# ANTERIOR CINGULATE CORTEX DYSFUNCTION IN FRAGILE X SYNDROME

By

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ABSTRACT

Fragile X syndrome (FXS) is often accompanied by sensory hyperarousal and hypersensitivity, leading to cognitive impairments in attention, learning, and memory. To better understand the neural mechanisms underlying hypersensitivity to sensory stimuli, we investigated how these atypical sensory processes influence goal-directed behavior by using an animal model of FXS- *Fmr1* Knockout (*FMR1* KO) mice. Compared to wild-type (WT) mice, *Fmr1* KO mice displayed greater vulnerability to distracting auditory and visual stimuli when performing the same visual discrimination task, suggesting hypersensitivity and inability to ignore distractors. Prior studies have found that the anterior cingulate cortex (ACC) is involved in increasing cortical responses to behaviorally relevant information (Zhang et al, 2014; Fiser et al., 2016; Norman et al. 2021). We propose that dysfunction in inputs from ACC→V1 may contribute to the ability to ignore sensory distractors and selectively attend to behaviorally relevant stimuli in *Fmr1 KO* mice. This is supported by in vivo two-photon calcium imaging of ACC axon terminals in V1, which shows reduced modulation of ACC→V1 input during distractor susceptibility in *Fmr1* KO. Inactivation of ACC prevents WT mice from overcoming distractors and elevating ACC function using Methylphenidate showed trends for reduced distractibility in *Fmr1* KO mice. Identifying disruptions in these long-range inputs to V1 will provide knowledge about the mechanistic understanding of sensory hypersensitivity in a range of neurodevelopmental disorders.

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# INTRODUCTION

Fragile X Syndrome (FXS) is a sex-linked genetic disorder—on the X chromosome—due to the absence of the FMRP protein, which is crucial for brain development. Individuals with FXS tend to have a deficiency in learning, memorizing, and hypersensitivity to sensory stimuli (Theodore et al, 2023). Moreover, boys are twice as likely to be diagnosed with FXS compared to girls, but women are more likely to be carriers of FXS because they obtain a double X chromosome (Theodore et al, 2023). There are programs made to help people with FXS, such as the Fragile X Program in New South Wales (Turner et al. 1997). The Fragile X Program was a form of informing and educating others about FXS; for instance, the researchers would ask parents for consent to test their child on FXS via a blood sample, and then they would inform the parents on how to proceed and what are the best methods (Turner et al. 1997).

Such programs are not the only way to support people and families with FXS; interventions are also encouraged. Interventions, such as the Head Sprout Early Reading Program, help the child find strategies and adjust to the different ways of learning (Theodore et al, 2023). Furthermore, these intervention programs work best when the child is young because they can easily adapt, learn, and grow with these strategies as they get older; whereas it might be harder for them to adjust when they become more mature. Therefore, it is important to attempt and try to understand what is occurring in the brains of individuals with FXS while they are learning.

Based on studies, FXS may be due to dysfunction in anterior cingulate cortex (ACC), a region in the frontal lobe. The ACC is crucial for decision-making and reward positivity—it is activated during reward delivery but also during errors, which highlights how the ACC guides action selection for certain actions (Umemoto et al., 2017). Moreover, some evidence suggests

that the reward signals correlate with continuing to do the action that gives a reward (Umemoto et al., 2017). ACC sends robust inputs to the primary visual cortex (V1). In V1, it activates multiple interneurons, including vasoactive intestinal polypeptide (VIP) interneurons. We have recently identified disruption in VIP cells in a mouse model of FXS. Although it remains to be tested whether ACC→V1-VIP dysfunction impairs learning and distractibility in FXS.

Many experimental studies have used an FXS mouse model to study the effects of Fragile X Syndrome in the brain. The mice being used have the *Fmr1* gene knocked out; thus, they lack the resulting FMRP protein. Mouse models are used so invasive neural recordings can be coupled with behavior and then resulting insights inform human symptoms. Using a mouse model for FXS (*Fmr1* KO mouse) we examined if vasoactive intestinal polypeptide (VIP) neurons play a significant role in hypersensitivity to sensory stimuli. We were able to implement analogous tasks in humans with FXS (Goel et al., 2018). In this experiment, it has been found that in *Fmr1* KO mice, compared to wild-type mice, VIP neurons did not modulate in error trials as much (Rahmatullah et al., 2023). We also found that Pyramidal cell selectivity was disrupted resulting in distractor susceptibility (Rahmatullah et al., 2023).

Rahmatullah et al. also investigated *Fmr1*knockout mice to be a model for Fragile X Syndrome (Rahmatullah et al., 2023). The research team used a go/no go task and two-photon calcium imaging and found that *Fmr1* knockout mice had impaired visual discrimination compared to wild-type mice (Rahmatullah et al., 2023). In the end, our work suggested that there might be a way to manipulate inhibition to help with sensory processing in Fragile X syndrome.

Methylphenidate (MPH) is commonly used and known as Ritalin, which is a prescribed drug for people with attention-deficit/hyperactivity disorder (ADHD). MPH blocks dopamine transporters, which in turn stops the reuptake of dopamine into the presynaptic neuron (Gotlieb,

2001). This allows for dopamine to stay in the synaptic cleft for a longer time, which increases attention and decreases attention to distractions (Gotlieb, 2001). It has also been found that methylphenidate can increase motor activity due to the larger amount of dopamine present in the synaptic cleft (Wrenn et al., 2015). This suggests that dopamine levels in individuals with FXS are low and this reduction in dopamine might affect attention and inability to overcome distractors. Importantly, the effects of this drug on the cells and neural circuits are unknown. Therefore, we wanted to investigate if MPH administration in *Fmr1* KO mice can rescue delayed learning and attention deficits.

ACC has been shown to be important in attentive tasks and elevating behaviorally relevant neural responses. Further, we see an elevation in ACC→V1 activity during the distractor task. To show that indeed ACC was required to overcome the distractor challenge, we used Muscimol to inactivate ACC and see if that manipulation prevents WT mice from overcoming distractors. Muscimol binds to GABAA receptors and promotes inhibition (Beaumont et al., 1978). Therefore, there would not be many signals being transported from the ACC to VIP interneurons to activate the other downstream neurons—STT, PV, and pyramidal neurons—that promote learning. The reason to test this drug is to examine whether inactivating the ACC is an important part of the learning and attention process. If there is a decline in the attention or learning of the mouse, then we can assume that the ACC is important for learning and attention. Then, these insights can be used to target therapeutic interventions in humans with FXS.

Based on preliminary data, the goal will be to enhance ACC to V1 inputs using Methylphenidate to improve visual discrimination performance, and susceptibility to distractors, in FXS. From this experiment, we can find out, specifically, what neurological pathways need more assistance, and could potentially find a drug to help those with Fragile X Syndrome. In this

field of study, we are focusing on how we can improve sending signals from the ACC to interneurons to aid learning and memory.

## METHODS

### *Experimental Animals*

In this experiment, the mice that were used were wild-type (WT) mice, which are normal functioning mice, and *Fmr1* Knockout (KO) mice, which are mice that model FXS. The mice will undergo different stages during the experiment: surgery, habituation, pretraining, task, distractor task, and control task. Overall, the mice will be on top of a Styrofoam ball in front of a screen, which displays different visual discrimination tasks. The mice will be stable on the Styrofoam ball with a headrest that will be placed comfortably on them. In front of them will be a licking port that only dispenses water as a reward for a specific visual discrimination task.

In terms of approvals, the IACUC does checks with our PI and the rest of the lab members need to complete certain trainings and requirements from the IACUC to work in Dr. Goel's lab. These pieces of training consist of attaining AALAS certification through AALAS training, Animal Handler's and User's Medical Questionnaire, and video training on how to handle mice.

### *Surgical Procedure*

Around 6 to 8 weeks of age, the mice undergo surgery by the lab's graduate students. First, the mice were anesthetized by being put in the induction chamber with 5% isoflurane. Then, after they were fully anesthetized, they were moved onto a stereotaxic frame with a nose cone, which gave the mouse 1.5% isoflurane for maintenance, while being on top of a heat blanket (38 degrees Celsius) to keep the mice at body temperature. Once the skull was exposed, a

U-shaped aluminum bar was placed using dental cement to be able to head restrain the mice for behavioral tasks in the future. Once the head bar is placed, the mice undergo a week of post-operative care to fully recover.

*Handling and Habituation*

Approximately a week after the surgery, the experimenter will begin to handle the mice so they can get used to the experimenter. The handling stage is about three to five days, depending on how comfortable the experimenter and mice are with one another. During days one and two, the experimenter would put their hand inside their cage for five minutes to allow the mice to sniff them. For days three through five, the experimenter will scoop the mice onto their hand and hold them for five minutes. As the mice are in the experimenter's hands, they try to have the mice tread from one hand to the other to get them used to listening to different commands. Moreover, the experimenter will give the mice a sunflower seed at the end of each handling session as a reward.

After the handling phase, the mice begin the habituation phase and their water deprivation. The habituation phase is a three-day phase that lasts for 15 minutes, and after each habituation session, the mice are given about 1-2mL of water based on the percentage of weight loss. On day one, the mice are brought into the behavior rig room where they are head-restrained and put on top of a Styrofoam ball inside a Styrofoam bowl that ejects air to allow the ball to move and allows the mice to run on it. The mice are inside the behavior rig for 15 minutes with only the fans on and with a red light. On day two, they are still head-restrained and put on the ball, but the introduction of visual stimuli is present along with the lick port being visible to them. Lastly, on day three, the lick port is put closer to their mouth—approximately 5-6mm—to get them habituated to having it close to them during pretrials and the main task.

*Pretraining*

At this point of the procedure, the mice should have about a 15 to 20 percent weight loss from when water deprivation occurred. During this experiment stage, the mice are put on the Styrofoam ball and the lick port is approximately 1 to 2mm from their mouth. The computer screen in front of them will display preferred visual stimuli—45 degrees—for 3 seconds with no punishment time. Between the second and third second, the lick port will dispense a tiny droplet of water. For the first couple of days, the experimenter will coax the mouse with water using a pipet during the time window—2-3 seconds—every 30 trials until the mouse associates the water with the lick port and begins licking by itself. Moreover, on the first day of pretrial, there are 150 trials, and then starting from the second day there are 250 trials. The pretrial stage lasts about four to five days for wild-type mice and about four to six days for K.O. *Fmr1* mice; it lasts until the mouse achieves a licking percentage of 80% or higher for two days.

*Visual Discrimination Task*

After pretraining, the mice have now associated water with the lick port, so we can begin the visual discrimination task, which is a go-/no-go task. During the visual discrimination task, the mouse is shown two different visual stimuli randomly: a visual stimulus tilted 45 degrees (preferred stimulus) and one tilted 135 degrees (non-preferred stimulus). The preferred stimulus is tilted 45 degrees and is the go task; therefore, the lick port will dispense water between the second and third second. The non-preferred stimulus is tilted 135 degrees and is the no-go task; therefore, it will not dispense water during the allotted time. The mouse will have to learn how to discriminate between these two stimuli as well as when to lick during the preferred stimuli—between the second and third second.

When the mouse does not lick during the allotted while the preferred stimulus is present it is identified as a 'Miss,' but when the mouse does lick during the allotted time it is identified as a 'Hit.' However, when the mouse does not lick during the non-preferred stimulus it is identified as a 'Correct Response (CR),' but when the mouse does lick it is identified as a 'False Alarm (FA).' When the mice get a 'Miss' or an 'FA,' the screen will turn grey for 6.5 seconds as a form of punishment.

The first day of training consists of 250 trials and the training after consists of 350 trials. The amount of time for each training varies on how well the mice do on the task—approximately 46 to 56 minutes. To determine if the mice can properly discriminate between preferred and non-preferred stimuli, we use the discriminability index (*d'*). The equation we used to calculate *d'* is:

$$d' = norminv(Hits/Hits+Misses) - norminv(FAs/FAs+CRs)$$

*Norminv* is a function in Matlab that returns the inverse of the normal cumulative distribution function.

To determine if mice are knowledgeable of discrimination, they must receive a *d'* of 2 for two days. This stage of the experiment could last for about three to four days for wild-type mice, and *Fmr1* KO will take either more or less time.

*Visual Discrimination Task with the Presence of Auditory Distractors*

During this stage of the experiment, the mice still undergo the visual discrimination task with the preferred and non-preferred stimuli; however, they are also presented with an auditory distractor stimulus for half the trials. This auditory distractor stimulus is a single tone that increases the sound inside the rig up to 90 decibels for each training. The single tone lasts for 1.5 seconds while the visual stimuli are presented on the screen. Each training session is 200 trials

long, and the amount of time it lasts depends on how well the mice do—approximately 20 to 30 minutes. To determine the mice as 'expert' mice, they must get a *d'* of 2 for two days; wild-type mice may achieve this *d'* in about 2-3 days while *Fmr1* KO mice may take less or more time. The *d'* is determined using the same equation used for the Visual Discrimination Task.

*Two-photon Calcium Imaging*

During the visual discrimination task with auditory distractor, the mice are imaged under a two-photon microscope (Hyper scope by Scientifica). The mice undergo the same process as stated above while being imaged. The microscope images axon terminals that are being activated through calcium modulated receptors. All the mice are genetically mutated to obtain calcium modulated receptors within their neurons because when a postsynaptic neuron is being signaled by another neuron, there is an influx of calcium. This influx allows the calcium to bind to these receptors and change its conformation, emitting fluorescence. This fluorescence is captured using the two-photon calcium imaging.

*Visual task Re-test and Control Task*

Once the mice have completed the Auditory Distractor Task and have achieved a *d'* of 2 for two days, they move on to the extra task. The extra task is the visual discrimination task, but only for 200 trials, to make sure the mice are 'expert' mice and do know how to discriminate between the preferred and non-preferred stimuli. Most of the time, the mouse will achieve a *d'* of 2 or higher demonstrating that they are indeed 'expert' mice. After the extra task, the mice are given a control task of 100 trials. The control task is the visual discrimination task, but the monitor inside the behavior rig is turned off, meaning that the mice will not be able to

discriminate between the two stimuli and know when the water droplet will be released from the lick port. The mice should get a low *d'* to demonstrate that they are using the visual stimuli to identify when the water droplet is being released. If the mice get a high *d'*, then they are probably cheating and not using the visual stimuli to figure out when the water droplet is given.

*Pharmacological interventions: Methylphenidate*

For this experiment, the mice undergo the same steps as a regular behavioral experiment. However, the only difference is that the mice were injected with methylphenidate hydrochloride (MPH), which is a drug that reduces hyperactivity and impulsivity while also enhancing attention and working memory. Therefore, the mice being used were strictly *Fmr1* KO mice because we wanted to see if the MPH would work. Before injecting the mice with MPH, they had to be anesthetized. To anesthetize the mice, they were put in an induction chamber with 5% isoflurane until they were fully anesthetized. After anesthetization, the mice were given a subcutaneous injection with 0.035mL of 10mg/mL MPH 30 minutes before the task—pretrials, visual discrimination, and visual discrimination with the auditory task. This was done to allow the effects of MPH to take place and for the anesthesia to wear off.

*Pharmacological interventions: Muscimol*

For this experiment, the mice undergo the same steps as a described in "Visual Discrimination Task." However, before the visual discrimination auditory task, the mice were injected with Muscimol, which is a drug that increases inhibition, thus reversibly inactivating ACC. These experiments were performed on wild-type (WT) mice. Before injecting the mice, the mice were anesthetized by being put in the induction chamber with 5% isoflurane. Then,

after they were fully anesthetized, they were moved onto a stereotaxic frame with a nose cone, which gave the mouse 1.5% isoflurane for maintenance, while being on top of a heat blanket (38 degrees Celsius) to keep the mice at body temperature. After putting them on the nose cone, we placed a piece of resin about the ACC area 0.3mM anterior of Bregma and then drilled a hole at this point. From there, the mouse was injected with 0.5 ul of 2mM Muscimol at a depth of 0.9mM (900 microns), and the hole was covered using super glue. We waited 30 minutes for the effects of Muscimol to take place and for the anesthesia to wear off before performing the visual discrimination with auditory distractors task.

## RESULTS

### *Fmr1 KO Mice demonstrate delayed learning on visual discrimination task*

As stated above in the Methods section, the wild-type (WT) and *Fmr1* KO mice were trained in a visual discrimination task (go/no-go task) while being water restricted. The mice were shown two different visual stimuli. The preferred stimulus moved across the screen at a 45-degree angle and provided water. On the other hand, the non-preferred stimulus moved 135-degrees and did not provide water. (Fig. 1A). Specifically, the mouse had to learn when to lick for water during the preferred stimulus and when not to lick for the non-preferred stimulus. Correct behavioral responses included a 'hit' and 'CR' while the incorrect behavioral responses included a 'FA' and 'miss,' which would result in a 6.5 second punishment time. (Fig. 1A). To obtain task performance, the discriminability index (*d'*) was used (see above, Methods). To determine if a mouse was an 'expert,' they would have to attain a $d' > 2$.

*Fmr1* KO mice showed a significant delay in learning how to discriminate between the preferred and non-preferred stimuli compared to WT mice (on average, 4.5 ± 0.3 days for WT mice vs 6.0 ± 0.4 days for *Fmr1* KO mice; $p = 0.002$, Mann-Whitney test; Fig. 1C) (Rahmatullah

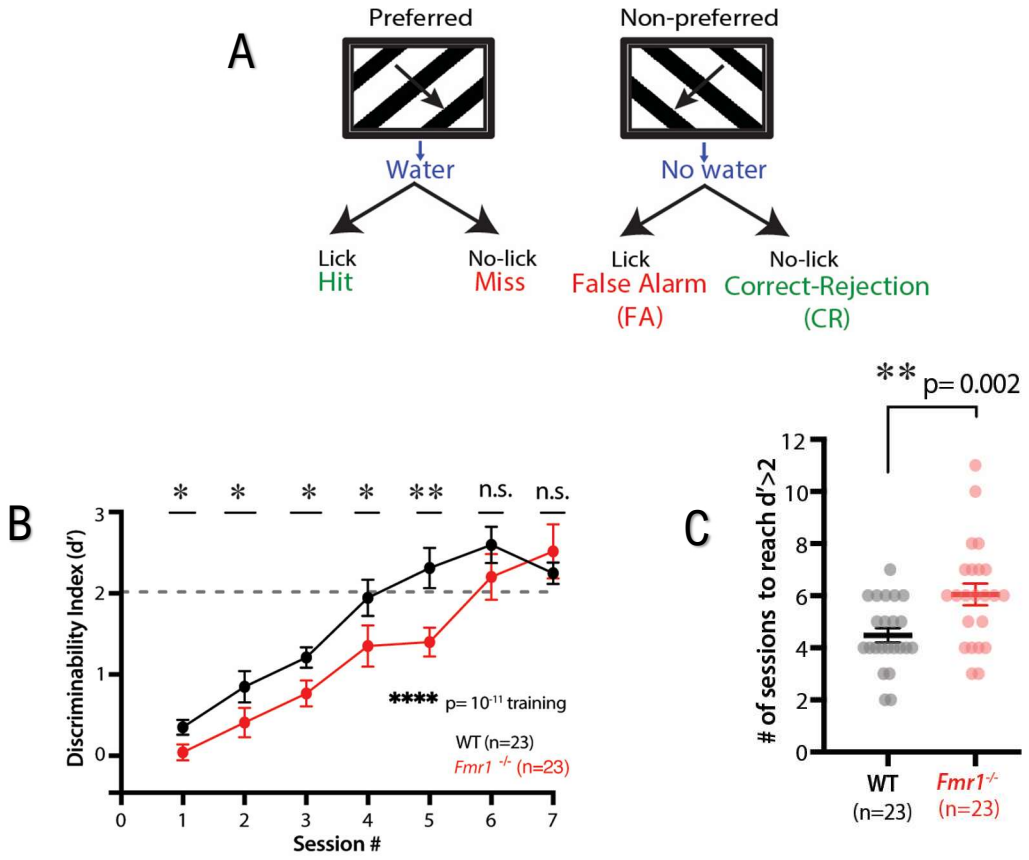


**Figure 1.** *Fmr1* KO mice showed a decline in performance on the visual discrimination task. ***A***, Illustration of the behavior paradigm for visual discrimination task (45 degress tilt for preferred and 135 degree tilt for non-preferred stimuli). CR, correct rejection; FA, false alarm. ***B***, *Fmr1* KO mice showed a delay in learning visual discrimination task. Performance is measured by the discriminability index (*d'*), where *d'*>2 is an 'expert' mouse. ***C***, *Fmr1* KO mice showed a delay in achieving a *d'*>2 compared to WT mice (on average, 4.5 ± 0.3 days for WT mice vs 6.0 ± 0.4 days for *Fmr1* KO mice; p = 0.002, Mann-Whitney test) [Adapted from Noorhan et al 2023]

et al., 2023). This may be due to the attentional deficiencies and impulsivity in FXS. Perhaps, *Fmr1* KO mice are taking longer to learn this task because they are too impulsive and continuously lick during the non-preferred stimulus. We also found that *Fmr1* KO mice have a lower percentage of CR responses and a higher percentage of FA responses (Rahmatullah et al., 2023). This contributes to the longer delay of time to learn the task and achieve a *d'* greater than 2.

*Auditory distractor task worsens the performance of Fmr1 KO mice on visual discrimination task*

After all the mice have achieved a *d'* > 2 for 2 consecutive days, they moved on to the auditory distractor task, which is when a tone would randomly ring for 50% of the trials (Fig. 2A) (See above in Methods). We have found that, although the *Fmr1* KO mice are now experts in the task, there is a decrease in performance when auditory distractors are present (Fig. 2B). Moreover, we observed that not many WT mice were affected by the auditory distractor compared to *Fmr1* KO mice. *Fmr1* KO mice would perform a *d'* > 2 for trials that did not include an auditory distractor but would have a performance decline on the tasks that did have an auditory distractor (Rahmatullah et al., 2023). We found that 2% of the WT mice and 20% of the *Fmr1* KO mice had a decrease in *d'* on the auditory distractor task (data not shown) (Rahmatullah et al., 2023). However, when the *Fmr1* KO mice retested on the visual discrimination task without distractors, they proved to be experts indistinguishable from that of WT mice (Rahmatullah et al., 2023).

Not only did *Fmr1* KO's *d'* decline significantly, but they also took more trials to achieve and maintain a *d'* > 2 for two consecutive days (Fig. 2C). This corresponds with the higher percentage of FA and lower percentage of CR discussed in the previous section. Although the *Fmr1* KO mice are now 'experts,' they could not withhold their licking when preferred and non-preferred stimuli were present compared to WT mice (Rahmatullah et al., 2023). This may have occurred due to the auditory distractors distracting them. These analyses imply that the presence of distractors negatively impact the performance of *Fmr1* KO mice.

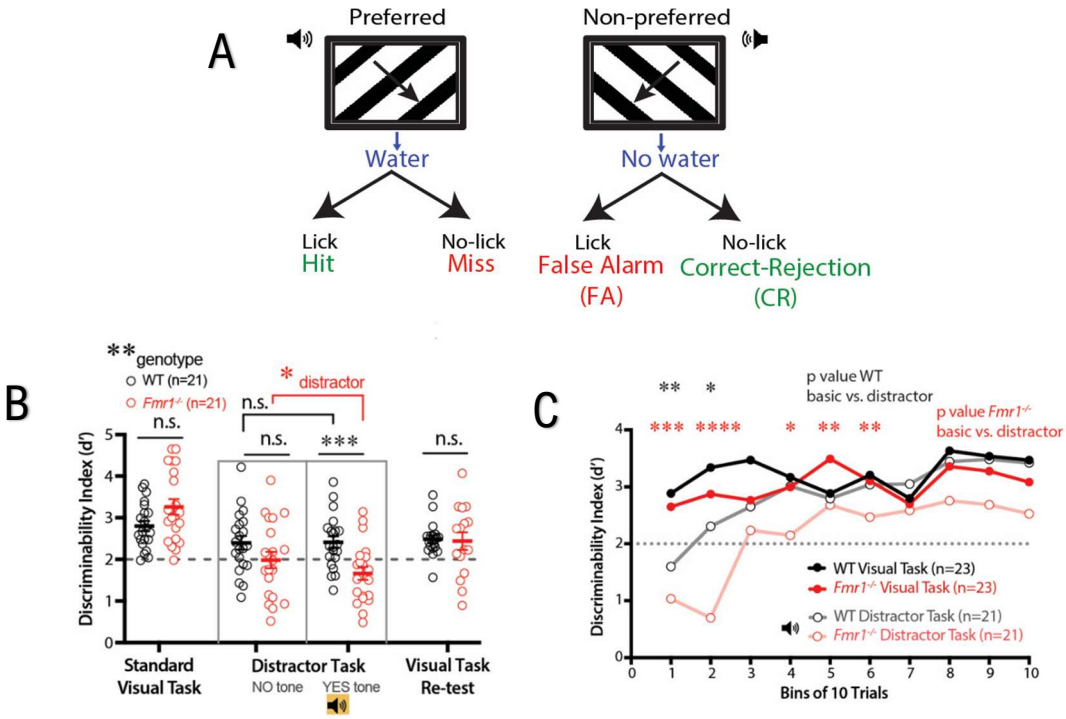


**Figure 2.** *Fmr1* KO mice showed a decline in performance on the visual discrimination task with auditory distractors present. ***A***, Illustration the behavior paradigm for visual discrimination task with auditory distractor (45 degress tilt for preferred and 135 degree tilt for non-preferred stimuli). CR, correct rejection; FA, false alarm. ***B***, *Fmr1* KO mice exhibit a worse performance when the auditory distractor tone is on compared to when it is off, They also had a more significant decline in performance compared to WT mice. Whereas WT mice had no significant change. ***C***, *d'* was calculated and tracked throughout the auditory distractor task (in bins of 10) . *Fmr1* KO mice took a longer amount of time to achieve an expert level compared to WT mice. [Adapted from Noorhan et al 2023]

*Disruptions from Anterior Cingulate Cortex (ACC) signaling to Visual Primary Cortex (V1)*

After viewing how *Fmr1* KO mice have a harder time learning the visual discrimination task and the auditory distractor task, we wanted view what the anterior cingulate cortex (ACC) was doing during the distractor task (See above, Methods). Specifically, we wanted to test if there was a difference between inputs from the ACC to the visual primary cortex (V1) between *Fmr1* KO and WT mice. All mice have a genetically encoded calcium receptor that when calcium binds to the encoded calcium receptor, changes conformation and elicits fluorescence. Therefore, when an action potential reaches the axon terminal and activates voltage-gated

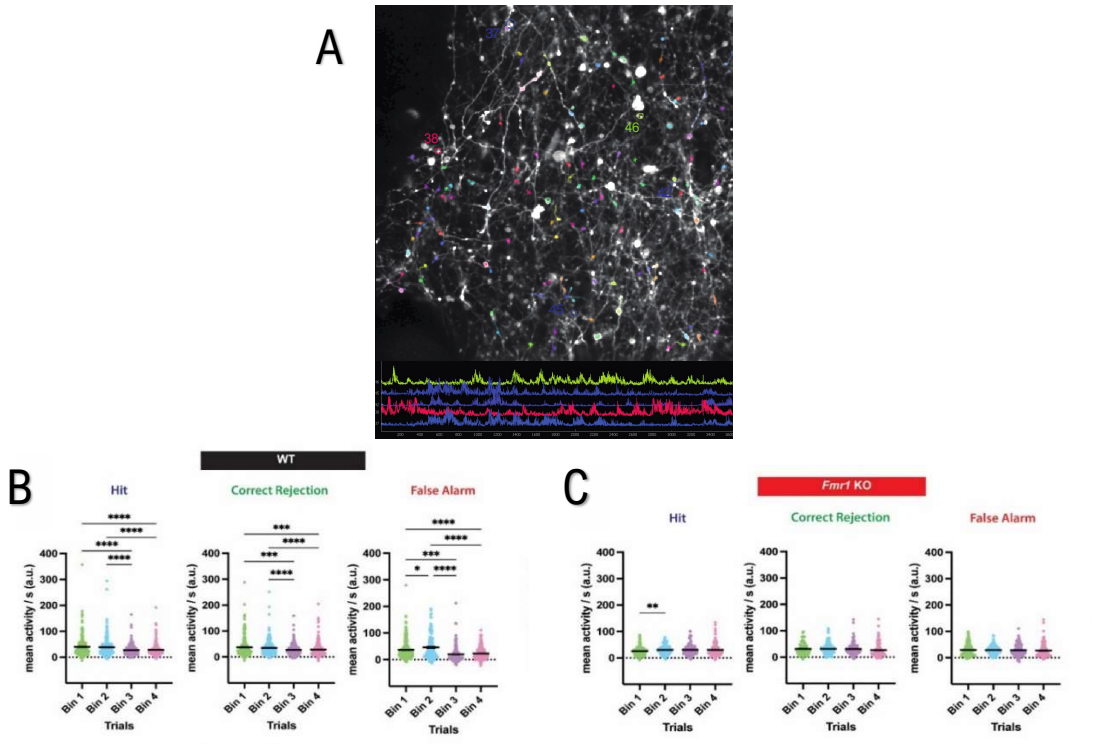


**Figure 3.** *Fmr1* KO mice do not exhibit an enhancement from anterior cingulate cortex (ACC) to the visual primary cortex (V1) during auditory distractor task. ***A***, Illustration of ACC axon terminals in V1 using a two-calcium photon imaging rig. ***B***, WT exhibit an elevation in ACC during false alarms, suggesting that ACC inputs keeps track of what is occurring early in distractor task to provide an attentive signal to overcome it later. ***C***, *Fmr1* KO mice do not have any changes in ACC activity, suggesting that ACC to V1 is not changing, which means attentive signal is lost in *Fmr1* KO mice.

calcium channels, the calcium will also bind to the encoded calcium receptor and elicit a fluorescence to display that there is an activation in this synapse from one neuron to another.

Using two-photon calcium imaging, we were able to image axonal varicosities in V1 (Fig. 3A) during the auditory distractor task. As seen in Figure 3, axonal activity is quantified in 4 bins containing 25 trials. When comparing bins 1 and 2 to bins 3 and 4 for WT mice, there is an enhancement of activity between ACC →V1 during error trials (FA) (Fig. 3B), suggesting that ACC inputs keep track of events that are happening early in the distractor task to provide an attentive signal to overcome it in the future. However, the enhancement between ACC →V1 is absent for *Fmr1* KO mice (Fig. 3C). Instead, there is no change in activation of ACC, suggesting

that ACC →V1is not changing like the attenuative signal is lost in *Fmr1* KO mice. These results suggest that ACC input to V1 is disrupted in *Fmr1* KO mice.

*Muscimol inactivation of ACC prevents WT mice from overcoming auditory distractors*

After viewing the differences in ACC between WT and *Fmr1* KO mice, we wanted to view the effects of Muscimol in the ACC of WT mice (See above, Methods). Muscimol is a drug that temporarily inhibits its surroundings by binding to GabaA receptors, leaving them open for longer periods of time (Fig. 4A). Subsequently, more GABA, an inhibitory neurotransmitter, will enter the postsynaptic neuron and inhibit its activity by hyperpolarizing it (Fig. 4A). When we injected Muscimol in WT mice, 30 minutes before the distractor task, they were not as attentive; they had decreased licking and did significantly worse on the distractor task with a $d' < 2$ (Fig. 4B). However, the days before and after the Muscimol injection, the mice did well with a $d' > 2$ brain, suggesting that they are 'expert' mice and the Muscimol was the main effect for them doing poorly (Fig. 4B). Consequently, when we inject Musicmol around the ACC, the ACC would not function properly due to increased inhibition, and the mice would not be able to discriminate between preferred and non-preferred stimuli properly. These analyses suggest that activity from the ACC is important to learning to discriminate between stimuli.

*Methylphenidate aids Fmr1 KO mice with the Visual Discrimination Task*

Now that we know that Muscimol negatively affects the ACC, we thought Methylphenidate (MPH) may enhance the ACC's activity (See above, Methods). MPH is a common medication used to treat symptoms that are common in FXS and attention-deficit hyperactivity disorder: difficulties in attention, learning and hypersensitivity. MPH used because

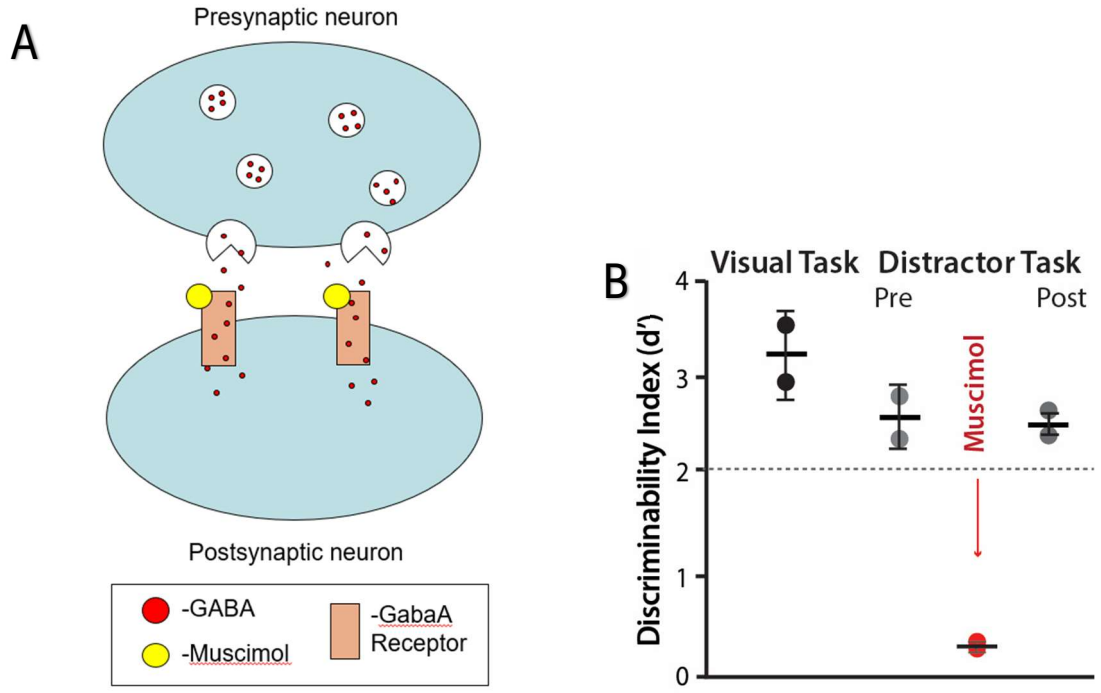


**Figure 4.** The effects of Muscimol within the brain. ***A***, Illustration of how Muscimol binds to GabaA receptors within the brain, prolonging their activity to stay open and take in more GABA neurotransmitters (an inhibitory neurotransmitter). ***B***, WT mice with Muscimol perform worse on the distractor task. Their performance was at expert level before and after the muscimol injection, suggesting ACC is required for overcoming distractors.

it increases dopaminergic activity and elevates anterior cingulate function by blocking dopamine transporters and thus inhibiting the reuptake of dopamine (Fig. 5A). This allows dopamine to remain in the synaptic cleft for a longer period and promotes more activity (Fig. 5A). How MPH modulates neural function during visual perceptual learning in FXS is unknown. We will address this question by examining ACC→V1 network activity during our visual task in mouse.

We have found that when *Fmr1* KO mice are injected with MPH, 30 minutes before every task, they learn how to discriminate between the preferred and non-preferred stimuli on the visual discrimination task faster than *Fmr1* KO without MPH (Fig. 5B). Normally, as stated above, it would take about 6 days for a *Fmr1* KO mouse to achieve a $d' > 2$ whereas *Fmr1* KO mice took respectively 4 to 5 days to achieve a $d' > 2$. (Fig. 5B). Moreover, *Fmr1* KO mice with

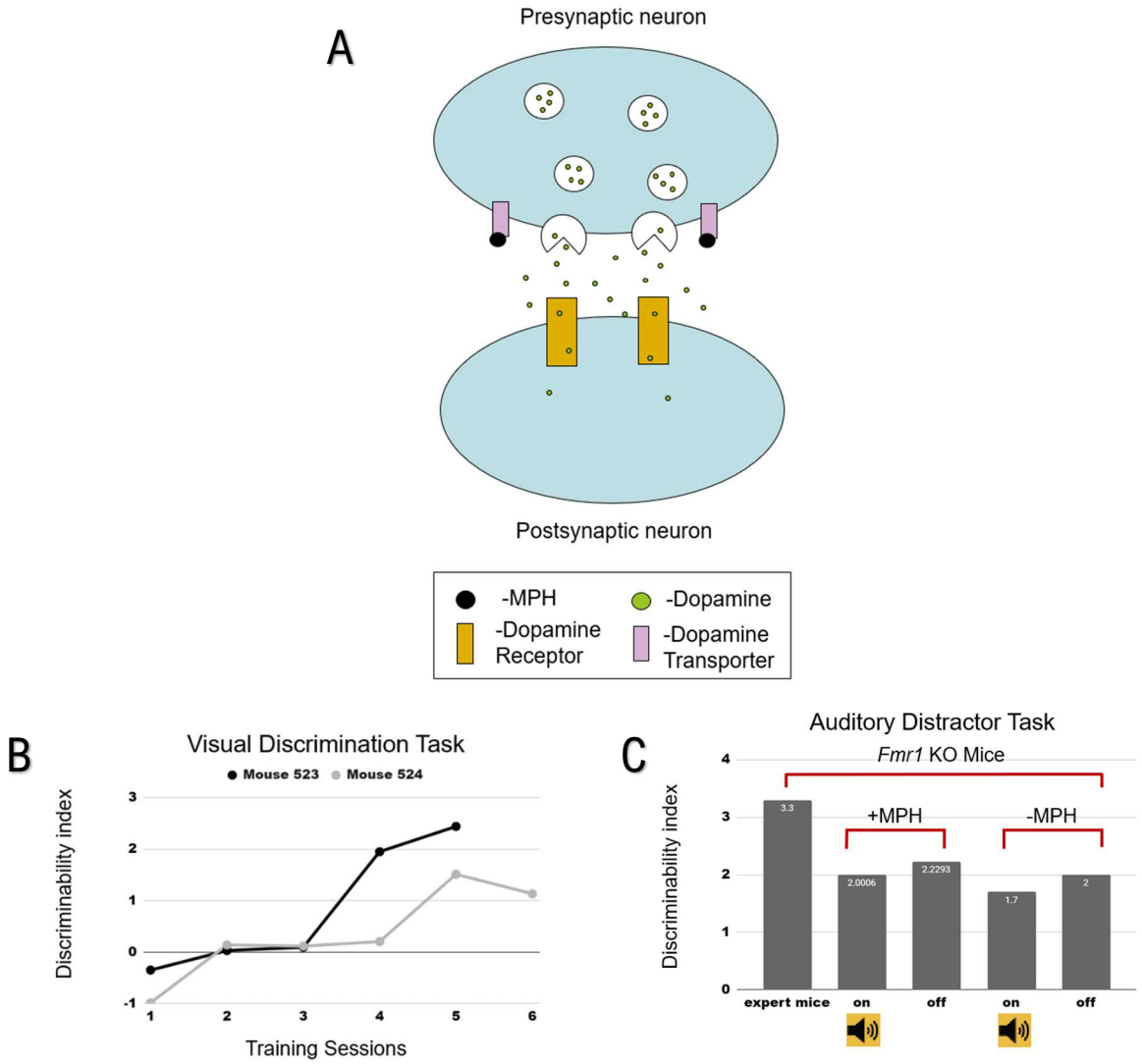


**Figure 5.** The effects of methylphenidate (MPH) within the brain. ***A***, Illustration of how MPH blocks dopamine transporters, which prevents the reuptake of of dopamine into the presynaptic neuron, allowing for a higher dopamine concentration to be used for the postsynaptic neuron. ***B***, *Fmr1* KO mice that are injected with MPH (523 and 524) achieve a *d'*>2 in a short amount of training sessions (~4-5 days). ***C***, *Fmr1* KO mice perform better when they are injected with MPH compared to *Fmr1* KO that are not injected with MPH.

MPH achieved higher *d'* on the distractor task compared to *Fmr1* KO mice without MPH (Fig. 5C). *Fmr1* KO mice with MPH scored a *d'* of 2 with the auditory distractor on while those without MPH scored a *d'* of 1.7(Fig. 5C), suggesting that the MPH aided in avoiding the distractibility of the tone. However, not only did the *Fmr1* KO with MPH do better with the auditory distractor on, but also did better when the auditory distractor was off with a *d'* of

approximately 2.23 compared to *Fmr1* KO mice without MPH, who had a *d'* of 2 (Fig. 5C), suggesting that MPH also aided in discriminating between the two visual stimuli. These results suggest that MPH is enhancing activity in the brain that allows *Fmr1* KO mice to learn faster and not be as distracted.

## DISCUSSION

Fragile X Syndrome (FXS), the leading known genetic cause of atypical behaviors associated with autism spectrum disorders (ASD) (Chudley and Hagerman, 1987; Yu and Berry-Kravis, 2014; Niu et al., 2017), arises due to the reduced expression or loss of the Fragile X Mental Retardation Protein (FMRP). Individuals with ASD experience atypical sensory processing, often in many sensory modalities including taste, touch, audition, smell and vision (Robertson and Baron-Cohen, 2017). The consequence of altered sensory processing is debilitating, resulting in impairment in sensory discrimination and an inability to ignore irrelevant sensory stimuli such as innocuous sounds, smells, sights, or touches. This hyperarousal to sensory stimuli leads to hypersensitivity, tactile defensiveness, ADD and eventually impairments in learning. We set out to investigate the differences of learning in FXS using a mouse model—WT mice and *Fmr1* KO mice. We had WT and *Fmr1* mice undergo a visual discrimination task while being water restricted, and we found that *Fmr1* KO had a harder time learning how to discriminate between preferred and non-preferred visual stimuli to receive water.

Moreover, we also found that there is more activation from the anterior cingulate cortex (ACC) to the visual primary cortex (V1) in WT mice. From there we wanted to establish a causal relationship between ACC and task performance. Therefore, we inhibited input from the ACC using Muscimol, which diminished behavioral performance. Then, we questioned what would occur if we enhanced input from the ACC using methylphenidate (MPH), which resulted in

better behavioral performance. Our findings clearly demonstrate the ACC is an important part of the brain that has a strong role in the deficiencies in FXS.

Potential technical difficulties we had to be wary of is if the screen or lick port for the visual discrimination task did not work properly; consequently, we did a practice run before the mouse was in the rig. Moreover, other limitations would be if we do not conduct the exact same procedures for each section—before and after MPH/Muscimol—for the experiment, then, we will not be able to state a causal effect of MPH/Muscimol. Continually, if we do not know how to properly hold the mice as we transport them to the lab area, then we could possibly stress out the mice and skew our lab results. Therefore, any potential roadblocks would be that the mice are fatigued, stressed, or distracted. Especially with *Fmr1* KO mice, a limitation is that they are seizure prone which can hinder learning.

Insights from our study in *Fmr1* KO mice will provide the fundamental knowledge needed to design circuit based therapeutic strategies for humans with FXS. This is because the *Fmr1* KO mouse that is an established FXS model since it shows functional alterations that are similar to humans and hypersensitivity phenotypes in mice resonate with human symtoms (Consortium, 1994; Kazdoba et al., 2014). Other similarities in the phenotypes between mice and humans include delayed learning. Several recent studies suggest that sensory issues and atypical sensory processing can be predictive of and contribute to abnormal anxiety and other cognitive and social deficits (Green and Ben-Sasson, 2010; Robertson and Simmons, 2013; Tavassoli et al., 2014; Wheeler et al., 2016; Kojovic et al., 2019). Thus, enhancement in ACC will likely affect not only learning, memory, and attention, but also social skills such as communication. Lastly, we can aid in the findings for FXS and can better understand the neurological processes that occur in individuals with brain disorders.

Future goals include performing two-photon calcium imaging to examine the effects of Methylphenidate on ACC→V1 inputs, and other cell types in V1. This will provide much needed information on the action of MPH and its effects on different circuits in the brain.

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