Behavioral Effects of Administering CTEP Treatment in a Mouse Model of Fragile X Syndrome

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ABSTRACT

Fragile X Syndrome (FXS) is a genetic neurodevelopmental disorder that causes autism and intellectual disabilities: exhibiting hyperactivity, elevated anxiety, and impaired cognitive/sensory processing. These deficits result from mutations in the X-linked gene Fragile X messenger ribonucleoprotein 1 (*Fmr1*). *Fmr1*-knock-out (KO) mouse models have shown consistency with observations in humans, displaying seizures and sensory processing deficits. Utilizing *Fmr1*-KO mice to identify a potential treatment for these symptoms, we administered a drug called 2-chloro-4-((2,5-dimethyl-1-(4-trifluoromethoxy)phenyl)-1H-imidazole-4-yl) ethynyl)pyridine (CTEP) to KO mice and measured behavioral changes. CTEP inhibits metabotropic glutamate receptor pathways, which are upregulated in FXS. Two types of experiments were run: open field test (OFT) and elevated plus maze (EPM), commonly used to study anxiety and hyperactivity. Experimental mice with higher anxiety depict decreased exploration and more time spent near the arena's wall or closed arms. We found that CTEP reduces the distance traveled in the OFT across both wild-type (WT) and KO groups, suggesting reduced locomotion. There were no statistically significant differences in time spent in EPM closed arms between WT and KO mice, indicating no treatment of anxiety. These results suggest that more effective intervention is needed to target anxiety deficits related to FXS.

Keywords: Fragile X Syndrome, anxiety, hyperactivity, CTEP, mGluR5

FACULTY MENTOR - Dr. Khaleel Razak, Department of Psychology & Neuroscience

Dr. Razak earned a bachelor's degree in Electronics and Communications Engineering (B.E.) from the College of Engineering, Anna University, Chennai, India (1992) and a Ph.D. in Zoology/Physiology/Neuroscience from the University of Wyoming (2001). He held postdoctoral associate positions at Georgia State University (2001-2003) and at the University of Wyoming (2004-2007). Dr. Razak's research at UCR is focused on auditory processing and brain plasticity.

Diane Le

Attending University of California, Riverside, Diane Le is a second year Neuroscience B.S. major with dreams of becoming a pediatrician. Diane works in Dr. Khaleel Abdulrazak's lab studying Fragile X Syndrome (FXS), a genetic cause of autism. A symptom of FXS, sensory hypersensitivity, leads to delayed development in early childhood. Searching for effective treatments, Diane is studying to understand the underlying mechanisms of this condition to better understand how adolescence is affected by neurological disorders.

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INTRODUCTION

Fragile X Syndrome (FXS) is caused by a mutation in the Fragile X Messenger Ribonucleoprotein 1 (*Fmr1*) gene. Located on the X chromosome, it produces a protein called Fragile X Messenger Ribonucleoprotein (FMRP), which plays a crucial role in cell function[1].

Resulting in hyperactivity, impaired cognitive function, and sensory processing deficits[2], the mutation of *Fmr1* causes a loss of FMRP, resulting in increased levels of protein synthesis, influencing brain plasticity[3]. *Fmr1*-knockout (KO) mouse models have shown consistency with observations in humans, displaying symptoms like altered startle responses, high anxiety, and processing deficits. These similarities suggest that animal models of FXS may provide a secure foundation for understanding the underlying mechanisms of FXS and may facilitate new approaches to understanding the mechanisms of basic sensory processing behaviors in humans. In this study, we used the drug 2-**c**hloro-4-((2,5-dimethyl-1-(4-(**t**rifluoromethoxy)phenyl)-1H-imidazol-4-yl) **e**thynyl)**p**yridine (CTEP), a long-acting allosteric inhibitor of metabotropic glutamate receptor unit 5 (mGluR5)[10] to test whether chronic pharmacological mGluR5 inhibition reverses FXS phenotypes in a fully developed mouse brain[9]. In Fragile X Syndrome (FXS), mGluR5 is upregulated, altering brain circuitry and elevating hyperexcitability. The signaling of mGluR5 activates a phosphatidylinositol-calcium second messenger system, which may be involved in regulating neural network activity and synaptic plasticity[11]. Using CTEP, we are testing whether its treatment effects extend to behavioral assays as shown by Hamilton et al., (2016), whose findings suggest that CTEP or its analog Basimglutant may potentially be an effective treatment for patients with impaired sensory cognition. Our question is the following: how does CTEP treatment impact anxiety and hyperactivity-related behavioral phenotypes? We utilized *Fmr1*-KO mice to test whether CTEP alleviates hyperactivity and anxiety symptoms by measuring behavior changes and comparing these changes to wild-type (WT) mice.

Two types of behavioral experiments were run: open field test (OFT) and elevated plus maze (EPM), which are commonly used to study anxiety-related behaviors in rodents[5, 6, 7]. Experimental mice with lower rates of anxiety typically depict less exploration and higher thigmotaxis (i.e., near the walls of the arena) in the OFT or spend more time in the closed arms of the EPM. By analyzing behavioral assays of CTEP effects on *Fmr1* KO mice, we gain insight into the impact of direct treatment on behavioral deficits of hyperactivity and anxiety.

MATERIALS & METHODS

Ethics Statement. All experiments and animal care/use protocols were approved by the Institutional Animal Care and Use Committee at the University of California, Riverside, and were carried out in accordance with the NIH "Guide for the Care and Use of Laboratory Animals."

Subjects. Mice were obtained by breeding wild-type male and *fmr1*$^{+/-}$female C57/Bl6J mice. Pups were weaned at P21 and group-housed until experimentation. The study used C57/Bl6J male mice of the *fmr1*-KO line and wild-type littermates at postnatal age P50-P90. They were provided with food and water *ad libitum*.

Genotyping. A standard tail snip was performed on mice for genotyping. Mice were anesthetized with a short-acting anesthetic (e.g., isoflurane). Tail tip removal was performed using sharp, sterile scalpel blades or scissors. Approximately 0.1-0.5 cm of tissue was removed.

EXPERIMENTAL DESIGN

Open Field Test. An open field test (OFT) consisting of four activity chambers was used and conducted in a standard-lit room. Before testing, mice were habituated to the room for 1 hour. Each mouse was placed in an open-box arena (43 x 43 x 43 cm) and roamed for 10 minutes during testing and video recording. Using SMART Video Tracking Software from PanLab Apparatus, we analyzed subject behavior during

the first 5 minutes of arena exploration. Arena designs are digitally overlaid on the recorded arena from TopScanLite, a software used to track the movement of the mouse models. Two aspects of behavior were characterized: **(1)** Total distance covered (mm) during the timed portion of the experiment; **(2)** Thigmotaxis, or percent time that subjects remain adjacent to the outer wall (depiction of anxiety)[6].

Elevated Plus Maze. An elevated plus maze (EPM) consists of four perpendicular arenas with open and closed walls. Before testing, mice were habituated to the room for 1 hour. Each mouse was placed in the arena, roaming free for 10 minutes during testing while video recording took place. All data were manually recorded using a timer to start and stop when the mouse was in and out of the closed arms. Bouts were recorded each time a mouse entered the arena with the closed walls. Two aspects of the elevated plus maze were characterized using this protocol: **(1)** Total time spent in closed arms during the timed portion of the experiment; **(2)** How many times the mouse models entered and left the closed arenas.

Data Analysis. Statistical comparisons were run using GraphPad Prism, on which we ran: **1)** Two-way ANOVA to find the main effects and mixed effects of genotype, treatment, and condition. **2)** Student's t-test to compare two groups at a time. To determine statistically significant differences between groups/treatments, a two-way ANOVA was used with Tukey's *post hoc* test to correct multiple comparisons.

Drug Administration. Stock solutions of CTEP (MP Biomedicals) were prepared regularly and frozen until use, when they were thawed, vortexed, and then administered. All drugs and vehicle control solutions were administered daily for 10 days by a stainless steel curved oral gavage tip (Cadence Science, product #7910). Gavage tips were soaked in ethanol, rinsed with DI water, and autoclaved before each use. Mice received 2mg/kg CTEP per 48 hours for 10 days.

5-minute recordings of mouse behavior in the respective arenas were analyzed prior to 10-day CTEP treatment (precondition) and following treatment (post-condition).

RESULTS:

The main goal of this study was to determine if 10 days of daily CTEP treatment, compared to the vehicle, reversed phenological symptoms of hyperactivity and anxiety in an adult *Fmr1*-KO mouse model. Looking at OFT % time in the thigmotaxis and center, there was no difference between groups in time spent in each region for genotype and treatment. Running a two-way ANOVA test, mice generally spent more time in outer regions compared to inner regions. Studying distance traveled for OFT, the figures depict distance traveled over two main areas: thigmotaxis and center. No statistically significant data was seen between groups using a two-way ANOVA test. In EPM of % time in closed arms, there was statistically significant data between CTEP KO pretreatment (pre) and CTEP KO post-treatment (post) depicting that there was an increase from pre to post. A t-test was used to determine the differences between groups and treatments, and a Tukey's *post hoc* test was used to correct for multiple comparisons.

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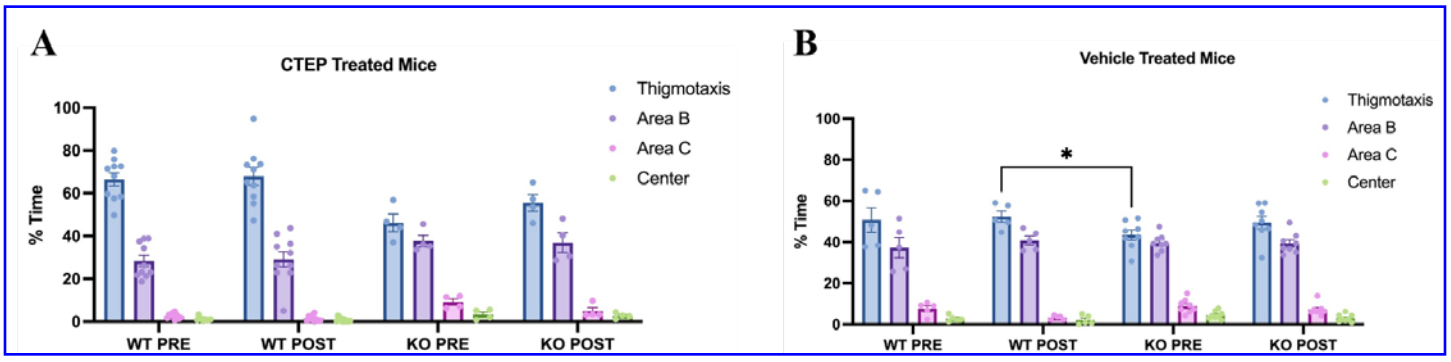

Figure 1. Graphs show the effects of CTEP and vehicle (Veh) treatment in pre- and post-wild type and knockout mouse models in the different arenas. **(A)** There was no difference between groups in how much time they spent in each region. Generally, mice spent more time in outer regions compared to inner regions. WT Pre: n=10, WT Post: n=10, KO Pre: n=4, KO Post: n=4. **(B)** The graph shows statistically significant differences between WT post-treatment with vehicle (control treatment) & KO pre-treatment. The thigmotaxis and Area B have the highest % of time spent. Area C and Center zone have the least % of time spent. WT Pre: n=5, WT Post: n=5, KO Pre: n=8, KO Post: n=8, * indicates $p \leq 0.05$. In both graphs, the y-axis depicts % time that the mice spent in each area. A two-way ANOVA was used to determine statistically significant differences between groups and treatment, and a Tukey's *post hoc* test was used to correct for multiple comparisons. In both graphs, the y-axis depicts % time that the mice spent in each area. A two-way ANOVA was used to determine statistically significant differences between groups and treatment, and a Tukey's *post hoc* test was used to correct for multiple comparisons.

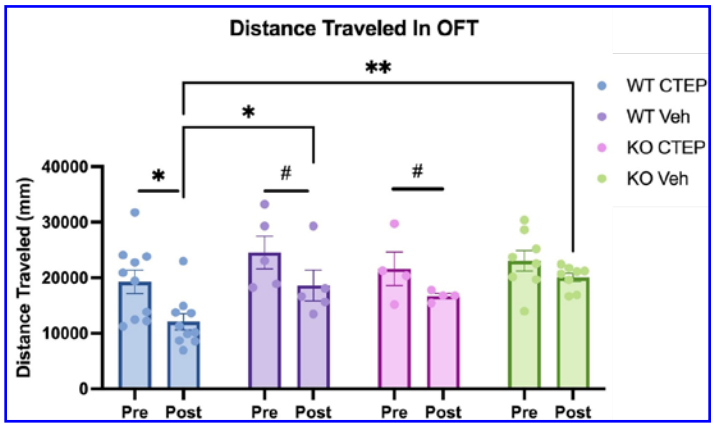

Figure 2. The y-axis depicts the total distance that mice traversed. The graph shows reduced distance traveled between pre and post in WT treated with CTEP (significant), WT treated with vehicle (trending), and KO treated with CTEP (trending). A two-way ANOVA was used to determine statistically significant differences between groups and treatment, and a Tukey's *post hoc* test was used to correct for multiple comparisons. WT CTEP: n=10, WT Veh: n=5, KO CTEP: n=4, KO Veh: n=8, * indicates $p \leq 0.05$, ** indicates $p \leq 0.01$, # indicates $p \leq .076$ (trending)

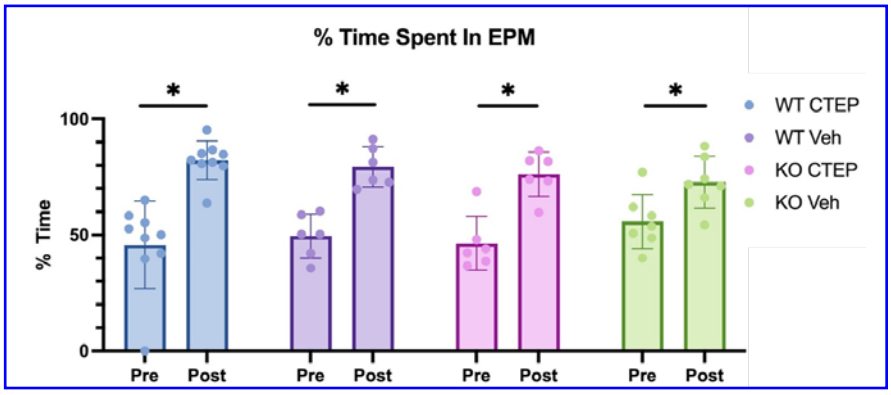

Figure 3. This graph shows % time spent in EPM in closed arms by comparing WT and KO CTEP and Veh mouse models. The y-axis depicts % time spent in the closed arms of the EPM. Generally, mice spent more time in the closed arms in the post-condition compared to pre condition, regardless of genotype and treatment type. A two-way ANOVA was used to determine statistically significant differences between groups and treatment, and a Tukey's *post hoc* test was used to correct for multiple comparisons. WT CTEP: n=9, WT Veh: n=6, KO CTEP: n=6, KO Veh: n=7, * indicates $p \leq 0.05$

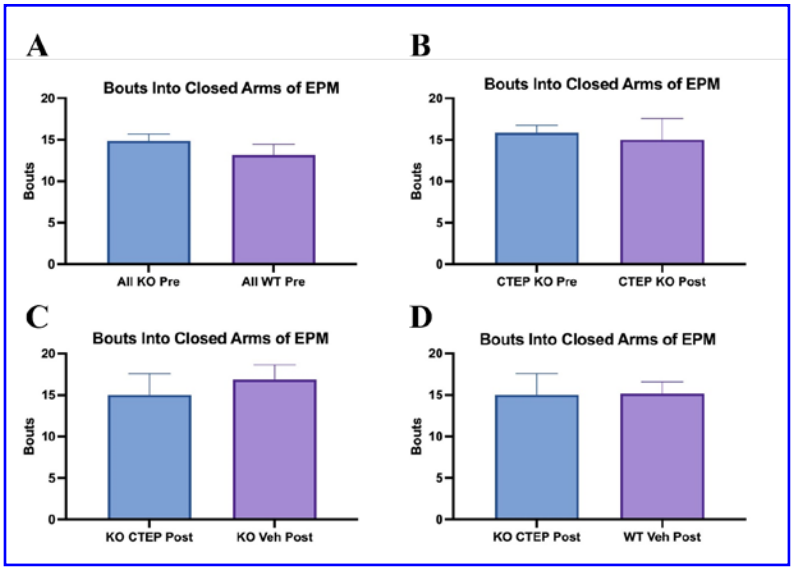

Figure 4. The graphs shows the number of times the mouse models entered the closed arms in EPM (bouts), comparing it to the different combinations of genotypes. **A)** All WT Pre: n=15, All KO Pre: n=12. **B)** CTEP KO Pre: n=4, CTEP KO Post: n=4. **C)** CTEP KO Post: n=4, Veh KO Post: n=8. **D)** CTEP KO Post: n=4, Veh WT Post: 5 Among the graphs, there were no statistically significant differences between groups in how much time they spent in each region. Generally, mice spent more time in outer regions compared to inner regions. The y-axis depicts the bouts that the mice spent in each area. A t-test was used to determine statistically significant differences between groups and treatments, and a Tukey's *post hoc* test was used to correct for multiple comparisons.

DISCUSSION

By using the mouse model of FXS, the *Fmr1* WT and KO mouse models are used to assess if behavioral symptoms of FXS have been reduced through the treatment of CTEP. Administering CTEP to *Fmr1*-KO and WT mice affected their behavior in OFT and EPM in the following ways: **1.** Both genotypes reduced distance traveled following treatment with CTEP (**Figure 2**), **2.** Only WT showed a reduction in distance traveled with Veh (**Figure 2**), **3.** KO that received the vehicle did not change the distance traveled with OFT (**Figure 2**), **4.** Between WT & KO mice and between CTEP treatment and vehicle, there were no significant differences in % of time spent in closed arms of EPM (**Figure 3**).

Studying the effects of 10-day CTEP treatment, locomotion in both genotypes was reduced, but there are no physical signs that hyperactivity and anxiety were decreased. Treatment was not improved significantly in alleviating these symptoms of hyperactivity and anxiety between CTEP and Veh mouse models. The behavioral assays of WT and KO may not have been sensitive enough to detect the differences in genotype because the WT mouse models used were glutamates, born in the same cage as the KO mouse models. Environmentally, WT models are influenced by KO, which is a huge factor in how the experiment is affected. With no statistically significant differences in % time spent in closed arms following CTEP treatment compared to vehicle, there was no indication of treatment of anxiety. Reducing hyperactivity, CTEP was shown to reduce the distance traveled in OFT of both WT and KO mouse models, suggesting reduced locomotion but no alleviation of KO-specific deficits.

Given that the mouse models were treated by oral gavage, this may have contributed to elevated anxiety in the subjects of the study. A future step that will address this is to administer treatment through water consumed regularly by mice to limit anxiety given by oral gavage. CTEP did not alleviate knockout-specific hyperactivity in this study; we suspect tolerance to CTEP may have been acquired[7]. They have shown that tolerance is built through continual CTEP treatment, as seen in clinical studies that show a lack of improvement of symptoms in humans with FXS and in rodent models. In the future, a single acute dose of CTEP will be provided instead of a chronic 10-day treatment. Using mouse models, we will assign each mouse a single dose at a younger age (~P28) and test their behavioral phenotype when they are older (~P60). As shown by Lovelace et al. (2020), there was an improvement in the *Fmr1*-KO mouse model of Fragile X Syndrome through treatment using minocycline. Administering the minocycline using a similar 10-day treatment protocol as that used in this study,

led to improvements as shown in electroencephalogram (EEG) measures[12]. Continuing this experiment, we will use EEG biomarkers to identify potential electrophysiological improvements following acute CTEP treatment. It is vital to continue searching for effective treatments for humans with FXS to decrease sensory symptoms of FXS and to further understand the underlying mechanisms of this condition.

CLINICAL RELEVANCE

A consistent symptom of FXS, sensory and auditory hypersensitivity is a prominent symptom noted in clinical and parent reports. Leading to increased anxiety and delayed language development, early childhood sensory processing abnormalities can lead to further disruptions in development. Taken together with studies that have shown alleviation in symptoms of mouse models with FXS through the treatment of CTEP, these studies point to mGlu5 inhibition as a therapeutic avenue for humans with FXS[13].

In neurodevelopmental disorders research and drug development, correlations have been made with the treatment of CTEP to suggest that it effectively reduces behavioral symptoms of sociability deficits, increased anxiety, hyperactivity, and sensory hyperexcitability[15]. CTEP has been tested as a potential therapeutic in the neurodevelopmental disorder: Fragile X Syndrome. A further evaluation of how CTEP affects a broad range of phenotypes displayed in mouse models is required before designing outcome measures for humans.

REFERENCES

[2] Dansie, L.E., et al. "Long-Lasting Effects of Minocycline on Behavior in Young but Not Adult Fragile X Mice." *Neuroscience*, Pergamon, 7 May 2013,

https://www.sciencedirect.com/science/article/abs/pii/S0306452213003916.

[1] "Fragile X Syndrome." *NORD (National Organization for Rare Disorders)*, 18 Sept. 2017, https://rarediseases.org/rare-diseases/fragile-x-syndrome/#:~:text=Affected%20Populations,and %201%3A1000%20males.

[11] "GRM5 Glutamate Metabotropic Receptor 5 [Homo Sapiens (Human)] - Gene - NCBI." *National Center for Biotechnology Information*, U.S. National Library of Medicine, https://www.ncbi.nlm.nih.gov/gene/2915.

[16] Hamilton A; Vasefi M;Vander Tuin C;McQuaid RJ;Anisman H;Ferguson SS; "Chronic Pharmacological mglur5 Inhibition Prevents Cognitive Impairment and Reduces Pathogenesis in an Alzheimer Disease Mouse Model." *Cell Reports*, U.S. National Library of Medicine, https://pubmed.ncbi.nlm.nih.gov/27210751/.

[7] Komada, Munekazu, et al. "Elevated plus Maze for Mice." *Journal of Visualized Experiments : JoVE*, MyJove Corporation, 22 Dec. 2008, Stoppel, David C., et al.

[5] Kazdoba, Tatiana M, et al. "Modeling Fragile X Syndrome in the FMR1 Knockout Mouse." *Intractable & Rare Diseases Research*, International Research and Cooperation Association for Bio & Socio-Sciences Advancement, Nov. 2014,

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4298642/.

[10] Lindemann L, Jaeschke G, Michalon A, Vieira E, Honer M, Spooren W, Porter R, Hartung T, Kolczewski S, Büttelmann B, et al. (2011). CTEP: a novel, potent, long-acting, and orally bioavailable metabotropic glutamate receptor 5 inhibitor. *J. Pharmacol. Exp. Ther* 339, 474–486.

https://molecularbrain.biomedcentral.com/articles/10.1186/1756-6606-6-15#:~:text=FMRP%20 was%20first%20characterized%20in,including%20intellectual%20disability%20and%20autism.

[12] Lovelace, Jonathan W., et al. "Minocycline Treatment Reverses Sound Evoked EEG Abnormalities in a Mouse Model of Fragile X Syndrome." *Frontiers*, Frontiers, 1 Jan. 1AD, https://www.frontiersin.org/articles/10.3389/fnins.2020.00771/full.

[8] "MGluR5 Negative Modulators for Fragile X: Treatment Resistance and Persistence." *Frontiers*, Frontiers,-1Jan.1AD, https://www.frontiersin.org/articles/10.3389/fpsyt.2021.718953/full.

[9 & 14] Michalon A;Sidorov M;Ballard TM;Ozmen L;Spooren W;Wettstein JG;Jaeschke G;Bear MF;Lindemann L; "Chronic Pharmacological mglu5 Inhibition Corrects Fragile X in Adult Mice." *Neuron*, U.S. National Library of Medicine, https://pubmed.ncbi.nlm.nih.gov/22500629/.

[13] Michalon, Aubin, et al. "Chronic Pharmacological mglu5 Inhibition Corrects Fragile X in Adult Mice." *Neuron*, U.S. National Library of Medicine, 12 Apr. 2012,

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8822597/.

[15] Pirbhoy, Patricia S, et al. "Increased 2-Arachidonoyl-Sn-Glycerol Levels Normalize Cortical Responses to Sound and Improve Behaviors in fmr1 Ko Mice." *Journal of Neurodevelopmental Disorders*, BioMed Central, 13 Oct. 2021, https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8513313/./

[4] *Rais M, Binder DK, Razak KA, Ethell IM. Sensory Processing Phenotypes in Fragile X Syndrome. ASN Neuro. 2018;10. doi:10.1177/1759091418801092*

[6] Seibenhener, Michael L, and Michael C Wooten. "Use of the Open Field Maze to Measure Locomotor and Anxiety-like Behavior in Mice." *Journal of Visualized Experiments : JoVE*, MyJove Corporation, 6 Feb. 2015, https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4354627/.

[3] Sidorov, Michael S, et al. "Fragile X Mental Retardation Protein and Synaptic Plasticity - Molecular Brain." *BioMed Central*, BioMed Central, 8 Apr. 2013,