STEREOTYPICAL BEHAVIORS IN *Fmr1* KNOCKOUT MICE

An Undergraduate Research Scholars Thesis

by

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TABLE OF CONTENTS

ABSTRACT

Stereotypical Behaviors in *Fmr1* Knockout Mice

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Fragile X syndrome (FXS) is an inherited neurodevelopmental disorder in humans that results from the lack of fragile X mental retardation protein (FMRP) in the individual. Many individuals with FXS experience restricted interests and sensory motor behaviors, the severity of which have been linked to a brain region known as the striatum. The striatum is an essential part of the basal ganglia and is, in part, responsible for motor function, interpreting messages from the cerebral cortex and translating them into movement. There is evidence suggesting that stereotypic behaviors are strongly influenced by an increase of dopamine in this region. Over the course of this study, cocaine was administered to both *Fmr1* knockout (KO) mice, which are a model for FXS, and their wild type (WT) counterparts to determine vulnerability to the development of stereotypical behaviors elicited under various conditions. Before, during, and following exposure to cocaine, a series of tests was performed that included hole board testing, locomotor analysis, and stereotypy analysis. The results of this experiment support the idea that cocaine increases the manifestation of stereotypies in both WT and *Fmr1* KO animals. However, while the data are suggestive of KO animals being more susceptible to this behavior, we as of yet

lack conclusive evidence. Additionally, the results of the experiment presented surprising data regarding the effects of cocaine exposure on reward pathways of WT mice.

NOMENCLATURE

ANOVA	Analysis of Variance
COC	Cocaine
FMRP	Fragile X Mental Retardation Protein
FXS	Fragile X Syndrome
IP	Intraperitoneal
KO	Knockout
MC	Multiple Comparisons
RM	Repeated Measures
RNP	Repeat Nose Pokes
SAL	Saline
TNP	Total Nose Pokes
WT	Wild Type

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CHAPTER I

INTRODUCTION

Fragile X syndrome (FXS) is the most common form of inherited intellectual disability and results from the silencing of the *Fmr1* gene on the X chromosome and loss of expression of the fragile X mental retardation protein (FMRP). FXS accounts for approximately 5% of autism spectrum disorder cases. The overall manifestation of this disorder in humans produces a variety of intellectual and behavioral disabilities and promotes the development of restricted interests and behaviors. The restricted behaviors, including repetitive or stereotypical motor movements, presumably result from altered striatal function and are observable in humans with FXS and in the mouse model for FXS, the *Fmr1* knockout (KO) mouse. In this study, *Fmr1* KO and normal littermate mice are used to determine the role that FMRP, or its absence, plays on the development of restricted motor behaviors.

Genetic Basis of Fragile X Syndrome

This inherited X-linked dominant disorder, results from the lack of FMRP in an individual (Garber et al, 2008). This protein is coded for by the *Fmr1* gene located at position Xq27.3 (Krawczun et al, 1985). It has been found that an increased number of CGG repeats, which are usually present in small quantities in the noncoding region of the gene, cause transcriptional silencing (Verkerk et al, 1991). In particular, the full mutation is associated with methylation of the area, which recruits histone deacetylases to "close" the chromatin and physically block the access of transcription factors that drive the synthesis of FMRP (Kumari et al., 2001). Due to the dominant transmission of FXS, it could be expected to occur much more frequently than it does. However, it has reduced penetrance for two reasons: 1) the *Fmr1* gene is

X-linked and the females are less severely affected than males (Garber et al, 2008), and 2) the premutation (an intermediate number of CGG repeats) can be present in the genome in females and males with delayed or absent phenotypic expression. Nonetheless, FXS is still one of the most prevalent forms of inherited mental retardation.

The *Fmr1* KO mouse model used in this study, much like the human counterpart, does not express *Fmr1* mRNA or any FMRP (Bakker et al.,1994). Additionally, similar clinical symptoms of FXS, including cognitive and social deficits, and a vulnerability for restricted or repetitive behaviors have been observed in *Fmr1* KO mice. However, the model is not perfect and some have argued that using mice instead of human hematopoietic stem cells is a less effective means of studying FXS as it relates to humans (Telias et al., 2019). The argument has been made that examining stem cells in the absence of FMRP allows for a better understanding of X-inactivation, as well as the effects of the protein on cellular development (Bhakar et. al, 2012). Nonetheless, the mouse model allows for a more holistic view of the disorder and the impact that loss of FMRP has systemically. This study focuses specifically on behaviors elicited as a result of a lack of FMRP, and thus, the mouse model is most suitable.

The Role of the Striatum

The striatum is an essential part of the basal ganglia and, as a whole, coordinates information related to motor and reward function. It consists of three structures called the caudate, the putamen and the nucleus accumbens. The caudate and the putamen compose the dorsal striatum and play pivotal roles in voluntary and goal-directed movement, as well as habit-learning. The nucleus accumbens, on the other hand, is commonly referred to as part of the ventral striatum and is known for its role in reward- and aversion-related perception. Recently, striatal function has been implicated in the development of behavioral inhibition, leading certain

individuals to exhibit restricted behaviors (Helfenstein et al., 2012). This is especially prevalent in individuals with forms of autism or FXS.

It can be difficult to monitor and record these repetitive behaviors in mice and other small rodents. However, there is evidence that stereotypic behaviors are strongly influenced by an increase of dopamine in the extracellular matrix of the striatum (Fulks et al., 2010). Thus by increasing dopamine levels in the brain, specifically through the use of a psychostimulant drug, these behaviors are magnified and thus more easily studied. Our group has previously shown a vulnerability for enhanced stereotypical behavior in mice lacking FMRP when exposed repeatedly to cocaine. In this work, after the first injection, both the WT and the *Fmr1* KO mice showed similar quantities of stereotypical behaviors. However, after a few days of receiving high doses, the *Fmr1* KO mice were much more inclined to show signs of stereotypy, while they exhibited less locomotion (Smith et al., 2014). These findings indicate that FMRP plays a role in limiting the development of stereotypy, allowing the competing behavior of locomotor sensitization to take precedence. Preferential sensitization of stereotypies over locomotion may relate to impairments in the release and uptake of dopamine seen in the dorsal striatum of *Fmr1* KO mice (Fulks et al, 2010).

The difference between the behaviors expressed in *Fmr1* KO mice compared to their WT counterparts has many implications for the specific role of FMRP in the brain. Using this mouse model, our goal is to determine whether *Fmr1* KO mice show enhanced vulnerability to cocaine-induced stereotypy in response to a different (lower) dose of the drug. We hypothesize that *Fmr1* KO mice will show higher scores on the scale and greater individual behaviors associated with cocaine-induced stereotypical movements, such as behavioral cycling. We will also measure *Fmr1* KO and WT mouse behavior in a hole board apparatus before and after cocaine to further

understand behavioral manifestations of restricted behaviors in mice and better use them as a model for FXS. We hypothesize 1) that *Fmr1* KO mice treated with cocaine will show greater total and repeated nose pokes than WT cocaine-treated mice and that, 2) while WT mice will shift their attention to the familiarized stimulus (chocolate chip) during the post-test, KO mice will not make this shift and will continue to prefer their pre-test nose-poking locations.

CHAPTER II

METHODS

Animals and Dosing

Fmr1 KO mice and WT male littermate mice were randomly assigned to treatment groups. Of the KO animals, two were given intraperitoneal (IP) saline (SAL) injections and six received cocaine (COC) IP. Additionally, six WT animals received cocaine and six received saline. Cocaine was prepared (1.5 mg/mL) in saline (0.9%), and saline (0.9%) was used for control injections. Injection volume was 0.1 mL per 10 g body weight.

Locomotor Testing

Each mouse underwent an eleven-day dosing period, receiving intraperitoneal (IP) injections once daily. Following four consecutive days of baseline injections (saline), mice of each genotype received either cocaine (15 mg/kg) or saline (control) for seven additional days. On the last baseline injection day (Day 4), as well as the first and fourth day of variable dosing (Day 5 and Day 8, respectively), each mouse was video recorded in an activity arena outfitted with photobeams for 30 minutes after injection. In this test, the overall distance travelled (cm) was monitored in addition to the analysis of behavioral patterns and traits.

Stereotypy Analysis

Videos from the activity trials were analyzed in one-minute bins separated by two-minute intervals of no analysis, beginning two minutes after placement of the mouse into the arena. During the first ten seconds of every bin, the overall behavior of the animal was scored on two different published stereotypy scales (Spangler et al., 1997, Kelley et al., 1998; see Appendices

A-B). Specific stereotypic behaviors were scored as present or absent as observed for each one-minute time interval (see Appendix C).

Hole Board Preference Testing

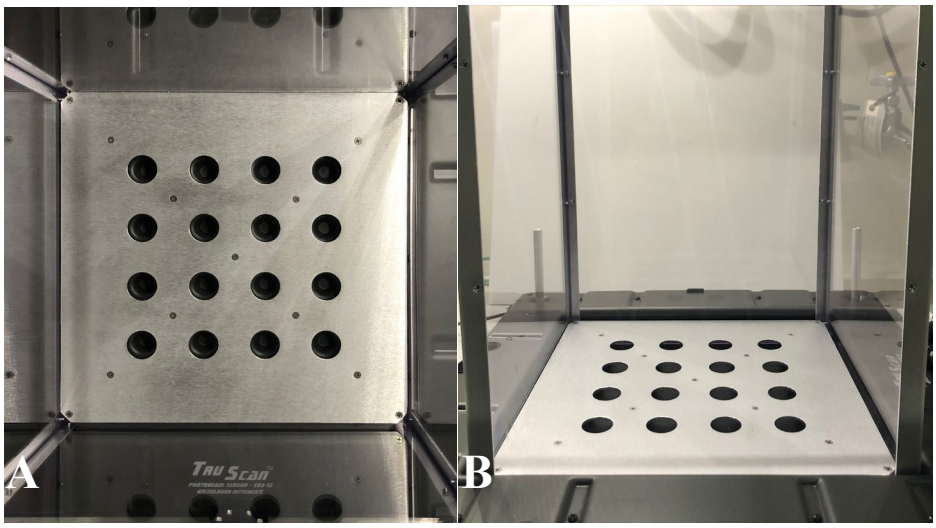

Figure 1. Hole board (a) top view and (b) side view.

Two forms of hole board testing, where normal flooring in the locomotor arena was replaced by a four x four grid of uniform holes (Fig. 1a, b), were conducted: unbaited (one pre-test and two post-tests at different time points) and baited. The unbaited pretest was conducted 5 days prior to the initiation of saline dosing. Each animal was placed in an individual arena for one hour and the number of instances in which their nose entered each hole was measured. Additionally, the instances in which they returned to the same hole without visiting another one was also counted (repeat nose pokes). The animals were again given an unbaited test on the day following the final day of variable dosing. Baited hole board began 4 days after the final day of variable dosing, where distinct reinforcers or stimuli were placed into the four center holes of the

grid and secured by wire mesh. In this particular experiment, one hole remained empty, and others contained a chocolate chip, a plain cheerio, or a small amount of clean, familiar bedding. Different bait arrangements (Fig. 2), which changed any given bait position between pre- and post-tests within the four-hole center block, were randomly used. Baited hole visits were measured in two tests (Fig. 2) separated by a 48 hour familiarization period, during which time mice were given daily home-cage access to one of the food stimuli (chocolate chips). A final unbaited hole board test was conducted approximately 31 days after the final day of variable dosing (timing varied between cohorts), and followed the same protocol as the initial unbaited hole board. Immediately following this last test, brain tissue was retrieved from the animals.

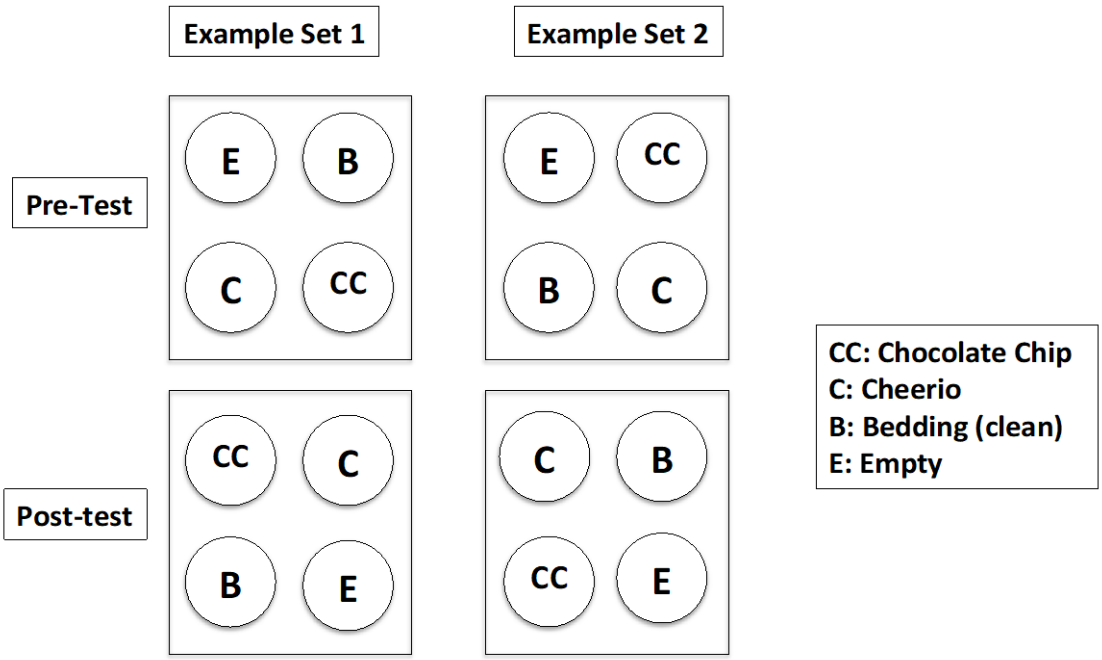

Figure 2. Example baited hole board arrangements.

Statistics

Stereotypy analyses were conducted by separate Two-Way Repeated Measures (RM) Analysis of Variance (ANOVA) over days, followed by Bonferroni post hoc or Tukey's multiple comparisons (MC). Each hole board test was analyzed by Two-Way RM ANOVA, as well as a Multiple Variable ANOVA, Tukey's MC and Bonferroni's multiple comparison (MC). Statistical analyses were performed either in GraphPad Prism or SPSS. Alpha was set at 0.05, and Greenhouse Geisser corrections are reported for RM ANOVA when tests for violation of sphericity showed statistical significance. Due to the complexity of these tests, here we focus on a subset of outcomes most relevant to the stated hypotheses.

CHAPTER III

RESULTS

Stereotypy Analysis

Stereotypical behavior was scored on the Kelley scale, for which we observed a main effect of Group (RM ANOVA, $F_{3, 31} = 4.2$, $p < 0.05$). While all four groups of mice exhibited similar behaviors on the fourth day of saline dosing (Day 4), where the average score was close to 1 ("active") for all groups, by Day 8 WT mice receiving cocaine had significantly greater stereotypy scores than WT mice receiving saline (Fig. 3a; Tukey's multiple comparison test, WT SAL vs. WT COC on Day 8, $p<0.05$). However, there was not a significant difference between the manifestation of stereotypies between the *Fmr1* KO group and the WT counterpart.

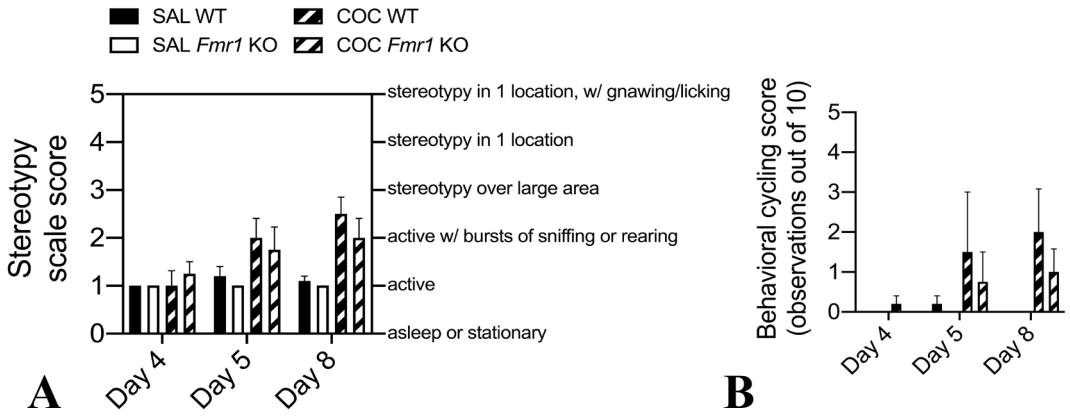

Figure 3. (a) Kelley stereotypy score and (b) behavioral cycling

In addition to the analysis of the overall impact of stereotypies on a scale for each mouse, there was specific interest placed on a variety of individual behaviors as listed in Appendix C. One of particular note was the presence of actions that our lab has termed "behavioral cycling."

This category refers to a cyclic set of a few behaviors that is repeated over part of the trial, which we observed overwhelmingly in mice that were exposed to cocaine (Fig. 3b). However, with our limited sample size in this study so far, we did not observe a significant change in groups or over time for behavioral cycling.

Unbaited Hole Board

As outlined in the methods, there were two variations of the hole board test performed. In the first of these, holes were unbaited, and tests were conducted at three different time points. At each time point, total nose pokes (TNP) were monitored, as well as repeat nose pokes (RNP), or the number of times over the trial that mice visited any given hole more than once consecutively. We used RM ANOVAs to analyze both TNP and RNP over the first two tests only. For TNP, we observed a significant group x test interaction (Fig. 4; $F_{3,14} = 9.74$, $p=0.001$), while for RNP, we observed a significant main effect of test only ($F_{1,14} = 8.00$, $p<0.01$). Follow-up analyses to the significant interaction for TNP showed, in Test 1, no differences among groups. However, for Test 2, there were significant differences between saline- and cocaine-treated WT mice (Tukey MC, $p<0.05$), saline- and cocaine-treated *Fmr1* KO mice ($p<0.001$), as well as between both cocaine-treatment groups ($p<0.05$). Animals that received drug also showed a drastic increase in poking behavior between Test 1 and 2, as illustrated by a significant increase of TNP for both WT and *Fmr1* KO cocaine-treated mice (Bonferroni MC, $p<0.05$ and 0.0001, respectively). A significant simple main effect of test was also observed for *Fmr1* KO mice (Bonferroni MC, $p<0.01$). Additionally, there seemed to be a more significant increase in the KO animals after exposure to cocaine when compared to the KO animals that only received saline. Finally, the last day of unbaited hole board show a return to normalcy amongst the TNP and a leveling out of RNP.

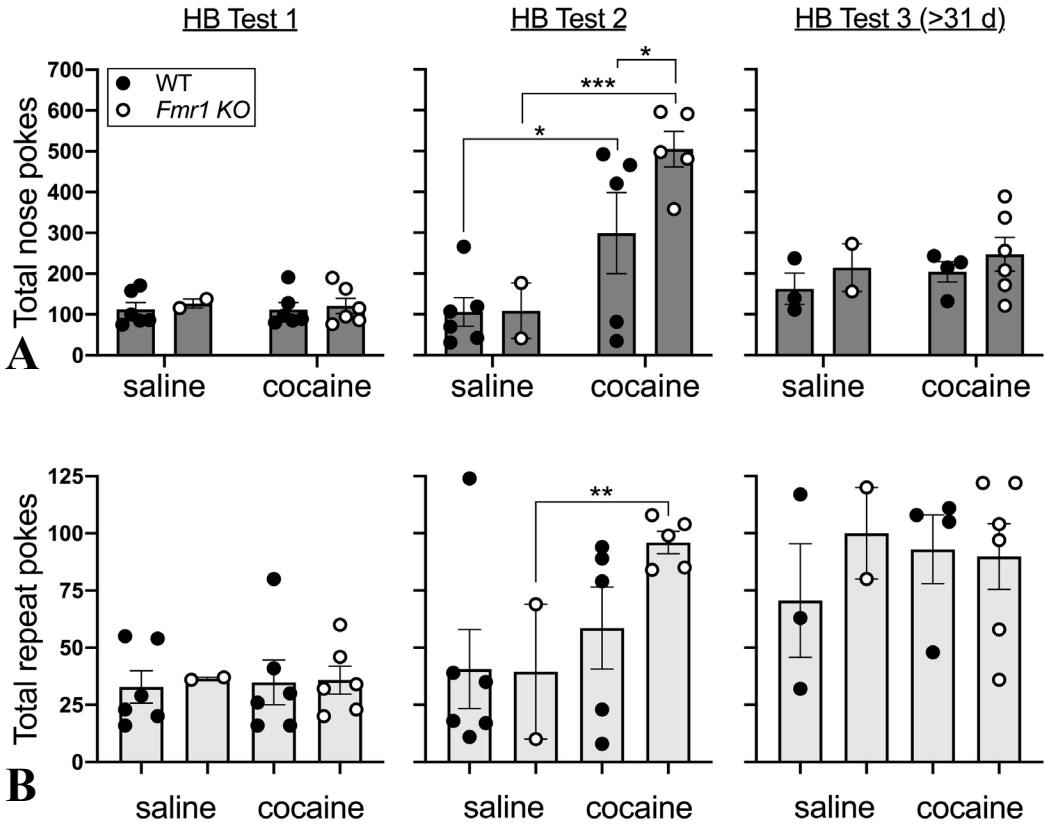

Figure 4. Unbaited hole board trials 1-3 (a) Total nose pokes and (b) Repeat nose pokes

Baited Hole Board

The baited hole board was run a total of two times with a 48-hour familiarization period for chocolate chips in between. As seen in Figure 5, group, bait type, and test (pre- versus post-) significantly affected hole board nose-poking (3-Way interaction between test, bait and group, $F_{4.4,23.5}$ =4.08, $p<0.05$). Following the interaction, we examined each bait (the chocolate chip, bedding, cheerio and the empty hole) and found significant test x group interactions for the empty hole ($F_{3,16}$ = 4.69, $p<0.05$) and the bedding, in particular ($F_{3,16}$ = 4.14, $p<0.05$). Additionally, there was an emerging trend toward a significant test x group interaction for the chocolate chip stimuli ($F_{3,16}$ = 2.98, $p=0.063$). To follow these two-way tests, we conducted a

MV ANOVA for both the pre- and post-tests. In analysis of the pre-test, we found simple main effects of group for the empty hole ($F_{3,19} = 4.17$, $p<0.05$), bedding ($F_{3,19} = 3.63$, $p<0.05$), and chocolate chip ($F_{3,19} = 4.83$, $p<0.05$) holes. Furthermore, consequent Bonferroni Pairwise Comparisons revealed significant differences with regards to the empty hole between WT SAL and KO SAL groups ($p<0.05$), as well as between KO SAL and KO COC ($p<0.05$). The same analysis also revealed significant differences for bedding between WT SAL and KO SAL ($p<0.05$), and for the chocolate chip between WT SAL and WT COC ($p<0.05$).

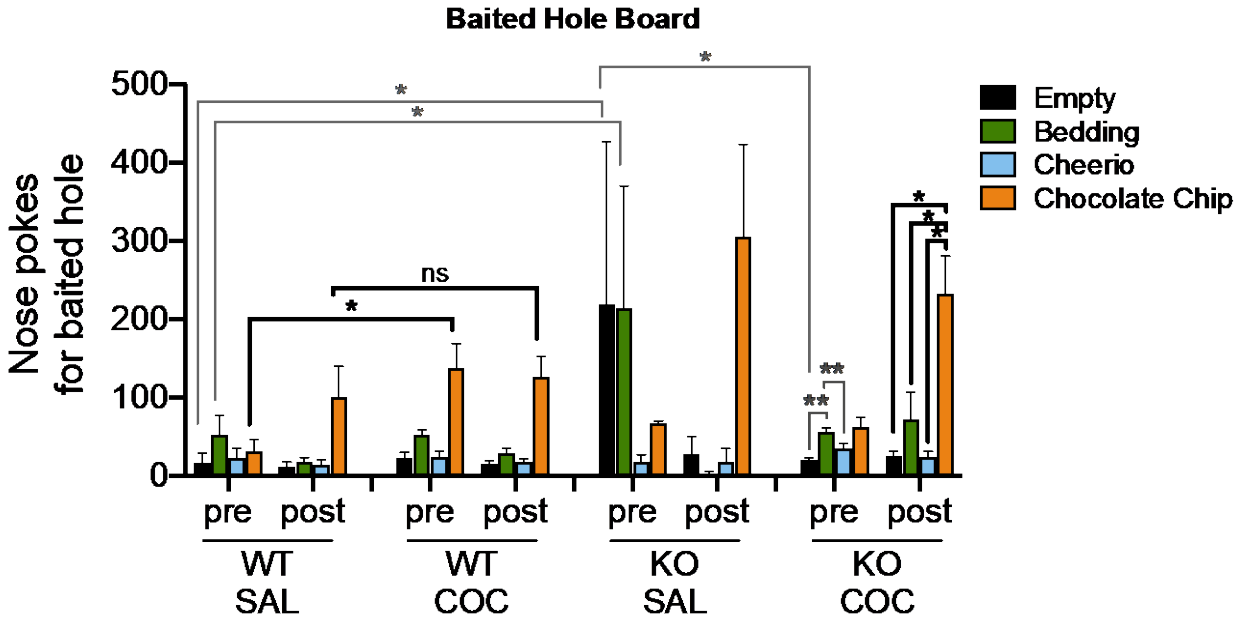

Figure 5. Baited hole board preference testing.

The MV ANOVA, when performed on the post-test, only expressed a significant simple main effect of group for the chocolate chip ($F_{3,19} = 3.25$, $p<0.05$). However, no further significance was observed in following comparisons. Additional follow-up to the overall three-way interaction using a two-way RM ANOVA assessed each group individually. The KO COC

group was the only group that revealed a significant test x bait interaction ($F_{3,15} = 9.08$, $p<0.01$). A consequent univariate ANOVA showed a significant simple main effect of bait during the pre-test. ($F_{3,15} = 9.06$, $p<0.01$), as well as for the post-test ($F_{3,15} = 16.82$, $p<0.01$). Tukey's MC of the pre-test expressed significant differences between the empty hole and the bedding stimulus ($p<0.01$), as well as between the bedding and cheerio stimuli ($p<0.01$). For the post-test, significant differences (Tukey's MC) between the empty hole and the chocolate chip stimulus ($p<0.05$), between the bedding and chocolate chip stimuli ($p<0.05$), and between the cheerio and chocolate chip stimuli ($p<0.05$).

CHAPTER IV

CONCLUSION

FMRP is a versatile protein, the lack of which results in the manifestation of FXS. Although several general functions of the protein are understood, little is known about the specific mechanisms by which it affects behavior or the complete implications that it might have on the normal functioning of an individual. Here, we begin to address FMRP's role in regard to the processing of striatal motor behaviors and reward, and find evidence that lack of FMRP enhances the development certain stereotypical actions. Additionally, we find that presence of FMRP may enhance detection of or interest in higher-value reinforcers following exposure to a drug of abuse, but it is not necessary for normal shifts toward higher-value reinforcers following familiarization.

Based on a prior study, we expected that stereotypical behaviors in mice that model FXS would be enhanced by the presence of cocaine; however, not all of the results of this experiment reveal a difference between *Fmr1* KO animals and the WT animals. In particular, the results of the Kelley scale and the behavioral cycling analysis do not indicate much of a difference between genotypes. However, there was statistical significance found in the unbaited hole board test implicating FMRP in the dampening of stereotypies (e.g., TNP and RNP) following exposure to cocaine, supporting our hypothesis. Our baited hole board hypothesis, that *Fmr1* KO mice would fail to shift attention to the familiarized stimulus, was not supported. With our limited sample size, if anything, it appears that KO mice may have developed greater interest in the chocolate chip stimulus than WT mice, but the study will need to be completed to determine any significant effect.

In addition, an unusual discovery emerged as a result of the baited hole board trial, pointing to a role for FMRP in the attraction to, or possibly detection of, natural reward after exposure to cocaine. The premature interest in the chocolate chip that was exhibited by the WT cocaine, but not in the KO cocaine, group suggests that the presence of FMRP in conjunction with drug exposure leads to alterations in the reward pathways, presumably of the basal ganglia. This finding presents interesting groundwork for further research into the field of addiction and the effects of cocaine on the brain.

This study, while possessing potential for future research, has a handful of limitations that affect its interpretation. The data presented above is representative of a rather small sample size; meaning that while the emerging trends are compelling, there is still much work to be done in order to obtain more conclusive results. Additionally, due to unforeseen circumstances, the final unbaited hole board test was not able to be conducted for the third cohort, adding to the issue of a smaller sample size. In the future, work can be done to increase the sample size and thus add more credibility to the results. Furthermore, the methods of behavioral measurement were not apt for measuring the effects of FMRP on locomotion, and would have been beneficial to our interpretations.

Overall, our results support the hypothesis that presence of FMRP mutes the development of stereotypical behaviors, though mechanics of this process will have to be explored further. Interestingly, this process seemed to be highlighted better in the hole board than activity field. Through this study, more was learned about the role that FMRP plays in development of stereotypical behaviors that emerge under a variety of conditions. These findings help to provide a framework that could potentially be used in further FXS research. However, the experiment also provided unanticipated insight into the combined role of FMRP and cocaine in relation to

different aspects of neural functioning. Hopefully in the future more can be done in order to identify just how this protein alters the basal ganglia in order to achieve the expressed behaviors observed in this experiment.

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APPENDIX A

Stereotypy Scale (Modified) From (Spangler et al., 1997)

(1) asleep, inactive

(2) alert, actively grooming

(3) increased sniffing

(4) intermittent rearing and sniffing

(5) increased locomotion

(6) intense sniffing in one location

(7) continuous pivoting and sniffing

(8) continuous rearing and sniffing

(9) maintained rearing and sniffing

(10) splayed hind limbs

APPENDIX B

Kelley Score (Kelley et al., 1998)

(0) asleep or stationary

(1) active

(2) predominately active with bursts of sniffing or rearing

(3) stereotyped activity predominately sniffing/rearing over large area of the cage

(4) stereotyped behavior maintained in one location

(5) stereotyped behavior in one location with bursts of gnawing/licking

APPENDIX C

Behaviors of Interest (Smith et al., 2014)

- Still: lying still with no movement- count of 5s
- Still/Affected: lying still with eyes open but behavior seems abnormal- count of 5s
- Pause: animal pauses for 1-3s
- Groom: head or body grooming
- Locomotor: mouse crosses the midline of the chamber at least once
- Rear: standing on hind legs
- Repeated Rear: standing on hind legs at least 5 separate times over a 10s interval
- Head-up Sniff: continuous sniffing with head pointed to top of cage for at least 5s
- Head-down Sniff: continuous sniffing with head pointed at the ground for at least 5s
- Behavioral Cycling: cycle of behaviors repeated in the same order at least once
- Jerk: quick jumpy movements followed by brief freezing (mouse seems startled)
- Mouth Movements: oral movements, tongue protrusions etc…
- Hop: short rabbit-like hops producing locomotion
- Jaw Tremor: involuntary shaking of jaw/cheek area
- Bite: biting cage floor or other objects
- Self-gnaw: intense repetitive gnawing of their own body parts (foot, leg etc…)
- Taffy Pull: repetitive paw to mouth movements
- Paws to Mouth: holding paws cupped under their chin
- Yawn: typical yawning behavior
- Head Sway: rhythmic, lateral swaying of their head (might be quite quick)
- Head Bob: repeated, vertical movement of their head