CONTRIBUTION OF CHOLINERGIC DYSFUNCTION TO LEARNING DEFICITS IN FRAGILE X SYNDROME

By

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ABSTRACT

Fragile X Syndrome (FXS), a genetic mutation, is a leading cause of attention and learning related disorders such as Autism Spectrum Disorder (ASD). However, the question of how dysfunctional mechanisms contribute to symptoms related to FXS remains unclear. Prior studies have shown that mice with FXS have an increase in cholinergic signaling. Cholinergic input allows sensory responses to occur in a more reliable way, and therefore when elevated atypically can lead to hyperarousal and deficits in learning. Our research goal is to examine the circuit defects that cause an increase in cholinergic input and determine if a reduction in cholinergic signaling to the primary visual cortex (V1) will rescue visual perceptual learning. We will use *Fmr1*$^{-/-}$ knockout (KO) mice, which is an established mouse model of FXS and shares similarities in sensory and learning issues observed in humans with FXS. Wild type (WT) mice, with neurotypical brain circuits will be used as controls. We will use a genetically encoded calcium indicator in combination with two photon microscopy to examine changes in cholinergic function. We will also observe locomotion and pupil dilation of the mouse during stimulus presentation to determine arousal and engagement. Our hypothesis is that an elevation in basal levels of cholinergic input from subcortical areas to V1 in *Fmr1*$^{-/-}$ mice will prevent V1 neurons from responding selectively to visual stimuli, thus contributing to impaired learning. We also expect to see an increase in engagement and arousal as measured by locomotion and pupil diameter in *Fmr1*$^{-/-}$ mice. The results from this study will inform therapies that can be targeted to specific circuits in ASD and FXS symptoms.

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INTRODUCTION

Fragile X Syndrome (FXS) is a genetic mutation that produces symptoms similar to those seen in people with Autism Spectrum Disorder (ASD) such as hypersensitivity and difficulties learning and paying attention (Kraan et al, 2019). FXS is caused by the repeats of 200 CGG nucleotides on the *FMR1* gene on the X chromosome (Bailey et al, 2008). The overactivity of vasoactive intestinal polypeptide (VIP) neurons in the primary visual cortex due to high levels of cholinergic input from the nucleus basalis (NB) and glutamatergic input from the anterior cingulate cortex (ACC) may be the cause of a lack of attention in those with FXS. The hyperexcitability of the neural circuits associated with FXS may contribute to the cause of hypersensitivity traits in those with FXS (Contractor et al, 2016).

My project will use the well established model of *Fmr1* knockout (*Fmr1*$^{-/-}$) mice as the experimental group against the control group of wild type (WT) mice which are neurotypical. We choose FXS to model autism because it produces many symptoms that overlap with the wide spectrum of symptoms those with autism experience. FXS also does not cause major anatomical defects, which allows the integrity of the brain structure to not interfere with our results (Goel et al). The *Fmr1*$^{-/-}$ mouse model consists of genetically modified mice that exhibit hypersensitivity symptoms similar to those seen in humans with FXS. Humans with FXS have abnormal DNA nucleotide sequences in the region of DNA that is associated with the *FMR1* gene. The homolog of this gene is removed in mice to model the abnormalities in the expression of the *FMR1* gene in humans with FXS (Bakker et al 1994). Prior studies have shown that *Fmr1*$^{-/-}$ mice may have higher levels of base level cholinergic signaling based on the evidence that another similar type of knockout mice, *Lynx1* KO mice, had dysfunctionalities in cholinergic input (Falk et al, 2021).

The anterior cingulate cortex (ACC) and the nucleus basalis (NB) are known to project to the primary visual cortex (V1) and are involved in attention and arousal. Cholinergic input allows sensory responses to occur in a more reliable way, however if its levels are too high it can lead to hyperarousal and deficits in learning. Hyperarousal can be characterized by not being able to focus on one task because the brain is trying to focus on multiple stimuli at once. Cholinergic input from NB activates VIP neurons and interneurons in layer 1 of the entire cortex, which includes V1 (Alitto, 2013). VIP cells inhibit parvalbumin (PV) and somatostatin (SST) expressing neurons in the primary visual cortex, which ultimately leads to the activation of pyramidal neurons through the inhibition of the inhibitory neurons. Vasoactive intestinal polypeptide (VIP) neurons are found in V1 and they influence task engagement and learning through their activation or deactivation (Lundberg et al, 1980). The activation of these neurons suggests that learning is occurring and one is building connections to improve upon their mistakes. The dysfunction of cholinergic input from NB in individuals with FXS is predicted to cause a ceiling effect in which base levels of VIP cell activity is increased to the point where further modulation will not change the activity of the circuit. The ACC provides inputs to V1 that are known to help modulate the processing of visual stimuli and behavior of the individual (Sidorov et al, 2020). The glutamatergic input from the ACC to V1 also impacts the glutamatergic circuit of VIP, PV, and SST neurons in V1 by activating VIP neurons to lead to reduced disynaptic inhibition of pyramidal neurons by PV and SST neurons (Zhang et al, 2014). We predict that the input from ACC to V1 will be decreased in individuals with FXS, causing reduced selectivity of the pyramidal cells due to disruption in inhibition.

Along with neural circuit level changes, individuals with Fragile X Syndrome also exhibit changes in behavior. In a previous study observing the behavior of *Fmr1*$^{-/-}$ mice while

performing a water maze task, the mice exhibited levels of learning below that of wild type mice and displayed deficits in motor performance (D'Hooge et al, 1997). Based on this prior research we will observe locomotion levels of the experimental mice from both groups during gray screen presentation, visual stimulus presentation, and auditory stimulus presentation. This aspect of behavior is significant to analyze because altered locomotion can be debilitating to individuals with FXS, making the finding of a therapeutic for the condition more urgent. We will observe the locomotion behaviors of the mouse to record activity levels and observe pupil dilation to record arousal during the experiments. We expect to see that the *Fmr1*$^{-/-}$ mice will exhibit increased levels of locomotion and pupil diameter during the visual and auditory stimuli. We expect to see increased levels of pupil diameter because hyperarousal is a commonly observed phenotype in Fragile X Syndrome, varying in levels of severity but still prominent in disrupting attention and learning (Lachiewicz et al, 2023). We also expect to see that the high levels of arousal in *Fmr1*$^{-/-}$ mice remain relatively constant during the experiment compared to WT mice.

METHODS

Experimental Animals

All experiments were conducted at University of California, Riverside and followed the guidelines set by the Institutional Animal Care and Use Committee under an animal use protocol (IACUC #95). The experimental animals included adult FVB wild type (WT) and *Fmr1*$^{-/-}$ mice between the ages of 8 to 16 weeks. The mice were housed in a vivarium with a 12 hour on and off light cycle and the experiments were conducted during the light cycle at the same time during each experimental day. We choose FXS and *Fmr1*$^{-/-}$ to model autism because it produces many symptoms that overlap with the wide spectrum of human symptoms. FXS also does not cause major anatomical defects, which allows the integrity of the brain structure to not interfere with our results (Kazdoba et al, 2014). This allows us to both behaviorally and neurally analyze FXS in a mouse model to determine which deficits are present.

Surgical Procedure and Viral Injections

All experimental mice underwent a craniotomy and headbar procedure. Mice were anesthetized using 5% isoflurane for induction and 1.5-2% isoflurane for sustained anesthesia during the procedure. The anesthetized mice were head restrained to a stereotaxic frame and sterile conditions were maintained during the procedure. The mice were placed on a heating blanket set to 38 C during the procedure to help the mouse maintain their body temperature. Toe pinches were used to ensure the mouse was fully anesthetized before proceeding with the surgery. After exposing the skull and scraping away the periosteum, a burr hole was drilled at 0.3 mm anterior and 0.5 mm lateral to Bregma to target the anterior cingulate cortex (ACC) for one group of mice. For another group of mice, a burr hole was drilled at 0.5 mm posterior and 1.8

mm lateral to Bregma to target the nucleus basalis (NB). In their respective burr holes, ~180 nanoliters of pAAV9-hSynapsin1-axon- GCaMP8s-P2A-mRuby3 (Addgene) were injected at a depth of 0.9 mm to target the ACC and a depth of 3.8 mm to target NB. Both injections were done using a nanoliter injector called the Nanoject (Drummond Scientific). A 4 mm diameter cranial window was drilled over the primary visual cortex (V1) and a glass coverslip was placed over the window and secured using cyanoacrylate glue and dental cement. A U-shaped aluminum head bar was attached to the skull using cyanoacrylate glue and secured using dental cement. The mice were placed in a recovery cage and were observed for 30 minutes after the procedure to ensure that the effects of the anesthesia had worn off and that the mouse was able to move coherently. After the procedure, 0.053 mL of 0.3 mg/mL buprenorphine and 0.04 mL of 2 mg/mL dexamethasone were administered subcutaneously to all mice on the day of the surgery and every 24 hours for the two days after the surgery as analgesics and anti-inflammatories to help with the recovery of the cranial window.

Handling and Habituation

Beginning 2 weeks after the craniotomy procedure, the mice underwent a handling and habituation phase to prepare them for the imaging protocol. The mice were handled by the experimenter for 5 minutes at a time for 3-5 days depending on the behavior and docility of the mouse and how quickly it is acclimated to the experimenter. Handling consisted of scooping up the mouse and allowing the mouse to walk freely on the experimenter's gloved hands for 5 minutes at a time. The mice were then habituated to the imaging setup for 3 days during which the mouse was head restrained and placed on an air-suspended ball to allow the mice to run freely while holding their head in place. On day one of habituation, the mice were placed on the

air-suspended ball for 15 minutes with only the red light flashlights on and a large fabric cover placed between the mice and the experimenter so the mice were not distracted by the presence of a human. On day two of habituation, the mice were subject to the same conditions as day one, however we added the 20x magnification objective above the cranial window and turned on a visual stimulus on the screen in front of the mouse for the last 5 minutes of the habituation session. On day three of habituation, the mice were under the same conditions as day two except ultrasound gel was added on top of the cranial window and the 20x objective was lowered to be almost touching the cranial window as the setup would be during the experiment. The objective and its interface with the window was wrapped in tape and fabric to prevent light from entering the area and affecting the imaging results.

In Vivo 2-Photon Calcium Imaging and Visual Stimulus

After handling and habituation have been completed, the mouse is ready to be imaged under a 2-photon microscope (Hyperscope by Scientifica). At this point, a minimum of three weeks must have elapsed since the surgical procedure, allowing the mouse and cranial window ample time to recover from the procedure and to allow for expression of the viral construct to begin. Before beginning the imaging session, the clarity of the cranial window is visually inspected to ensure that inflammation or bone growth is not present in high levels as they would introduce noise into the imaging capture. It is imperative that noise be minimized in order to receive accurate results for fluorescence changes of the axon terminals. The mice were placed on the same imaging setup that they were habituated to and videos of axon terminals in the window above the primary visual cortex were collected using ScanImage. The videos were collected while the mouse viewed a gray screen, a visual grating, and listened to only an auditory stimulus.

The visual stimulus consisted of sinusoidal gratings of multiple orientations (0, 45, 135, 180 degrees). The auditory stimulus consisted of 20 trials of monotone sounds played at 90 decibels with an on time of 1.5 seconds and an off time of 0.1 seconds between each sound. During the imaging process the mice are allowed to run freely on the polystyrene ball, therefore videos of the locomotion of the mouse were also collected using Logitech cameras during the imaging acquisition to account for activity in the axon terminals that may have occurred due to movement. The mouse's pupils were also recorded using the Basler camera to monitor movement and dilation of the pupil, which could also contribute to activity V1.

Data Processing and Analysis

Videos collected using ScanImage were further processed using MATLAB software to motion correct the video in the x and y planes. The motion corrected videos were then processed by the Suite2p software in order to extract the traces for fluorescence in the axon terminals during the image capture. We selected regions of interest (ROIs) based on the general trace in order to perform quality control and make sure that we were not including noise in the data analysis. We quantified the fluorescence of each ROI for each frame of imaging capture using MATLAB software. We then calculated the mean activity and modulation index of the axon terminals. The mean activity was the average fluorescence of each axon terminal during the time that the stimulus was presented. The modulation index was calculated using the following equation:

$$\textit{modulation index} = \textit{(activity during visual stimuli) / (baseline activity)}$$

The modulation index allows us to take the baseline activity into account and focus on the changes of modulations in mean activity to determine if there was an increase or decrease in

activity upon stimulus presentation. Videos collected of the mouse locomotion were analyzed using OpenCV software in order to determine the velocity of the mouse movement on the polystyrene ball. The script calculated the speed of the mouse by tracking the movement of black dots on the polystyrene ball and determining the velocity of said dots. The automatic detection of the black dots was controlled by measuring the velocity of dots that were unobstructed by the mouse in order to prevent phantom ROIs from being detected and corrupting our data. Plots were generated of the velocity of the ball during every 4 frames of capture in order to see how the locomotion of the mouse changes with stimulus presentation. We chose to use every 4th frame to allow the locomotion video which was filmed in 60 frames per second to line up more with the imaging capture, which had a frame rate of about 15.2 frames per second. Images of the pupil diameter were also analyzed using OpenCV software. Plots were generated of the pupil diameter during the entirety of the imaging capture and then it was determined at which time points the stimulus was presented in order to create an average pupil diameter during the stimulus presentation. The averages were calculated for each of the four orientations in the visual stimulus and compared the pupil diameter between *Fmr1*$^{-/-}$ mice and WT mice.

RESULTS

Neural Activity of the ACC to V1 Projections in Neurotypical circuits in response to Visual and Auditory Stimuli

To examine the neural activity of the ACC to V1 inputs in response to visual and auditory stimuli, the cranial windows in WT mice (n=1) were imaged during stimulus presentation. Prior to the imaging sessions the mice had not been exposed to the visual or auditory stimuli, allowing for habituation to the imaging set up but not to the distractors. The naive mice passively viewed the visual gratings and listened to the auditory stimulus in the experimental condition. In addition we collected data during the visual stimulus presentation that consisted of a gray screen. This served as a control for the stimulus evoked activity. Based on the traces of fluorescence for each frame of imaging capture, we clearly observed changes in activity during the presentation of both the visual and auditory stimuli (Figures 1a and 1b). These modulations suggest that ACC is activated during both visual and auditory stimulus presentations. ACC plays a role in sensory processing for visual and auditory stimuli.

Using Suite2p software the fluorescence of each axon terminal (N= 53 axon terminals) was extracted during the course of the entire imaging trial. Then the average fluorescence of the axon terminal only during the presentation of the visual stimulus was calculated. The average fluorescence change suggested mean changes in activity and were calculated for each stimulus. (Figure 1c). The activity for the gray screen presentation has a higher spread than that for the visual and auditory stimuli. This indicates that the auditory and visual stimuli produced more coherent and synchronized inputs from the ACC to V1. We also calculated the modulation index for each axon terminal during visual stimulus presentation. A modulation index above 1 indicates

that the presentation of the visual stimulus led to an increase in activity that was different from baseline activity.

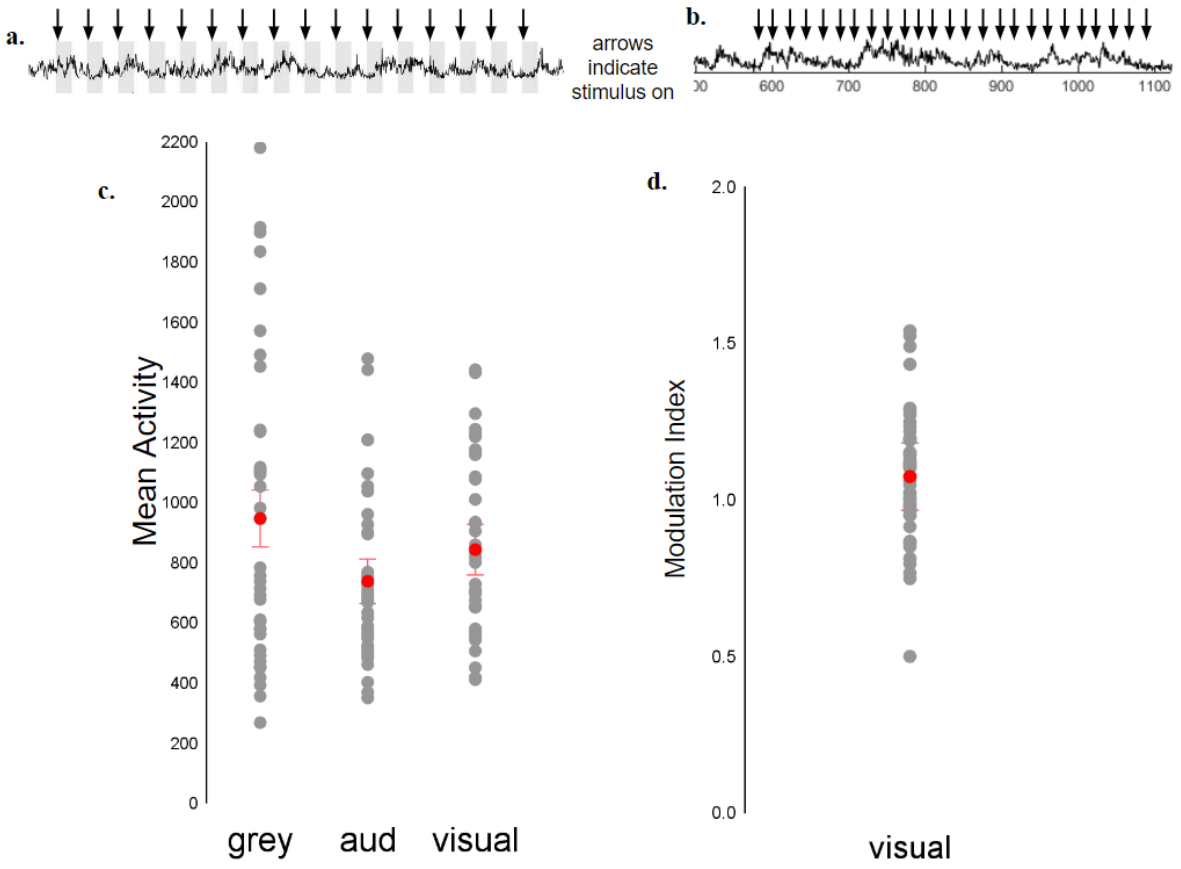

Figure 1. a. Sample trace of axon terminal activity from ACC input during visual stimulus presentation. **b.** Sample trace of axon terminal activity from ACC input during auditory stimulus presentation. **c.** Mean activity of each axon terminal during stimulus presentation measured by raw fluorescence. **d.** Modulation index of axon terminal activity during visual stimulus presentation.

Comparison of NB to V1 Axonal Activity in Response to Visual and Auditory Stimuli

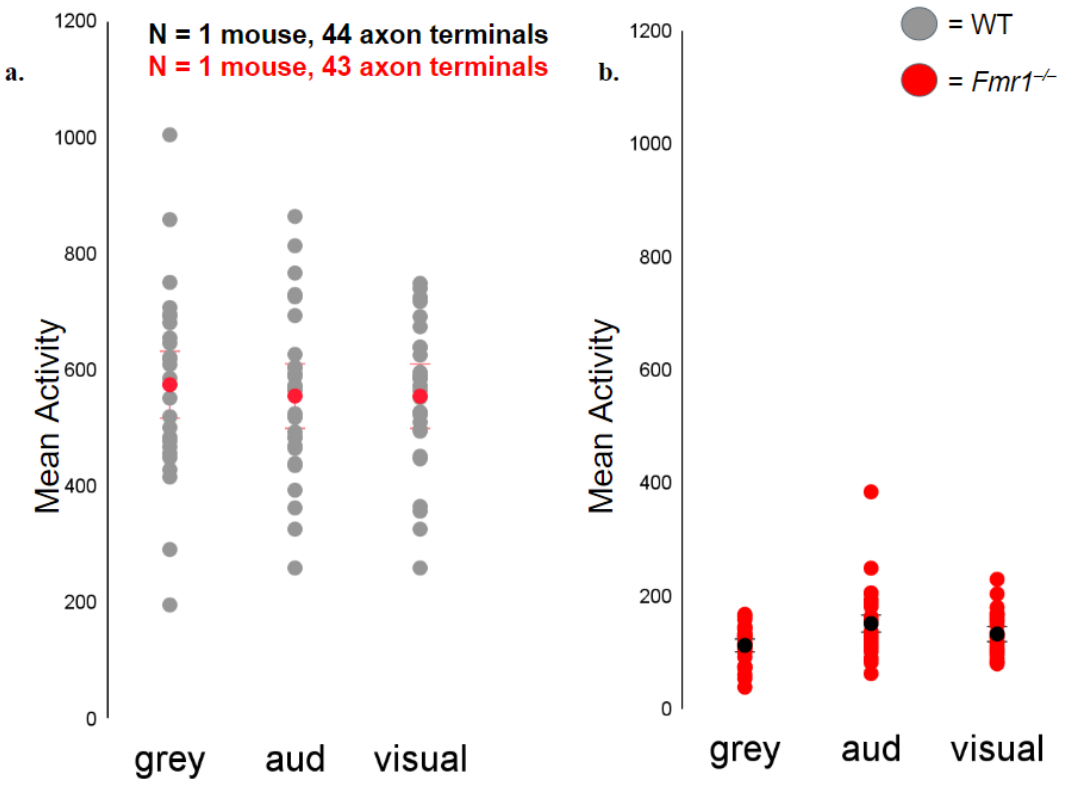

Figure 2. a. Mean activity of each axon terminal during stimulus presentation for WT mice. **b.** Mean activity of each axon terminal during stimulus presentation for *Fmr1*$^{-/-}$ mice

The mean activity during stimulus presentation was calculated by determining the average fluorescence change when the stimulus presented. Figure 2a shows the mean activity in a WT mouse with N= 44 axon terminals. Figure 1b shows the mean activity in a *Fmr1*$^{-/-}$ mouse with N= 43 axon terminals. Based on the raw fluorescence data, the *Fmr1*$^{-/-}$ mouse exhibits significantly decreased levels of mean activity when compared to the WT mouse. This decrease in activity during stimulus presentation indicates a hypofunction in NB→V1 input in *Fmr1*$^{-/-}$ mice. The spread of data between gray screen presentation and auditory and visual stimulus presentation for the *Fmr1*$^{-/-}$ mice are similar, supporting the idea that there is a ceiling effect of

NB input. This can cause a decrease in the ability to increase activity as it has already reached maximum levels.

Changes in Locomotion in Response to Stimuli

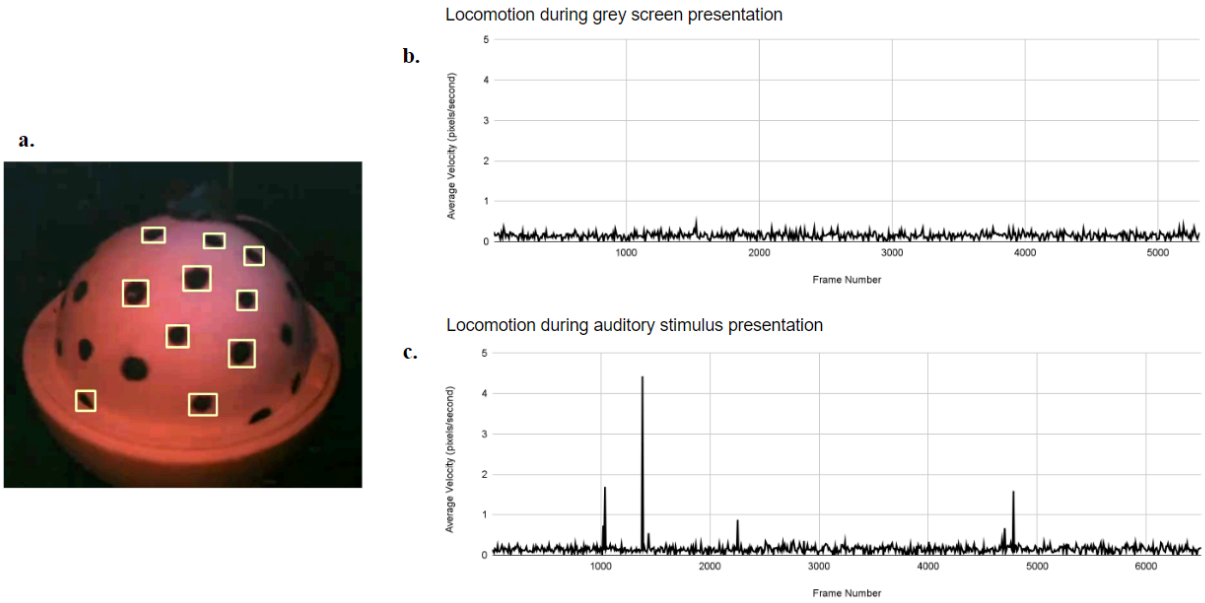

Figure 3. a. Sample frame from locomotion tracking software. Velocity of boxes across video used to measure locomotion of mouse on the polystyrene ball. **b.** Average velocity of ball per frame of imaging capture during grey screen presentation. **c.** Average velocity of ball per frame of imaging capture during auditory stimulus presentation.

We tracked the locomotion levels of the ball by labeling the polystyrene ball upon which the mouse ran with black dots. These black dots are tracked using OpenCV software that selects the black dot as an ROI and tracks its movement per frame of imaging capture (Figure 3a). The average of each ROI's velocity per frame was calculated to give us the average velocity of the mouse per frame. We plotted the average velocity for each frame of imaging capture (Figures 3b and 3c). We can see that during the gray screen presentation, the WT mouse exhibits a relatively constant level of locomotion throughout the imaging capture. However, during the presentation of the auditory stimulus, there are spikes in locomotion that align with the onset of stimulus

presentation. This suggests that locomotion is impacted by stimulus presentation and may play a role in the levels of mean activity in the neural circuits examined.

Changes in Pupil Diameter in Response to Stimuli

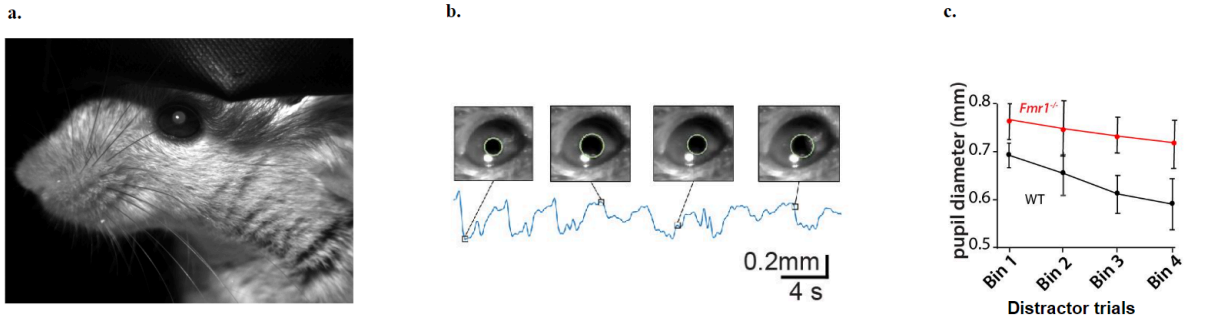

Figure 4. a. Sample frame of capture from Basler camera used to record pupil dilation and movement. **b.** Sample trace of pupil diameter during stimulus presentation along with close-up images of pupil tracking and diameter measurement. **c.** Comparison of pupil diameter during distractor task between *Fmr1*$^{-/-}$ and WT mice with each bin representing 25 trials.

We measured pupil diameter by recording videos of the mouse's eye during visual stimulus presentation (Figure 4a). Pupil diameter can be used as a measure of arousal in the mouse, with increased pupil diameter meaning increased levels of arousal. OpenCV software was used to track the pupil diameter for each frame of imaging capture (Figure 4b). The pupil diameter was averaged during the distractor task in which mice viewed the visual stimulus with random intervals of auditory stimuli. We plotted the data for average pupil diameter for every 25 trials of the distractor task (Figure 4c). From this plot we can see that the *Fmr1*$^{-/-}$ mice exhibited higher levels of pupil dilation overall as opposed to the WT mice. We also see that pupil diameter decreased over time in the WT mice whereas the pupil diameter remained higher for the *Fmr1*$^{-/-}$ mice. This supports the hypothesis that *Fmr1*$^{-/-}$ mice experience hyperarousal in response to visual stimuli.

DISCUSSION

Auditory and visual distractors are present in our day to day life, whether it be the sound of a car alarm or the flash of a camera. While these stimuli can be ignored by neurotypical individuals, they can be overwhelming for those with Fragile X Syndrome (FXS). These FXS individuals exhibit hypersensitivity to visual and auditory stimuli which can make sights and sounds more distracting than they are to neurotypical individuals (Contractor et al, 2016). This project is to further investigate the deficits in neural circuitry that can lead to altered behavior and sensory perception in individuals with Fragile X Syndrome. We found that *Fmr1*$^{-/-}$ mice exhibit decreased levels of neural activity in response to visual stimuli. We also found that *Fmr1*$^{-/-}$ mice exhibit higher levels of arousal during a visual stimulus task as measured by pupil diameter.

As with all experiments, our experimental design was rigorous. However, there are some changes in experimental designs that can improve our interpretation of the data. Firstly, in our experiments, we did not selectively examine NB→V1 input that is cholinergic. Our premise is that hyperarousal and hypersensitivity in FXS is due to disruption in cholinergic input. Currently, our data is the collective change in all NB→V1 input. In the future we will use ChAT-cre mice to selectively examine cholinergic NB→V1 input. Secondly, the amount of virus injected can affect expression levels in V1. The amount of viral construct injected into the ACC and NB could have influenced the overall fluorescence of the axon terminals in V1 by saturating or not saturating the cells enough. We will normalize the number of visible axon terminals to the signal obtained from soma in NB and ACC. Third, it is important to compare mice that have been housed in similar conditions, to avoid any effects of social isolation. Mice that were weaned alone may exhibit more spontaneous behaviors due to lack of social interaction. This can make

WT mice more similar to the *Fmr1*$^{-/-}$ mice, which can impact locomotion and behavioral data for a genotypically WT mouse. Fourthly, a further lack of FMRP caused by the knockout of the *Fmr1* protein in our mouse model might affect the expression of the viral construct GCamp. Finally, to make these findings robust and rigorous we need to increase the sample size and perform detailed statistical analysis. Thus these are future experiments that will improve the rigor of our data.

The data from this project can be used to inform further research into the neural circuitry of Fragile X Syndrome. In order to fine tune the findings of this project, the next step would be to use ChATCre mice to examine exclusively cholinergic input. The Cre dependent mouse model causes specifically cholinergic neurons to be labeled and express the tdTomato protein, allowing us to identify only cholinergic inputs from NB to V1 (Beebe et al, 2020). This will allow us to get a more accurate representation of cholinergic input because the overall input may be interfered with by the activity of other neurotransmitters.

Further to really establish causality of the ACC and NB in change in V1 neural dynamics, ACC and NB can be optogenetically or chemogenetically inhibited in WT mice and the activity of VIP cells can be measured in V1. We should expect to see that disruption of the ACC and NB will produce results in WT mice that align more closely with those of *Fmr1*$^{-/-}$ mice. These findings would be significant in understanding if a neurological therapy that targets either the ACC or NB will be effective in reducing the symptoms of FXS.

The imaging sessions performed in this project were all passive sessions in which the mouse did not perform a task to receive a reward. The next step would be to train the mouse to a visual discrimination task in which there is a preferred and non-preferred stimulus upon which the mouse will receive water as a reward. During this task, we can observe the neural activity of

NB inputs to V1 to further our understanding of how NB is involved in attention and reward in response to visual stimuli. This will allow us to further understand the underlying mechanism of the neural circuit and make inferences about how the modulation of NB can impact behavior.

In order to further modulate the ACC and NB inputs we can examine the effects of drugs that are used for treating Attention Deficit Hyperactivity Disorder (ADHD) on *Fmr1*$^{-/-}$ mice. We expect to see that these drugs will rescue atypical behavior in *Fmr1*$^{-/-}$ mice and make their behavior more similar to that of WT mice. The locomotion and pupillometry assays that we have established in this project will allow measurement of arousal and engagements and the role of arousal on neural activity change. We can also examine the inputs from the ACC and NB after the drugs have been administered to understand the mechanism of how these drugs impact this neural circuit.

Research into FXS is significant because it is a condition that impacts many on a day to day basis. The presence of certain stimuli can be overwhelming to those with FXS and impact their social behaviors. Understanding the changes in neural circuitry caused by this mutation can help better inform patients and providers on potential therapies for FXS. As demonstrated by this project, FXS can have behavioral and neurological impacts, therefore the therapies used to treat this condition, if targeted to specific circuits, will likely provide more effective solutions for distractibility.

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