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Genetic reduction of group 1 metabotropic glutamate receptors alters select behaviors in a mouse model for fragile X syndrome

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Abstract

Introduction—Genetic heterogeneity likely contributes to variability in the symptoms among individuals with fragile X syndrome (FXS). Studies in the *Fmr1* knockout (KO) mouse model for FXS suggest that excessive signaling through group I metabotropic glutamate receptors (Gp1 mGluRs), comprised of subtypes mGluR1 and mGluR5, may play a role. Hence, Gp1 mGluRs may act as modifiers of FXS. Currently no studies have addressed whether manipulation of mGluR1 activity may alter *Fmr1* KO behavioral responses, and only a few have reported the effects of mGluR5 manipulation. Therefore, the goals for this study were to extend our understanding of the effects of modulating Gp1 mGluR activity on *Fmr1* KO behavioral responses.

Methods—The present study determined if genetically reducing mGluR1 or mGluR5 by 50% affects an extensive array of behaviors in the *Fmr1* KO.

Results—Reduction of mGluR1 moderately decreased *Fmr1* KO activity. Reduction of mGluR5 caused an analgesic response in the *Fmr1* KO and decreased active social behavior. Modulation of either mGluR1 or mGluR5 did not significantly alter audiogenic seizures, anxiety- and perseverative-related responses, sensorimotor gating, memory, or motor responses.

Conclusions—Genetic reduction of mGluR1 or mGluR5 modified a few select *Fmr1* KO behaviors, although these modifications appeared to be subtle in nature and/or limited to select behaviors. This may indicate that 50% reduction of either mGluR1 or mGluR5 is insufficient to produce behavioral changes, and therefore, these receptors may not be dominant modifiers of a number of *Fmr1* KO behavioral phenotypes.

Keywords

Fragile X syndrome; FMR1; Metabotropic glutamate receptor; Mouse model; Behavior

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1. Introduction

Fragile X syndrome (FXS) represents a genetic condition that is typically due to inactivation of the *FMR1* gene resulting from a CGG triple repeat expansion in the *FMR1* promoter region, thereby leading to a loss of the fragile X mental retardation protein (FMRP) [1]. Individuals with FXS may exhibit a wide number of physical and behavioral dysfunctions ranging in severity, some of which include problems with learning, hyperactivity, anxiety, childhood epilepsy, hypersensitivity, and social interactions [2]. The variability in symptom severity may be dependent on the length of the expanded CGG repeat [3,4], variation in FMRP expression [5–9], and environmental factors [8], but it may also be due in part to the heterogeneity of the genome between individuals. Therefore, while disruption of the *FMR1* gene may be the primary insult in FXS, the profile of other genes may alter the manifestation of symptoms. The identification of secondary genes will help us to better understand FXS and how to better design therapeutic agents.

Through a mutation in the *Fmr1* gene the *Fmr1* knockout (KO) mouse recapitulates the loss of FMRP [10]. We have recently shown that behavioral differences arise when the genetic background is altered in *Fmr1* KO mice, supporting a role for other genetic factors contributing to behavioral variance in this model [11]. Based on other studies in this model, an mGluR theory was proposed in which a loss of FMRP leads to excessive signaling through group I metabotropic glutamate receptors (Gp1 mGluRs) which may contribute to the phenotypes observed in the *Fmr1* KO [12]. This theory is supported in part by studies demonstrating abnormal Gp1 mGluR-mediated processes in the *Fmr1* KO including dysfunction in translating a number of mRNAs upon Gp1 mGluR activation [13], as well as exaggerated forms of long-term depression (LTD) mediated through Gp1 mGluRs [14,15]. Furthermore, Gp1 mGluR antagonists have the ability to alleviate or even normalize some abnormal phenotypes in various models of FXS [16–19]. Therefore, Gp1 mGluRs may serve as attractive candidates for modification of *Fmr1* KO phenotypes.

Within the family of mGluRs, group I is specifically comprised of the subtypes mGluR1 and mGluR5 due to similar structures and physiological activity [20]. While both receptors can be found in many regions throughout the brain, mGluR1 is highly expressed in the cerebellum, thalamus, and CA3 pyramidal cells of the hippocampus, and mGluR5 is highly expressed in the CA1 and CA3 pyramidal cells of the hippocampus, striatum and cortex [20–22]. This differential brain distribution for each subtype points to the possibility that differing functional roles may exist between these two receptors, and this may have important consequences for FXS. Currently there is evidence supporting a role for both receptor subtypes in the *Fmr1* KO. Early studies of hippocampal Gp1 mGluR-mediated LTD (exaggerated in the *Fmr1* KO) demonstrated that mice lacking mGluR5 exhibit a loss of this form of LTD [23] and the mGluR5 antagonist MPEP (2-methyl-6-phenylethynyl pyridine hydrochloride) can reduce or eliminate mGluR-LTD [24–27], together indicating that this LTD may specifically be an mGluR5-mediated event. However, a more recent study suggests that this hippocampal mGluR-LTD may actually be mediated by both mGluR1 and mGluR5 [28]. Furthermore, mGluR-mediated LTD found at the parallel fiber-Purkinje cell synapse in the cerebellum, which is also abnormal in the *Fmr1* KO [15], has been characterized as mGluR1-dependent [29]. These data therefore imply that both mGluR1 and mGluR5 may play a role in FXS.

If Gp1 mGluRs act to modify fragile X phenotypes then altering the expression or signaling through these receptors should result in changes in FXS phenotypes. The ability of MPEP to ameliorate abnormal phenotypes in *Drosophila* [16], zebrafish [18] and mouse [17,19] models of FXS corroborates this idea. For example, MPEP decreases the overabundance of immature spines in the *Fmr1* KO in addition to reducing the susceptibility to audiogenic

seizures and restoring normal eye-blink-mediated sensorimotor gating. Furthermore, 50% genetic reduction of mGluR5 in the *Fmr1* KO can rescue abnormal ocular dominance plasticity, dendritic spines, inhibitory avoidance extinction, and susceptibility to audiogenic seizures [30]. While these results provide exciting evidence for mGluR5 as a modifier of FXS phenotypes, currently little effort has been made to investigate whether or not mGluR1 may also serve as a modifier of *Fmr1* KO responses. Moreover, a more extensive approach to exploring mGluR5's role as a modifier of behavior has not been conducted, as only activity, audiogenic seizures, one type of learning and memory, and a non-traditional form of measuring sensorimotor gating (eye-blink vs. whole body flinch response) have been examined.

Therefore, the present study aimed to (1) address the absence of experiments addressing whether mGluR1 may act as a modifier of *Fmr1* KO behaviors and (2) test the ability of mGluR5 as a modifier on a more extensive behavioral battery to expand on what has previously been reported. In order to accomplish these goals, we chose to evaluate the effects of a 50% genetic reduction of either mGluR1 or mGluR5 on *Fmr1* KO behaviors. Taking a genetic approach over a pharmacological one allows us to be more confident that we are specifically modulating only a certain receptor (vs. potential nonspecific effects at other sites with a drug), as well as the ability by using 50% receptor reduction to ask if these receptors are *dominant* modifiers of *Fmr1* KO behaviors.

2. Experimental Design and Methods

2.1. Animals

We generated *Fmr1* knockout (KO) mice with 50% reduction of either mGluR1 or mGluR5. The mGluR1-*Fmr1* mouse line (mG1 line) was created from crosses between C57BL/6J (B6) congenic (N12-N13) *Fmr1* HT females and B6 congenic (N9-N10) mGluR1 HT males (The Jackson Laboratory, Bar Harbor, ME, USA), generating four male genotypes: wild-type (WT), *Fmr1* KO (*Fmr1*), mGluR1 HT (mG1), and mGluR1 HT+*Fmr1* KO (mG1.*Fmr1*). The mGluR5-*Fmr1* line (mG5 line) was created similarly from crosses between B6 congenic (N12-N13) *Fmr1* HT females and B6 congenic (N8-N9) mGluR5 HT males (The Jackson Laboratory), generating four male genotypes: WT, *Fmr1*, mGluR5 HT (mG5), and mGluR5 HT+*Fmr1* KO (mG5.*Fmr1*). Social experiments utilized B6 mice (The Jackson Laboratory) as social partners. Only males were tested at 2–4 months of age except for those tested for audiogenic seizures in which mice were tested at 20–22 days of age.

Mice were housed 2–5 per cage and maintained on a 12:12 light/dark cycle with food and water available ad libitum. Mice were acclimated to the testing room for 30 min prior to testing, which occurred between 8:00 AM and 1:30 PM. All procedures were approved by the Baylor Institutional Animal Care and Use Committee in accordance with NIH guidelines.

2.2. Genotyping

Screening on the *Fmr1* locus for the WT allele was performed using primers S1 (5'-GTG GTT AGC TAA AGT GAG GAT GAT-3') and S2 (5'-CAG GTT TGT TGG GAT TAA CAG ATC-3') and for the KO allele using primers S1 and N2 (5'-TGG GCT CTA TGG CTT CTG A-3'). PCR reactions for WT and KO alleles were run separately using the following protocol: 2 min at 94 °C; 35 cycles comprised of 30 sec at 94 °C, 30 sec at 58 °C, and 30 sec at 72 °C; 10 min at 72 °C. Screening on the mGluR1 gene locus for the WT allele was accomplished using primers F (5'-GGC CAA AGG CAC GAA GCG GCA AAA G-3') and R (5'-TCT CCG TCC ATT CTC GCC ACA-3') and for the KO allele using primers R and Neo1 (5'-GAC GAG TTC TTC TGA GGG GAT CGA TC-3'). PCR reactions for the

mGluR1 gene locus were run as follows: 3 min at 94 °C; 35 cycles comprised of 10 sec at 94 °C, 30 sec at 60 °C, 90 sec at 68 °C; 7 min at 68 °C. Screening on the mGluR5 gene locus for the WT allele was performed using primers W1 (5'-CAC ATG CCA GGT GAC ATC AT-3') and W2 (5'-CCA TGC TGG TTG CAG AGT AA-3') and for the KO alleles using primers W1 and Neo5 (5'-CAC GAG ACT AGT GAG ACG TG-3'). PCR reactions for the mGluR5 gene locus were run with the following protocol: 2 min at 94 °C; 35 cycles comprised of 30 sec at 94 °C, 30 sec at 54 °C, 1 min at 72 °C; 10 min at 72 °C.

2.3. Double Genetic Mutant Mice Testing Procedure

We tested subjects from the mG1 and mG5 lines using three sets of mice. One set passed through a battery of behavioral tests (each test separated by 1–3 days of no testing) including: two sequential days of open-field activity, light-dark exploration, marble burying, prepulse inhibition, fear conditioning, hot plate, and rotarod (only animals in the mG1 line were tested in the rotarod test). A behavioral battery was chosen for the following reasons: (1) to minimize the number of mice in the study by testing each mouse through multiple behaviors, and (2) a more comprehensive behavioral analysis can be achieved through testing across multiple domains of CNS function [31,32]. Another set was tested in the social interaction test. The third set was tested for audiogenic seizures.

2.4. Behaviors

2.4.1. Open-field activity (OFA)—Activity and anxiety-related responses were evaluated in the OFA assay as previously described [33]. Each mouse was placed into a clear Plexiglas (40 × 40 × 30 cm) arena. The VersaMax Animal Activity Monitoring System (AccuScan Instruments, Columbus, OH, USA) recorded animal activity. Testing occurred in the presence of overhead bright lights (750 lux) and white noise (55 dB). The total distance traveled (locomotor activity) and center distance ratio [anxiety-related measure [34] defined as the ratio between the distance traveled in the center (22.5 cm × 22.5 cm) vs. the total distance traveled] were examined over a 30 min exploratory period.

2.4.2. Light-dark exploration—Anxiety-related responses were evaluated using the light-dark exploration test as previously described [32]. Testing occurred in a Plexiglas box comprised of two unequally sized chambers separated by a small opening (8.5 × 5 cm). The large chamber (30 × 21 × 21 cm) was clear and brightly lit (750 lux) with an open top. The smaller chamber (15 × 21 × 21 cm) was black with a closed top. Mice were positioned in the light side and transitions between the chambers were recorded for 10 min. A transition was counted when all four paws of the subject transferred from one chamber to the next. White noise (55 dB) was present during testing.

2.4.3. Marble burying—Repetitive-like behavior was determined using the marble-burying assay as previously described [33]. Twenty black glass marbles (15 mm diameter) were arranged in a 4 × 5 pattern on top of bedding (4.5 cm SANI-CHIP) in clean, standard mouse cages (27 × 16.5 × 12.5 cm). One end of each cage was left clear of marbles (~ 5 cm) in order to place a mouse in the cage. Each mouse was allotted a 20-min exploration period after which the number of marbles buried (covered by >50% bedding) was recorded. Testing was performed in the presence of white noise (55 dB).

2.4.4. Startle response and prepulse inhibition (PPI)—The prepulse inhibition test was used to study sensorimotor gating as previously described [35]. The testing apparatus consisted of the SR-LABTM startle response system (San Diego Instruments, San Diego, CA, USA). Each mouse was placed in a clear, cylindrical holding tube within a sound attenuating chamber and habituated to white noise (70 dB) for 5 min immediately prior to testing. The test was comprised of six blocks, with each block containing the following eight trial types

presented in a pseudo-randomized order: no stimulus (70 dB), startle stimulus (120 dB, 40 ms), three prepulse only stimuli (74, 78, 82 dB; 20 ms), and three trials where the three prepulse stimuli were presented 100 ms before the startle stimulus (e.g. 74 dB prepulse + 120 dB startle). The inter-trial interval was 10–20 sec. Startle responses for each trial were detected as force changes within the holding tube and were recorded for 65 ms following stimulus presentation. Percent prepulse inhibition of the startle response was calculated for each prepulse as follows: $100 - [(response\ to\ trials\ with\ prepulse\ and\ startle\ stimulus / response\ to\ trials\ with\ startle\ stimulus\ alone) \times 100]$.

2.4.5. Conditioned fear—Abnormalities in learning and memory were examined using the conditioned fear test as previously described [32]. On the day of training, a mouse was transferred to a test chamber (Med Associates Inc., St. Albans, VT, USA) and received 2 CS-US pairings, each consisting of the following: 2 min no stimulus, 30 sec conditioned stimulus (CS, white noise at 80 dB), 2 sec unconditioned stimulus (US, mild foot shock, 0.75 mA). Ethanol (30%) was used to clean the apparatus between animals. The following day, mice were first tested for contextual fear by transferring them to the test chamber for 5 min (no sound or foot shock). Approximately one hour later, mice were tested for cued fear (“CS test”). The context was altered during the CS test by placing a white Plexiglas on the floor of each chamber as well as along a vertical diagonal from opposing corners (creating a triangular test area). One-teaspoon vanilla imitation extract was placed in the chamber behind the vertical Plexiglas and isopropanol (70%) was used to clean between animals in order to alter the odor. The CS test consisted of a 3-min pre-CS phase (no sound), followed by a 3-min CS-phase (CS presented). Freezing behavior for all tests was scored every 10 sec, and was defined as the absence of visible movement (except for respiration). Percentage freezing behavior was calculated as the percentage of observations in which a mouse froze. Freezing during the CS test (day 2) was analyzed by subtracting the % freezing during the pre-CS from that during the CS-phase in order to control for background freezing behavior.

2.4.6. Hot plate—Analgesia-related responses (e.g. sensitivity to a painful thermal stimulus) were measured using the hot-plate analgesia meter (Columbus Instruments, Columbus, OH, USA). A mouse was placed onto a heated plate (55 °C). The time to the first hind-limb response as defined by shaking, jumping, or licking was recorded. The test was terminated once a hindlimb response was observed or a maximum time of 45 sec.

2.4.7. Rotarod—Motor coordination and skill learning were assessed using the Ugo Basile Rota-Rod apparatus for mice (Ugo Basile, Comerio, VA, Italy) as previously described [32]. Mice were placed on a rotating drum (3 cm diameter) that accelerated from 4–40 rpm over a 5-min period. The time spent walking on top of the drum was recorded. Mice were given three trials/day over three consecutive days, with a 15 min inter-trial interval.

2.4.8. Direct social interaction test—Social behavior was evaluated using the direct social interaction test as previously described [36]. Test subjects were weight-matched with B6 male mice “partners”. Each subject and partner was placed into a standard mouse cage on opposite sides of a clear, perforated (0.6 cm diameter holes) partition that allowed mice to see and smell one another. Mice were given water and food ad libitum. Approximately 24 hours later, the cage lid was replaced with a clear, perforated lid and mice were habituated for 5 min. The partition was then removed and behavior was recorded for 10 min using an overhead video camera. Videos were subsequently scored for three types of behavior: active social interaction (subject initiates interaction with partner), passive social interaction (partner initiates interaction with subject), and nonsocial interaction (subject engaged in nonsocial behaviors such as digging, exploring, self grooming). Videos were scored using a

Psion handheld computer (Psion Teklogix, Inc., Mississauga, ON, Canada) with the Noldus OBSERVER program (Noldus Information Technology, Wageningen, Netherlands).

2.4.9. Audiogenic seizures—Mice (20–22 days old) were tested for their susceptibility to sound-induced seizures. Mice were placed in a clean, standard mouse cage. A clear Plexiglas lid containing two mini-personal alarms (SKU 49–728, RadioShack, Fort Worth, TX, USA) was placed on top of the cage. The alarms were affixed on the underside of the lid such that each was centered over one half of the cage. Upon activation, each alarm emitted a 140 dB sound. The cage was placed in an illuminated sound-attenuating chamber. We have found that most seizures in *Fmr1* KO mice in our lab do not occur during a single sound presentation, and therefore we employed an acute priming protocol based on one used in Yan et al. [17]. The test protocol involved two presentations of the following: 1 min with no sound, 2 min with alarms ON. The presence of a seizure, defined as wild running and/or myoclonic/myotonic convulsions, was recorded.

2.5. Western blotting

Following cervical dislocation, hippocampi and cerebella were rapidly removed and homogenized using glass, handheld homogenizers on ice with homogenization buffer (10 mM Hepes, 150 mM NaCl, 50 mM NaF, 1 mM EDTA, 1 mM EGTA, 1 mM $\text{Na}_4\text{P}_2\text{O}_7$, 0.1% Triton-X 100, 0.1% SDS, protease inhibitor cocktail (Sigma, Saint Louis, MO), phosphatase inhibitor cocktails 1 and 2 (Sigma)). Samples were centrifuged at $2,000 \times g$ to separate insoluble material. Protein concentrations were determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA). Equal protein amounts of each sample were size-separated using SDS-PAGE (7.5% gel). Samples were mixed 1:1 with a 2x Laemmli sample buffer (0.125 M Tris HCl, 4% SDS, 20% glycerol, 0.02% bromophenol blue, 0.3% 2-mercaptoethanol, 0.15 M DTT) and incubated at 98 °C for 5 min prior to loading. Protein was transferred to a PVDF membrane using a semi-dry transfer apparatus (Bio-Rad). Membranes were blocked with 5% non-fat dry milk, TTBS (Tris-buffered saline, 0.1% Tween-20) for one hour at room temperature. Membranes were incubated with primary antibodies overnight at 4 °C with gentle agitation. Primary antibodies were diluted as follows with 5% bovine serum albumin, TTBS: mGluR1 antibody (Abcam, Cambridge, MA), 1:1,000; mGluR5 antibody (Tocris), 1:10,000; FMRP antibody (Millipore, Billerica, MA), 1:5,000; β -actin antibody (Abcam), 1:50,000. Membranes were washed three times with TTBS, 10 min each, after which they were incubated at room temperature for one hour with appropriate secondary antibodies diluted with 5% milk, TTBS. After washing three times with TTBS, 10 min each, membranes were exposed to ECL Plus (GE Healthcare Bio-Sciences, Piscataway, NJ) for 5 min and then exposed to film. Bands were scanned and quantitated using Image J (NIH, Bethesda, MD, <http://rsb.info.nih.gov/ij/>). All band intensity levels are expressed relative to actin, the loading control. In order to compare samples run on different gels, an independent single protein sample (whole brain crude homogenate) was loaded on every gel, and therefore all experimental samples within a given gel are expressed relative to the independent sample.

2.6. Statistical Analyses

A one-way independent ANOVA (genotype) was performed for each experiment except for audiogenic seizures. An ANOVA with repeated measures was calculated for the rotarod experiments. Fisher's exact test was performed for all audiogenic seizure experiments. The Least Significant Difference test was used for all post-hoc analyses. All statistical analyses were performed separately within each genetic cross (i.e. ANOVAs and Fisher's tests for the mG1 line with any necessary post-hoc analysis comparing all four genotypes within the mG1 line were completed separately from that for the mG5 line).

3. Results

3.1. Reduction of mGluR1 or mGluR5 in the *Fmr1* KO

We created *Fmr1* KO mice with reductions in either mGluR1 or mGluR5. Western blot analysis confirmed approximately 50% reductions of these receptors in heterozygous mice (Fig. 1). A main effect for genotype was found for mGluR1 in the mG1 line [$F(3, 36)=7.96$, $p<0.001$] and mGluR5 in the mG5 line [$F(3, 43)=4.72$, $p=0.006$]. Compared to WT and *Fmr1*, levels for mGluR1 were significantly lower in mG1 ($p=0.001$ and 0.025 , respectively) and mG1.*Fmr1* mice ($p<0.001$, $p=0.005$), and mGluR5 levels were decreased in mG5 ($p=0.021$ and 0.009) and mG5.*Fmr1* mice ($p=0.014$ and 0.006). No differences in Gp1 mGluRs were observed between WT and *Fmr1* mice (p -values > 0.2).

3.2. Assessment of behavioral modification

The primary question for this study was whether or not mGluR reduction alters *Fmr1* KO behavior; therefore, modification was defined as a significant change in either the mG1.*Fmr1* or mG5.*Fmr1* compared to the *Fmr1* KO. Likewise, no modification is defined as no significant change from the *Fmr1* KO. Since abnormal behavior in the *Fmr1* KO is defined as a significant difference from WT, we define a more moderate or partial modification as a behavioral change that is not significant compared to the *Fmr1* KO, but is also no longer significant when compared to the WT.

3.2.1. Modification of *Fmr1* analgesic response by mGluR5—The ability to respond to a thermal stimulus on a hot plate (55 °C) was utilized to examine analgesia responses (Fig. 2). While there was no overall main effect for the latency to respond in the mG1 line [$F(3, 88)=1.89$, $p=0.137$], we did observe a main effect for the mG5 line [$F(3, 73)=2.95$, $p=0.038$]. In the mG5 line *Fmr1* and mG5 mice did not differ in their responses compared to WT; however, mG5.*Fmr1* double-mutant mice exhibited significantly longer response times than WT ($p=0.029$), *Fmr1* ($p=0.01$), and mG5 mice ($p=0.025$), suggesting that a reduction in mGluR5 in combination with a loss of FMRP caused a decrease in thermal sensitivity.

3.2.2. Moderate modification of *Fmr1* activity by mGluR1—Locomotor activity was assessed by measuring the total distance traveled in the open-field activity (OFA) assay. For the mG1 line, we observed an overall main effect of distance traveled [Fig. 3a; $F(3, 88)=3.47$, $p=0.019$]. Consistent with previous reports [10,11,37–39], follow-up analysis revealed increased activity in *Fmr1* mice compared to WT ($p=0.002$). Both mG1 and mG1.*Fmr1* mice did not differ in activity compared to either WT ($p=0.095$, 0.092 respectively) or *Fmr1* ($p=0.142$, 0.137), indicating that the activity of mG1.*Fmr1* mice was intermediate to that observed in WT and *Fmr1* mice.

We also observed an overall main effect of distance traveled for the mG5 line [Fig. 3b; $F(3, 73)=3.92$, $p=0.012$]. As observed with the mG1 line, *Fmr1* mice in this group also exhibited significantly elevated activity over WT mice ($p=0.036$). Compared to WT, mG5 mice were not different ($p=0.8$); however mG5.*Fmr1* mice displayed hyperactivity ($p=0.007$) that was comparable to *Fmr1* activity ($p=0.393$).

Habituation to the novelty of the open field was observed as reflected by a decrease in activity on day 2 compared to day 1, and no interactions were observed between day and genotype indicating that all genotypes habituated similarly within each line (data not shown).

3.2.3. Moderate modification of *Fmr1* social behavior by mGluR5—Our lab has previously demonstrated abnormal social behavior in *Fmr1* KO mice [36,40]. In a direct social interaction test with an unfamiliar mouse, *Fmr1* KO mice tend to initiate and engage in more interactions than WT mice. In the experiments for this study, we specifically evaluated two parameters: frequency (the number of times a behavior occurred) and duration (the time spent in the behavior).

In the mG1 line, we observed an overall main effect for the frequency of active social interactions [Fig. 4a; $F(3, 66)=5.89, p=0.001$]. *Fmr1* mice initiated more social interactions than WT mice ($p<0.001$). While mG1 behavior did not differ compared to WT ($p=0.163$), mG1.*Fmr1* behavior did not differ from *Fmr1* behavior ($p=0.714$) and was significantly higher than WT ($p=0.002$), indicating that mGluR1 reduction had no effect on the frequency of active *Fmr1* KO social behavior. There was also a trend towards an effect for the duration of these interactions, but it did not reach significance [Fig. 4c; $F(3, 66)=2.55, p=0.063$].

In the mG5 line, we observed overall main effects for both the frequency and duration of active social interactions [Fig. 4b, frequency: $F(3, 78)=6.47, p=0.001$; Fig. 4d, duration: $F(3, 78)=3.73, p=0.015$]. *Fmr1* mice in this line performed active behaviors with increased frequency and duration versus WT ($p=0.003, 0.001$ respectively). Behavior in mG5 and mG5.*Fmr1* mice did not differ compared to WT (mG5 and mG5.*Fmr1* respectively; frequency: $p=0.262, 0.083$; duration: $p=0.191, 0.068$). On the other hand, while mG5 mice behaved significantly different than *Fmr1* mice (frequency: $p<0.001$, duration: $p=0.035$), mG5.*Fmr1* mice were not different compared to not only WT but also not different than *Fmr1* mice (frequency: $p=0.128$, duration: $p=0.087$), thereby indicating that mGluR5 reduction only partially modifies *Fmr1* KO social behavior.

There were no differences in passive social interactions in either line for both the frequency [mG1 line: $F(3, 66)=0.39, p=0.760$; mG5 line: $F(3, 78)=0.92, p=0.437$] and duration [mG1 line: $F(3, 66)=0.60, p=0.618$; mG5 line: $F(3, 78)=1.78, p=0.158$] of these interactions.

3.2.4. Potential modification of *Fmr1* audiogenic seizure susceptibility by both mGluR1 and mGluR5—*Fmr1* KO mice exhibit an increased susceptibility to audiogenic seizures [41], and therefore we chose to evaluate the consequences of Gp1 mGluR reduction in the *Fmr1* KO in this behavioral paradigm. Using Fisher's exact test we found significant elevations in seizures in *Fmr1* mice over that of WT within both lines [Fig. 5; mG1 line: $p=0.04$; mG5 line: $p=0.005$]. While mice heterozygous for either mGluR1 or mGluR5 displayed a low incidence of seizures analogous to that of WT mice (mG1: $p=0.57$; mG5: $p=0.2$), double mutant mice for both lines exhibited significantly more seizures than WT mice (both lines: $p<0.001$). Reduction of either mGluR1 or mGluR5 in the *Fmr1* KO appeared to further exacerbate the seizure phenotype (mG1.*Fmr1* seized 88% more than *Fmr1*, mG5.*Fmr1* seized 53% more than *Fmr1*), however these increases did not reach significance.

3.2.5. No modification of *Fmr1* anxiety- or perseverative-related, sensorimotor gating, fear-based memory or motor coordination responses—The *Fmr1* KO mouse has previously been reported to exhibit decreased anxiety as measured during exploration of an open-field arena by the proportion of the total distance traveled that is spent in the center [37]. In the present study anxiety-related responses were assessed using two indices from two separate tests: the center ratio in OFA and transitions in the light-dark exploration test. While we observed no overall main effect for the center ratio in the mG1 line [Fig. 6a; $F(3, 88)=0.13, p=0.945$], there was a main effect in the mG5 line [Fig. 6b; $F(3, 73)=5.10, p=0.003$]. *Fmr1* mice in the mG5 line were more consistent with previous reports [11,37,42] since they exhibited increased center ratio values compared to WT mice

($p=0.018$), indicating decreased anxiety-related responses. Similarly, mG5.Fmr1 mice also traveled significantly more in the center compared to both WT ($p=0.001$) and mG5 mice ($p=0.005$). WT and mG5 mice did not significantly differ from one another ($p=0.574$), nor did Fmr1 mice differ from mG5.Fmr1 mice ($p=0.224$) indicating that mGluR5 reduction did not affect this anxiety-related phenomenon in the *Fmr1* KO. We observed no overall main effect for the number of light-dark transitions in either the mG1 [Fig. 6c; $F(3, 88)=1.86$, $p=0.142$] or mG5 line [Fig. 6d; $F(3, 73)=1.17$, $p=0.328$].

Marble burying may reflect a perseverative-type behavior in rodents [33,43,44], and it is also known to decrease upon administration of Gp1 mGluR antagonists [45]. We did not observe an overall main effect for the number of marbles buried for either the mG1 line [Fig. 6e; $F(3, 88)=1.73$, $p=0.166$] or the mG5 line [Fig. 6f; $F(3, 73)=1.27$, $p=0.29$]. Although we did not observe an overall effect for either line, the data do suggest that mice heterozygous for mGluR1 or mGluR5 appear to bury less than WT mice, consistent with published Gp1 mGluR antagonist effects as well as from experiments within our lab (data not shown). Although the overall ANOVA was not significant, based on a priori considerations (i.e. pharmacological reductions in marble burying following antagonist administration), we did perform Student's *t*-tests comparing either WT (mG1 line) and mG1 or WT (mG5 line) and mG5 and observed a significant reduction in marble burying by both mG1 ($p=0.036$) and mG5 ($p=0.044$). Again, while this reduction is subtle, yet consistent within the heterozygotes, a similar phenomenon does not appear to hold for either double mutant.

We examined sensorimotor gating responses using the prepulse inhibition (PPI) test (Fig. 7). Our lab and others have demonstrated reduced startle and elevated PPI in the *Fmr1* KO [11,35,46–48]. For both the mG1 and mG5 lines, we observed overall main effects for the startle response [mG1 line: $F(3, 88)=12.70$, $p<0.001$; mG5 line: $F(3, 73)=12.56$, $p<0.001$] and the average PPI response [mG1 line: $F(3, 88)=19.96$, $p<0.001$; mG5 line: $F(3, 73)=4.24$, $p=0.008$]. In both lines, Fmr1 mice exhibited behavior consistent with behaviors previously observed: reduced startle (both lines: $p<0.01$) and elevated PPI (mG1 line: $p<0.001$; mG5 line: $p=0.002$). For the mG1 line, even though mG1 mice exhibited significantly elevated startle responses compared to WT ($p=0.028$), mG1.Fmr1 mice behaved similar to the Fmr1 group with reduced startle compared to WT ($p=0.002$). The average PPI responses in mG1 and mG1.Fmr1 mice were significantly elevated compared to WT (for both: $p<0.001$). Fmr1 and mG1.Fmr1 mice did not significantly differ in their responses ($p=0.451$), but both were significantly higher than mG1 mice ($p=0.025$, 0.003 respectively). For the mG5 line, mG5 mice did not differ compared to WT mice in either their startle or PPI responses ($p=0.507$, 0.413 respectively). On the other hand, mG5.Fmr1 mice exhibited responses analogous to Fmr1 mice: reduced startle ($p<0.001$) and elevated PPI ($p=0.027$) relative to WT mice. Therefore, neither mGluR1 nor mGluR5 reduction resulted in changes in *Fmr1* KO PPI responses.

Fear-based memory was measured using conditioned fear. There was no overall main effect for freezing behavior during the context test for both the mG1 line [Fig. 8a; $F(3, 88)=0.40$, $p=0.756$] and the mG5 line [Fig. 8b; $F(3, 73)=1.52$, $p=0.217$]. We also did not observe an overall main effect of freezing behavior during the CS test for the mG1 line [Fig. 8c; $F(3, 88)=1.15$, $p=0.333$] and the mG5 line [Fig. 8d; $F(3, 73)=1.67$, $p=0.181$]. Our finding of no obvious impairment of conditioned fear responses in Fmr1 mice is consistent with previous reports [11,37].

Since mGluR1 is abundant in the cerebellum and critical for appropriate motor function we chose to evaluate motor coordination responses in the mG1 line using the rotarod assay. Over the course of a 3-day (3 trials/day) training paradigm, repeated ANOVA revealed an overall main effect of genotype for the time spent on the rotarod [Fig. 9; $F(3, 88)=11.16$,

$p < 0.001$]. Performance in mG1 mice was not affected as they performed equally well as WT ($p = 0.576$). On the other hand, both *Fmr1* and mG1.*Fmr1* mice performed worse than WT ($p = 0.001$, $p < 0.001$ respectively); *Fmr1* and mG1.*Fmr1* mice did not differ from one another ($p = 0.162$). This indicates that mGluR1 reduction had no impact on *Fmr1* KO motor coordination. While the time spent on the rotarod captures an index of motor coordination, we also wanted to assess motor learning, and therefore we calculated a learning index as the average time spent on the rod on the last day of testing – the time spent on the first day. For this measure, we did not observe an overall main effect [data not shown; $F(3, 88) = 0.21$, $p = 0.893$]. The rotarod test was not performed with the mG5 line because of the low-level expression of mGluR5 in the cerebellum [22].

3.3. No modification of *Fmr1* testicular phenotype

Macroorchidism is often observed in post adolescent males with FXS, and a similar phenotype is reported in *Fmr1* KO mice [1,10,49]. Since both mGluR1 and mGluR5 are expressed in rat and human testes [50], we weighed adult (2–5 months of age) testes in this study to determine if mGluR1 or mGluR5 reduction would affect testicular weight in the *Fmr1* KO. In order to control for body weight, we analyzed testes weights as expressed as a testes weight: body weight ratio (Fig. 10). We observed an overall effect of the testes ratio for both the mG1 [Fig. 10a; $F(3,35) = 7.02$; $p = 0.001$] and mG5 [Fig. 10b; $F(3,37) = 6.90$; $p = 0.001$] lines. *Fmr1* mice in both lines exhibited significantly enlarged testes (by 16–20%) compared to WT mice (mG1 line: $p = 0.002$; mG5 line: $p = 0.003$). Reduction of either mGluR1 or mGluR5 in the *Fmr1* KO did not affect testicular size: weights were comparable to KO mice and significantly enlarged (by 15–20%) compared to WT mice (mG1.*Fmr1*: $p = 0.026$; mG5.*Fmr1*: $p < 0.001$).

4. Discussion

The purpose of this study was to determine whether 50% genetic reduction of either mGluR1 or mGluR5 might modify a wide range of behavioral responses in the *Fmr1* KO. The hypothesis that we might expect to see behavioral modifications was based on studies spanning at least the past decade that have brought attention to the potential importance of Gp1 mGluRs in the pathogenesis of FXS (reviewed in [51]). The present series of experiments helps fill in a void where there exists a lack of information concerning whether mGluR1 also acts as a modifier of *Fmr1* KO phenotypes. Furthermore, the present study broadens our understanding of the extent that mGluR5 may modify *Fmr1* KO behaviors which may be of relevance considering the plethora of symptoms with which individuals with FXS may present. The behaviors incorporated within our behavioral battery not only cover a range of CNS domains, but also include several behaviors, not previously tested for Gp1 mGluR modification, in which *Fmr1* KO mice have previously been demonstrated to exhibit robust/consistent deficits. Here we report a lack of robust modifications of *Fmr1* KO behaviors by 50% reduction of either mGluR1 or mGluR5, and instead we describe modifications which are observed in a limited set of behaviors. From the numerous behavioral tests performed ranging across multiple domains of CNS function we observed clear modification of surprisingly only one behavior (hot plate) and more “moderate” modifications of three other behaviors (activity, social interaction, audiogenic seizures); for the most part each modification was accomplished by only one of the two Gp1 mGluRs.

4.1. Modification of *Fmr1* KO behavioral responses

While no study has specifically reported abnormalities in pain processing, self-injurious behavior is a distinct behavioral feature observed in FXS that may reflect aberrations in pain processing [1,52]. The hot plate test for thermal sensitivity was the only behavior in which we observed clear modification of an *Fmr1* KO response, and this was achieved only by

mGluR5 reduction. We found *Fmr1* KO mice to exhibit normal heat sensitivity, consistent with previous reports [53,54]. Even though mice heterozygous for mGluR5 also displayed normal behavior, this reduction in combination with a loss of FMRP caused an analgesic response in the double mutant, possibly indicating that FMRP may act to positively reinforce pain sensitivity. The presence of both FMRP and Gp1 mGluRs in peripheral sensory neurons [56,57] suggests a role for both in pain sensitivity. Injection of DHPG, a Gp1 mGluR agonist, can induce thermal hypersensitivity, while the mGluR5 antagonist MPEP decreases thermal sensitivity [55]. Some mGluR1 antagonists such as CPCCOEt and LY367385 also attenuate DHPG-induced thermal hypersensitivity [55], while others exhibit mild or no analgesic effects [57,58]. *Fmr1* KO mice demonstrate insensitivity to DHPG-induced thermal hypersensitivity, and it has been postulated that FMRP contributes to peripheral sensitization of nociceptors [54]. It is also important to note that although *Fmr1* KO mice did not display an increased latency to respond, we have recently shown that there is an overall effect of *Fmr1* deficiency on sensitivity in the hot-plate assay when six different genetic backgrounds were compared, suggesting that loss of FMRP does cause a decrease in pain sensitivity, but this effect is very small and only revealed with the very large N used in our previous study [11]. Collectively, it appears that loss of FMRP and reductions in mGluR5 synergize to decrease pain sensitivity as assayed by the hot-plate test. Other assays for pain sensitivity will be needed to determine if this phenomenon generalizes to other pain pathways. At this point it is unclear why we did not see a similar response with mGluR1 reduction, but the current findings indicate that either FMRP did not affect nociceptive signaling through mGluR1 or signaling through mGluR5 was more sensitive to a loss of FMRP.

4.2. “Moderate” modification of *Fmr1* KO behavioral responses

A high percentage of boys with FXS exhibit attention deficit hyperactivity disorder [1], and hyperactivity in the *Fmr1* KO when exploring an open-field arena has been observed in several labs [10,11,37–39] and was corroborated in this study. We observed a modest reduction in *Fmr1* KO activity with mGluR1 reduction. The direction of the modification is consistent with experiments in our lab (data not shown) as well as in other labs demonstrating reductions in locomotor activity with mGluR1 antagonists [59–61]. Since these mGluR1 antagonists are not generally reported to demonstrate robust effects on activity and mice heterozygous for mGluR1, similar to our mG1 mice, do not exhibit alterations in activity [62], this may partially explain why we observe only a modest modification. We did not observe any changes in activity upon mGluR5 manipulation which is consistent considering that an acute administration of MPEP often has no effect on locomotor activity (e.g. [63]). These findings suggest that mGluR5 is not a dominant modifier of activity.

Many individuals with FXS face a major challenge in dealing with difficulties in interacting within a social context, with clinical features often consisting of shyness and social anxiety [1]. Dysfunction in the development of normal social interaction behavior also represents one of the three core symptoms for autism spectrum disorder (ASD) [64]. There has been much discussion in recent years concerning the use of fragile X models to better understand ASD considering that (1) approximately one third of children diagnosed with FXS also meet the full criteria for ASD and (2) FXS is the most common single gene cause of ASD [65]. Therefore, understanding genetic modifiers of social behavior in the *Fmr1* KO may provide a better framework for understanding social dysfunction in these disorders. Aberrant social behavior in the *Fmr1* KO is a relatively robust and consistent phenomenon observed within our lab [36,40]. The cellular and molecular mechanisms underlying social behavior are largely unknown, although there are some indications that Gp1 mGluRs may play a role. For example, MTEP, an mGluR5 antagonist, can induce social isolation in rats [66] and MPEP

appears to produce an anti-aggressive effect on social interactions between male mice by increasing non-offensive social investigation as well as non social exploration [67]. We observed a modest reduction in *Fmr1* KO active social behavior upon mGluR5 reduction consistent with the finding that signaling through mGluR5 may contribute towards social behavior. Considering that our classification of “active” social behavior encompasses a wide range of possible mouse activities (e.g. sniffing, grooming, touching/crawling over, threats, attacks, following/chasing, wrestling), our analysis precludes us from determining the exact nature of the behavior that mGluR5 reduction modified. Our lab and others have previously suggested that there may likely be a social anxiety component to the *Fmr1* KO dysfunction [36,68]. If the observed reduction in overall active social behaviors reflects a decrease in social anxiety, this would be consistent with the view that mGluR5 antagonism results in anxiolytic-like effects [45,69–72]. On the other hand, while mGluR1 antagonists have also been found to be anxiolytic-like in nature [72], mGluR1 reduction does not appear to be a dominant modifier of *Fmr1* KO social behavior. While there are some indications that cerebellar dysfunction may compromise non-motor aspects of behavior [73], for example children with compromised cerebellar function exhibit varying degrees of abnormal social behaviors [74], it is still unclear whether the cerebellum or mGluR1 specifically plays a role in a social context (see [75]).

Gp1 mGluR antagonists demonstrate anticonvulsant activity in numerous seizure models (reviewed in [76]), and MPEP has been demonstrated to attenuate the heightened susceptibility to audiogenic seizures in *Fmr1* KO mice [17]. Our lab has also observed reductions in *Fmr1* KO audiogenic seizures with both the mGluR1 antagonist JNJ16259685 as well as MPEP (data not shown). Therefore we found it surprising that genetic reduction of either mGluR1 or mGluR5 in the *Fmr1* KO not only did not reduce audiogenic seizures but appeared to exacerbate the *Fmr1* KO phenomenon. The discrepancy between these pharmacologic and genetic manipulations may potentially be explained by the fact that drugs are administered as an acute mGluR reduction while genetic manipulation involves chronic mGluR reduction, spanning throughout brain development which could have longstanding implications regarding developing connections/networks.

However, our seizure findings also contradict those from Dolen et al. [30] that found normalization of *Fmr1* KO audiogenic seizures with 50% mGluR5 reduction. Both of our labs used similar presentations of an auditory stimulus (125dB vs. 140dB alarm), with the only major protocol difference being that we used two 2-min auditory presentations separated by a minute while Dolen used a single presentation. It is important to note that we utilize a two 2-min protocol because most of our mice do not display a seizure during a single sound stimulus. Since there is currently little evidence in the literature suggesting that this type of procedural difference would affect seizure manifestation, non-procedural differences may be more likely to be responsible. Variations in animal housing environments may affect behavior [77–79]. For example, differences in background noise levels can affect animal hearing or cause physiological stress [80–82]. Furthermore, differential breeding strategies can affect behavioral responses [83]. Moreover, various environmental factors have been demonstrated to affect seizure occurrence in animal models [84]. Therefore, if one genotype is more susceptible to these environmental factors, this may translate to behavioral changes specific to a particular genotype. Regardless, we do see an apparent modification of the audiogenic seizure phenotype, thereby lending support for the idea that both mGluR1 and mGluR5 modulation may alter this phenotype in the *Fmr1* KO.

4.3. No modification of *Fmr1* KO behavioral responses

There were several paradigms for which neither mGluR1 nor mGluR5 reduction altered *Fmr1* KO behavior. For some of these assays, including light-dark exploration, marble burying, and fear conditioning, *Fmr1* KO mice exhibited normal responses. However, for

others including the OFA center ratio (anxiety-related) and rotarod, *Fmr1* KO mice displayed abnormal responses. Regardless of the presence of an *Fmr1* KO phenotype, our finding that 50% Gp1 mGluR reduction failed to induce a response may indicate that they are not *dominant* modifiers of these particular behaviors in general as neither WT nor *Fmr1* KO behavior was affected.

For the PPI test, mGluR5 reduction did not alter responses in either WT or *Fmr1* KO mice. It has previously been demonstrated that MPEP can restore normal PPI in *Fmr1* KO mice [19], however this report employed an eye-blink conditioning assay to study PPI as opposed to the more conventional method using the whole-body flinch response. In fact baseline responses in the *Fmr1* KO are distinctly different in these two paradigms: de Vrij report decreased PPI, while elevated PPI is observed when using the whole-body flinch [11,35,46–48]. Therefore the inability for 50% mGluR5 reduction to alter *Fmr1* KO PPI behavior may either reflect that this is specific to the whole-body flinch method or that mGluR5 is simply not a *dominant* modifier.

On the other hand, 50% mGluR1 reduction did cause changes in the PPI test compared to WT: mG1 mice exhibited elevated startle responses and elevated PPI. Despite changes in WT behavior with mGluR1 reduction, no similar alterations in *Fmr1* KO behavior were observed as mG1.*Fmr1* exhibited similar phenotypes as *Fmr1* mice. This insensitivity to change may actually reflect a failure to modify PPI behavior specifically in the *Fmr1* KO. Interestingly, this phenomenon may also be more subtly suggested in marble burying whereby heterozygotes for mGluR1 and mGluR5 buried fewer marbles while both double mutants failed to exhibit similar reductions. While this indicates that Gp1 mGluR manipulation cannot alter *Fmr1* KO behavior in these specific paradigms, their possible insensitivity to this manipulation does support the notion of a disrupted Gp1 mGluR signaling pathway.

4.4 Conclusions

Over the course of this study, we anticipated more profound modifications of *Fmr1* KO behaviors and therefore were surprised to find only modest changes, particularly in light of the exciting results from Dolen et al. [30] demonstrating normalization of a number of *Fmr1* KO abnormalities following a similar genetic reduction of mGluR5. On one hand, one might argue that mGluR5 is the dominant “player” and for this reason with mGluR1 reduction we see only modest modification within a couple behavioral assays (activity and seizures). However, this does not explain why we also fail to see more robust changes with mGluR5 reduction. It is challenging to directly compare our results with Dolen since they report only two behaviors: audiogenic seizures and inhibitory avoidance extinction. We do not believe that the data presented here contradict that presented in Dolen, but perhaps when looking at a wider range of behavioral paradigms we are confronted with a more complicated picture regarding *Fmr1* KO behaviors.

The data presented here do indicate that genetic reduction of either mGluR1 or mGluR5 can potentially modulate behavioral responses in *Fmr1* KO mice, although these modifications appear to be subtle in nature and/or limited to select behaviors. The fact that we observed differential behavioral modifications between mGluR1 vs. mGluR5 reduction suggests the importance of further understanding the role between *both* mGluR1 and mGluR5 in relation to the pathogenesis of FXS. Furthermore, the lack of more robust modifications indicate a possibly more complex system whereby *Fmr1* KO behavioral modification is governed by a more complicated interaction between Gp1 mGluRs and FMRP or one that involves pathways/molecules outside that of the Gp1 mGluR signaling pathway.

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Highlights

- mGluR1 genetic reduction decreased *Fmr1* KO locomotor activity
- mGluR5 genetic reduction alters KO analgesic responses and active social behavior
- Seizures, sensorimotor gating, memory & coordination were not significantly altered

Fig. 1. Reduction of mGluR1 or mGluR5 in mice heterozygous for these alleles. Western blot analysis of mGluR1 in cerebellar homogenates (**a**, **b**) or mGluR5 in hippocampal homogenates (**c**, **d**). Representative blots (**a**, **c**) and quantitated results (**b**, **d**). Values plotted represent means $\pm$ SEM for intensity normalized to actin and an independent sample loaded on all gels ($n = 10-13$). * $p < 0.05$, as compared to WT

Fig 2. Modification of *Fmr1* KO analgesic response by mGluR5. Latency to demonstrate a hind limb response during the hot-plate test for the (a) mG1 and (b) mG5 line. Values plotted represent means $\pm$ SEM ($n = 15-25$). * $p < 0.05$

Fig 3. Moderate modification of *Fmr1* KO locomotor activity by mGluR1. Total distance traveled during exploration of an open-field arena for the (a) mG1 and (b) mG5 line. Values plotted represent means $\pm$ SEM ($n = 15-25$). * $p < 0.05$, as compared to WT

Fig 4.

Moderate modification of *Fmr1* KO social behavior by mGluR5. Frequency of active interactions (initiated by test subject) for the (a) mG1 and (b) mG5 line. Total duration of active interactions for the (c) mG1 and (d) mG5 line. Values plotted represent means $\pm$ SEM ($n = 14-24$). * $p < 0.05$, as compared to WT

Fig 5. Potential modification of *Fmr1* KO audiogenic seizure susceptibility by both mGluR1 and mGluR5. % subjects exhibiting a seizure (wild running or tonic-clonic) in response to two presentations of a 2-min 140 dB auditory stimulus for the (a) mG1 and (b) mG5 line. Values plotted represent mean percentages ($n = 10-27$). * $p < 0.05$, as compared to WT

Fig 6.

No modification of anxiety- or perseverative-related *Fmr1* KO behaviors. Center:total distance traveled ratio in the OFA assay for (a) mG1 and (b) mG5 lines. Number of transitions between light and dark compartments in the light-dark test for (c) mG1 and (d) mG5 lines. Number of marbles buried during the marble burying test for (e) mG1 and (f) mG5 lines. Values plotted represent means $\pm$ SEM ($n = 15-25$). * $p < 0.05$, as compared to WT

Fig 7.

No modification of *Fmr1* KO sensorimotor gating. Startle response to a 120 dB auditory stimulus for the (a) mG1 and (b) mG5 line. % prepulse inhibition (PPI) of the startle response for the (c) mG1 and (d) mG5 line. Values plotted represent means $\pm$ SEM ($n = 15-25$). * $p < 0.05$, compared to WT; # $p < 0.05$, compared to mG1

Fig 8.

No modification of *Fmr1* KO learning and memory during fear conditioning. % freezing during context test for the (a) mG1 and (b) mG5 line. % freezing during CS test for the (c) mG1 and (d) mG5 line. Values plotted represent means $\pm$ SEM ($n = 15-25$)

Fig 9. No modification of *Fmr1* KO motor coordination. Average daily time spent on the rotarod for the mG1 line during training for 3 days, 3 trials/day. Values plotted represent means $\pm$ SEM ($n = 22-24$)

Fig 10.

No modification of *Fmr1* KO testicular phenotype. Testes weight: body weight ratios for the (a) mG1 and (b) mG5 line. Values plotted represent means $\pm$ SEM ($n = 7-14$). * $p < 0.05$, as compared to WT