue these experiments over the next few months looking for a protocol in which we see adaptation and then will try to compare WT and *Fmr1* KO mice.

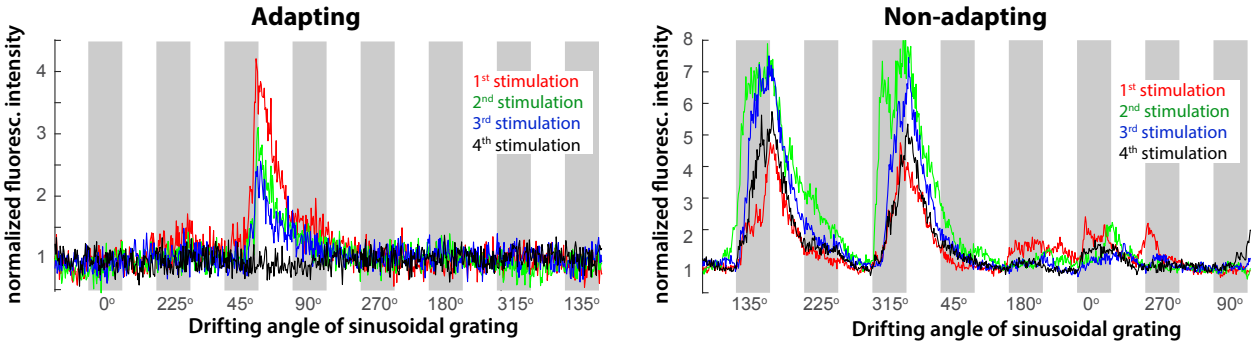


Figure 1: Visual stimulation leads to neuronal adaptation in some neurons in V1. Unanesthetized, head-restrained mice passively viewed gratings drifting in 8 different directions (3 s 'ON' / 3 s 'OFF' gray screen). **Left,** an example of an orientation selective L2/3 neuron that had progressively smaller responses to gratings drifting the 45º direction. **Right,** an example of a different orientation selective L2/3 neuron tuned to 135º and 315º that did not show any adaptation to successive presentations of these gratings.

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

In a paper we published in 2017 [1], we demonstrated that adult *Fmr1* KO mice perceive repetitive whisker stimulation as aversive, because they run preferentially away from the side of stimulation. 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 yet unpublished studies (carried out in Year 2 of funding), we now show that this maladaptive avoidance response correlates with the degree of neuronal adaptation, 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. 2**).

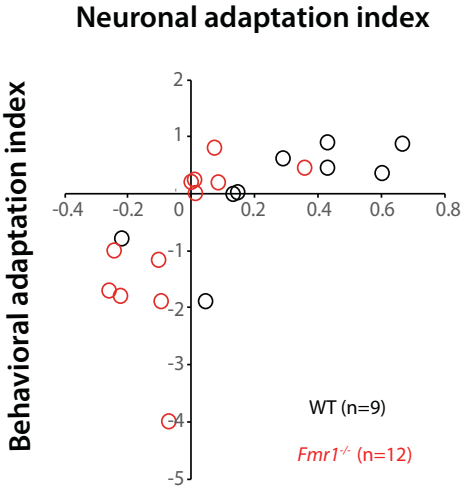
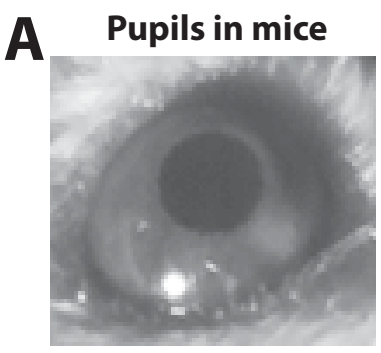


Figure 2: The degree of behavioral adaptation correlates with neuronal adaptation in L2/3. We found a significant correlation between the neuronal adaptation index (x-axis) and the behavioral adaptation index (y-axis). Positive values for neuronal adaptation indicate that firing of L2/3 neurons (as calculated 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 with the last 5 whisker stimulations than with the first 5. Note that, on average, *Fmr1*$^{-/-}$ mice showed less neuronal and less behavioral adaptation than WT mice.

We had also proposed to investigate conditional knockout mice in which Fmr1 is deleted in cortical neurons after the 2nd postnatal week. As stated in last year's progress report, we had obtained lox-STOP-lox-Fmr1 mice from at Baylor University and crossed them to the CamKII-Cre to obtain conditional Fmr1 KO mice. Unfortunately, these mice appeared to have germline recombination, as many neurons throughout the brain had no Fmr1 expression (not shown). This problem with CamKII-Cre mice has been described previously. We are in the process of finding alternatives to this approach, such as finding other Cre- driver lines for late postnatal forebrain expression.

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

The goal of this subtask 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 can be assessed and correlated in individual animals. This would allow us to track locomotion and potentially defensive forelimb movements with a camera, as well as pupil diameter with a high-speed camera, because pupil size can be used as a measure of the arousal state of the animal. As mentioned above (Subtask 2), we have been able to show that, in the same mouse, we can correlate maladaptive avoidance behaviors with the activity of pyramidal neurons in barrel cortex in WT and Fmr1 KO mice (**Fig. 3**). However, those calcium imaging recordings were conducted in sedated mice and the behavioral studies were not done at the same time. Thus, we still need to complete the experiments proposed in Subtask 3 in awake mice, which will allow us to determine whether Fmr1 KO mice show signs of anxiety and stress (e.g., dilated pupils). 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.



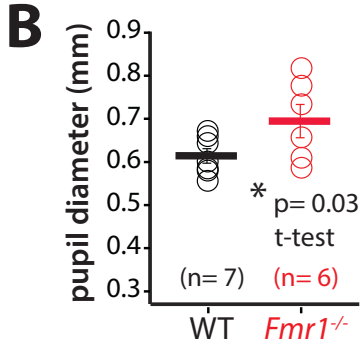


Figure 3: Larger pupils in *Fmr1*$^{-/-}$ mice. A) We monitor pupil diameter with high-speed videography at high-resolution. **B)** *Fmr1*$^{-/-}$ mice had significant larger pupils than WT mice throughout the training, a sign of hyperarousal. Interestingly, as WT mice learn the task, their pupil size increases.

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 L/23 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 [2]. We have started doing calcium imaging in PV interneurons in WT mice with the same whisker stimulation paradigm (**Fig. 4**).

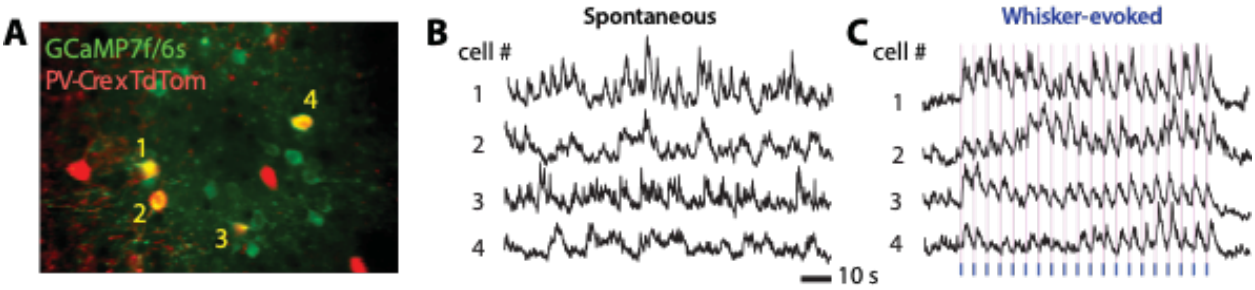


Figure 4: Calcium imaging in parvalbumin (PV) interneurons during repetitive whisker stimulation. A) 2-photon images of PV neurons expressing GCaMP7f (green) and Td-Tom (red). In this animal both a flex'd version of GCaMP7f and a regular rAAV-syn-GCaMP6s were co-injected into a PV-Cre x ai9 (TdTom) mouse, in order to visualize simultaneously pyramidal neurons and PV cells in L2.3. **B)** Representative traces of GCaMP signals for spontaneous activity in PV cells in a WT mouse. The paradigm of stimulation was the same as in our published paper (He et al., J Neurosci, 2017), with 20 sequential stimulations lasting 1 s (3 s inter-stim interval). **C)** Representative traces of GCaMP signals for whisker-evoked activity (vertical bars) in the same PV cells. Note that some cells show mild adaptation (#3), others show mild facilitation (#4) and others show neither.

These experiments will be conducted in Year 3 of the DoD grant.

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

The major goal of this Aim was to determine whether the connectivity and excitability of the major cell types in the somatosensory microcircuit was altered. As we reported last year the initial experiments suggest that there are changes in synaptic connectivity during early development.

Major Task 2: Determine the alteration in connectivity and function of synapses in the sensory microcircuit

Subtask 1: Determine whether there are disruption in the fine grain connectivity of interneuron subtypes and principal neurons in layer IV of the somatosensory cortex of Fmr1 KO mice.

In this year of the proposal we have begun to determine whether the local connectivity of the major cell types in the barrel are altered in the Fmr1KO mice. To do this we have made whole cell patch clamp recordings from either layer IV spiny stellate neurons or PV or SST interneurons which can be identified by expression of fluorescent marker. In each case the internal solution for recording was such that individual synaptic responses could be disaggregated as excitatory or inhibitory when the recorded cell was held using voltage clamp at a membrane potential of -70mV. In these recording we have begun to establish the technical parameters required to produce consistent and comparable profiles of connectivity to each of the neuronal subtypes by making comparisons across multiple slices and animals. Once a voltage clamp recording is established an extracellular solution containing a caged glutamate (MNI-glutamate) is bathed across the slice and photolysis achieved using a 405 nm laser. An uncaging stimulation grid centered on the recorded neuron and aligned to the pia is established and laser photolysis controlled by a set of uncaging galvanometer mirrors randomly over this grid. Figure 5 shows an example experiment. In Figure 5A the recording configuration is shown from one experiment in which a recording was made from a layer IV spiny stellate neuron and optical photolysis of MNI glutamate was made at points on the 16 x 16 axis. Responses recorded from direct glutamate activation of dendritic or somatic receptors on the recorded neuron were large and with short latency after stimulation (Figure 5B), In contrast the responses from depolarization of synaptically coupled neurons were small in size, had a delayed latency and kinetics that are in line with synaptically evoked responses (Figure 5B, red traces). Using this approach, we are able to construct a heat map that constitutes a connectivity map for the recorded neuron (Figure 5s) We are currently taking this approach to establish if this connectivity map is altered for any one of the cell types in layer IV (ie spiny stellate cells, PV interneurons and SST interneurons). Once a large data set of WT and KO recordings has been established these data will allow us to determine the extent of altered connectivity in the layer IV circuit in juvenile and adult *Fmr1* KO mice. Once we have completed this in the next period of the award we will work on Subtask 2 which takes a complementary approach to determine connectivity in layer II/III of the barrel cortex.

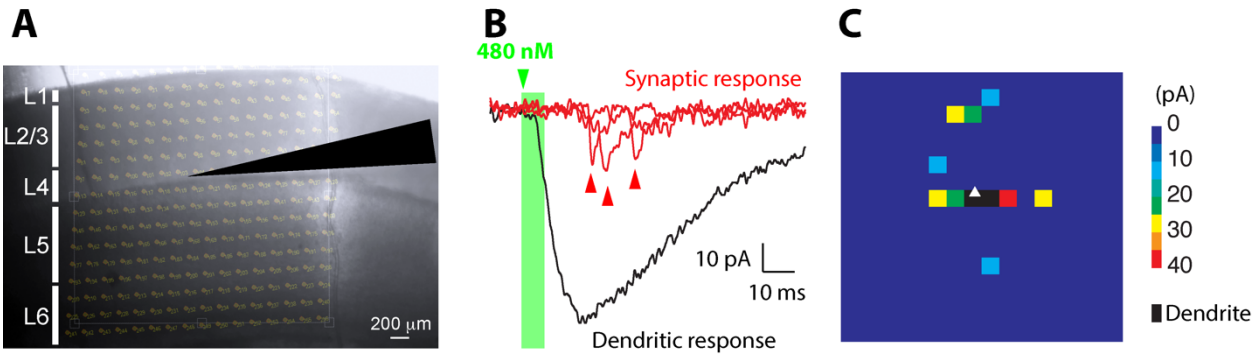


Figure 5 : Connectivity mapping by LSPS A) Recording configuration and representative image of the somatosensory cortex overlaid with a 16 x 16 grid for glutamate uncaging. Recording electrode is outlined by black shadow. Calibration; 200 µm B) Representative dendritic (black) or synaptic (red) responses evoked by glutamate uncaging. Note the longer latency of synaptic responses after light stimulation. 7 ms time window (orange shadow) is used to discriminate dendritic and synaptic responses. Calibration; 10 ms, 10 pA C) Heatmap of synaptic responses. Each pixel represents each uncaging grid. Black pixels indicate zones of dendritic responses. Soma position is shown as white triangle.

Subtask 3: Determine whether the development of extrinsic connectivity from thalamus is altered in Fmr1 KO mice. In this section we proposed to use optogenetic approaches to look at connectivity between thalamus and cortex. We expect to have these experiments completed in the next award cycle.

Subtask 4: Determine whether the dynamic properties of individual synaptic connections in the somatosensory cortex are altered in FXS mice. We have begun acquiring data for this subtask by recording from layer IV neurons and establishing how synaptic responses undergo short term plasticity during a train of activity. As yet we only have an incomplete data set and it is not clear whether deficits exist in the FXS mice, but we expect this subtask to be completes in the next year.

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 additional data acquisition will occur to complete Aims1-3

5. 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

6. PRODUCTS:

Publications, conference papers, and presentations:

He CX, EA Arroyo, DA Cantu, A Goel, and C Portera-Cailliau (2018). A versatile method for viral transfection of calcium indicators in the neonatal mouse brain. *Front Neural Circuits,* Front Neural Circuits. Jul 23

Ricard C, ED Arroyo, CX He, C Portera-Cailliau, G Lepousez, M Canepari, and D Fiole (2018) Two-photon probes for *in vivo* multicolor microscopy of the structure and signals of brain cells. *Brain Struct & Funct,* Sep; 223(7):3011-3043

Goel A, D Cantu, J Guilfoyle, GR Chaudhari, A Newadkar, B Todisco, D De Alba, N Kourdougli, LM Schmitt, E Pedapati, CA Erickson, and C Portera-Cailliau (2018). Impaired perceptual learning in a mouse model of Fragile X syndrome is mediated by parvalbumin neuron dysfunction in V1 and is reversible. *Nature Neuroscience*, 21, 1404-1411

7. PARTICIPANTS:

Name: Anis Contractor

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 (Complete only if the funding support is provided from other than this award.)

Name: Jian Xu
Project Role: Research Assistant Professor
Researcher Identifier (e.g. ORCID ID) :
Nearest person month worked: 6
Contribution to Project: Performed experiments and analyzed data
Funding Support: None (Complete only if the funding support is provided from other than this award.)

Name: Chrissy Remmers
Project Role: Graduate Student
Researcher Identifier (e.g. ORCID ID) :
Nearest person month worked: 9
Contribution to Project: Performed experiments and analyzed data
Funding Support: None(Complete only if the funding support is provided from other than this award.)

Name: Yiwen Zhu
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.)

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to report

What other organizations were involved as partners?

Nothing to report

8. REFERENCES

1. He, C.X. *et al.* Tactile Defensiveness and Impaired Adaptation of Neuronal Activity in the Fmr1 Knock-Out Mouse Model of Autism. *J Neurosci* **37**, 6475-6487 (2017).
2. Goel, A. *et al.* Impaired perceptual learning in a mouse model of Fragile X syndrome is mediated by parvalbumin neuron dysfunction and is reversible. *Nat Neurosci* **21**, 1404-1411 (2018).