

Published in final edited form as:

*Behav Brain Res.* 2012 March 1; 228(1): 1–8. doi:10.1016/j.bbr.2011.11.018.

## Genetic reduction of muscarinic M<sub>4</sub> receptor modulates analgesic response and acoustic startle response in a mouse model of Fragile × Syndrome (F×S)

Surabi Veeraragavan<sup>a</sup>, Deanna Graham<sup>a</sup>, Nghiem Bui<sup>a</sup>, Lisa A Yuva-Paylor<sup>a</sup>, Jürgen Wess<sup>c</sup>, and Richard Paylor<sup>a,b,†</sup>

<sup>a</sup>Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX 77030

<sup>b</sup>Department of Neuroscience, Baylor College of Medicine, Houston, TX 77030

<sup>c</sup>Laboratory of Bioorganic Chemistry, NIDDK, National Institutes of Health, Bethesda, MD 20892

### Abstract

**Introduction**—The G-protein coupled muscarinic acetylcholine receptors, widely expressed in the CNS, have been implicated in Fragile × Syndrome (F×S). Recent studies have reported an overactive signaling through the muscarinic receptors in the *Fmr1*KO mouse model. Hence, it was hypothesized that reducing muscarinic signaling might modulate behavioral phenotypes in the *Fmr1*KO mice. Pharmacological studies from our lab have provided evidence for this hypothesis, with subtype-preferring muscarinic M<sub>1</sub> and M<sub>4</sub> receptor antagonists modulating select behaviors in the *Fmr1*KO mice. Since the pharmacological antagonists were not highly specific, we investigated the specific role of M<sub>4</sub> receptors in the *Fmr1*KO mouse model, using a genetic approach.

**Methods**—We created a double mutant heterozygous for the M<sub>4</sub> receptor gene and hemizygous for the *Fmr1* gene and examined the mutants on various behaviors. Each animal was tested on a behavior battery comprising of open-field activity (activity), light-dark (anxiety), marble burying (perseverative behavior), prepulse inhibition (sensorimotor gating), rotarod (motor coordination), passive avoidance (learning and memory) and hotplate (analgesia). Animals were also tested on the audiogenic seizure protocol and testis weights were measured.

**Results**—Reduction of M<sub>4</sub> receptor expression in the heterozygotes completely rescued the analgesic response and partly rescued the acoustic startle response phenotype in the *Fmr1*KO mice. However, no modulation was observed in a number of behaviors including learning and memory, activity, perseverative behavior and audiogenic seizures.

**Conclusion**—Reducing M<sub>4</sub> receptor signaling altered only select behavioral phenotypes in the *Fmr1*KO mouse model, suggesting that other targets are involved in the modulation of fragile × behaviors.

### Keywords

Fragile × syndrome; muscarinic; M<sub>4</sub> receptors; analgesic response; behavior; sensorimotor gating

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<sup>†</sup>Corresponding Author: Richard Paylor, rpaylor@bcm.edu, Tel: 713-798-6124, Fax: 713-798-1445.

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## 1. Introduction

Fragile × syndrome (F×S) is the most common inherited cause of intellectual disability and in individuals with the full mutation (i.e. those with over 200 CGG repeats in the 5' untranslated region of the *Fmr1* gene) a wide range of behavioral abnormalities are manifested including hyperactivity, obsessive-compulsive behavior, aggression, self-injurious behavior, alterations in sensorimotor gating, cognitive impairments, abnormal social behavior, and autism [1]. An *Fmr1*KO mouse model was created [2] that recapitulates some of the phenotypes seen in patients including hyperactivity, altered social interaction, susceptibility to audiogenic seizures and performance impairments on some assays of learning and memory [3–11]. Recent studies have elucidated a potential role for the muscarinic cholinergic receptors (mAChR) in F×S [12,13]. The muscarinic agonist, carbachol has been shown to cause alterations in the synaptic response in the subiculum of the *Fmr1*KO mice [12]. Also, enhanced carbachol M<sub>1</sub>-mediated LTD has been reported in the *Fmr1*KO mice [13]. Increased expression of the downstream targets of FMRP, such as EF1a and CaMKII, following muscarinic activation by carbachol was observed in WT but not in the *Fmr1*KO mice, indicating the presence of an overactive muscarinic signaling in the KO mice [13]. Of the five molecularly distinct mAChR subtypes (M<sub>1</sub>–M<sub>5</sub>) [14] expressed throughout the brain, M<sub>1</sub> and M<sub>4</sub> receptors show high expression in the brain with distinct patterns. The M<sub>4</sub> receptor has been shown to be predominantly expressed in the striatum, which is part of the extrapyramidal network involved in the coordination of movement and also in the cortex, hippocampus and basal ganglia [15–21]. Data from M<sub>4</sub> knockout mice [19] suggested a role for the M<sub>4</sub> receptors in regulating sensorimotor gating [22], cognitive processes [23], locomotor activity [19] and analgesic response [24], all of which were altered in the *Fmr1*KO mouse model [2,3,5,6,25–28]. Based on the findings from a variety of sources (e.g. electrophysiological, biochemical, and behavioral) we hypothesized that reduction of M<sub>4</sub> activity might modulate F×S behavior. Recently, our lab reported that the M<sub>4</sub> receptor-preferring antagonist tropicamide modulated perseverative behavior, learning and memory and audiogenic seizures (AGS) in WT and *Fmr1*KO mice [29]. In a separate study we also reported that the M<sub>1</sub> receptor-preferring antagonist dicyclomine modulated select fragile × phenotypes including perseverative behavior and AGS in the *Fmr1*KO mouse model [30]. Taken together these recent pharmacological reports strengthen the hypothesis that reducing the activity of muscarinic receptors, in particular the M<sub>4</sub> and M<sub>1</sub> subtypes could potentially modulate phenotypes in *Fmr1*KO mice.

The goal of the present study was to further understand the specific role of M<sub>4</sub> receptors in modulating the F×S phenotypes using a genetic strategy rather than a pharmacological approach. Importantly, since we have shown that numerous *Fmr1*KO behavioral phenotypes were dependent on genetic background [28], and thus influenced by dominant genetic modifiers, the current study also addressed the potential that the M<sub>4</sub> receptor gene might be one of those dominant modifiers. Therefore, in the current study, we used a genetic approach to reduce M<sub>4</sub> receptor expression by 50% in the presence of the *Fmr1* mutation to further investigate the role of M<sub>4</sub> receptor as a modulator of *Fmr1*KO behaviors. Additionally, although pharmacological modulation of M<sub>4</sub> receptor signaling altered F×S phenotypes [29], the potential non-specific effects of the pharmacological agents used can be avoided using the receptor-specific genetic approach. Results from the current study demonstrated that genetically reducing M<sub>4</sub> receptor expression resulted in modulation of select fragile × behaviors. The double mutants, heterozygous for the M<sub>4</sub> gene and hemizygous for the *Fmr1* gene showed a complete reversal of the analgesic phenotype and a partial rescue of the acoustic startle response phenotype in the *Fmr1* KO mice.

## 2. Materials and Methods

### 2.1. Animals

Subjects for this study were generated by crossing heterozygous *Fmr1* female (*Fmr1*<sup>+/-</sup>) (C57BL/6J background backcrossed for 14–17 generations) and heterozygous M<sub>4</sub> (M<sub>4</sub><sup>+/-</sup>) male mice (Gomez et al., 1999) (backcrossed for over 10 generations to C57BL/6NTac mice). Therefore, it is important to note that the current mice are on a genetic background that is 'F1' between B6J and B6NTac, and although there should be few differences compared to our routine 'pure' B6J background (e.g. [30]) the current mice are not genetically the same as those *Fmr1*KO on a B6J background. The resultant progeny consisted of 4 genotypes: Wild type (WT), M<sub>4</sub> heterozygotes (M<sub>4</sub> Het), *Fmr1* hemizygous knockout (*Fmr1*KO) and the double mutant heterozygous for M<sub>4</sub> and hemizygous for *Fmr1* (DM). The total number of animals used in the study includes 8 wildtypes, 15 M<sub>4</sub> heterozygotes, 13 *Fmr1*KOs and 15 double mutants. Although using strict littermate helps control for between-litter variability, we were not necessarily able to obtain all the four genotypes in each litter. So we tested 2–3 litters together (in batches) to account for the variability. The animals were 2–3 months of age, except those used in the audiogenic seizure study, which were 20–22 days of age. Only male mice were used in this study. Mice were housed 2–5 per cage with access to food and water ad libitum. They were maintained in a 12 hr light: dark cycle. Prior to testing all the animals were transferred to the testing room followed by a 30-minute acclimatization period. The animal care and behavioral testing procedures were approved by the Baylor College of Medicine Animal Care and Use Committee and followed NIH guidelines.

### 2.2. Behavior testing assignment

All the animals went through a standard testing battery comprising of open-field activity (OFA) (day 1 and 2), Light dark (LD), marble-burying (MB), prepulse inhibition (PPI), rotarod, passive avoidance (PA) and hot plate. This testing battery enables us to test a wide range of behavioral assay and minimizes the number of animals used in the study [31,32]. However, for the audiogenic seizures we used a separate set of mice.

### 2.3. PCR techniques

Standard PCR techniques were used to genotype the animals [33,19]. For the WT allele of the *Fmr1* gene we used the following primers: Fmr1\_S1 (5'-GTG GTT AGC TAA AGT GAG GAT GAT-3') and Fmr1\_S2 (5'- CAG GTT TGT TGG GAT TAA CAG ATC- 3') and the expected band size was 527bp. For the KO allele of the *Fmr1* gene we used the following primers: Fmr1\_S1 and Fmr1\_N2 (5'-TGG GCT CTA TGG CTT CTG A- 3') and the band size was 501 bp. The following protocol was used for genotype the *Fmr1* locus: 94°C for 2 minutes, 94°C for 30 seconds followed by 58°C for 30 seconds and 72°C for 30 seconds. This cycle was repeated 30 times. Final extension was done at 72 for 10 minutes. The WT and KO PCRs were run separately using the same conditions. For the WT allele of the M<sub>4</sub> gene the following primers were used: M4-A (5'-GGA GAA GAA GGC CAA GAC TCT GG- 3') and M4-B (5'-GGC AGT CAC ACA TTC ACT GCC TG-3') and the expected band size was 367 bp. For the KO allele of the M<sub>4</sub> gene the following primers were used: M4-B and NEO-1 (5'-CAG CTC ATT CCT CCC ACT CAT GAT-3') and the expected band size was 480 bp. The following protocol was used to genotype the M<sub>4</sub> locus: 94°C for 10 minutes, 94°C for 30 seconds followed by 30 seconds of annealing at 60°C and 72°C for one minute. This cycle was repeated 30 times and the final extension was done at 72°C for 10 minutes. The WT and the KO reactions were run separately using the same protocol.

## 2.4. Behavior experiments

**2.4.1. Open-field activity**—Total distance travelled in an open field was used as a measure of activity. The testing chamber comprised of a plexiglass arena (40 × 40 × 30 cm) that was brightly lit with overhead lights (~ 800 lux). Recordings were made using the automated versamax animal activity monitoring system (Accuscan Instruments, Columbus OH). Throughout testing a white background noise was present (55 dB). Each test session lasted for about 30 minutes and the total distance travelled and the number of vertical rearing responses were recorded. We also measured anxiety-like behavior defined by the ratio of center distance, which is the distance travelled by the animal in the center (22.5 × 22.5 cm) of the chamber, to the total distance traveled. The same test was repeated on the second day to measure the response to a novel environment.

**2.4.2. Light dark**—Light- dark exploration assay was used to measure anxiety-like responses. The testing chamber comprised of a rectangular plexiglass chamber divided in to two unequal compartments, separately by a partition with an opening (8.5 cm × 5 cm) large enough to allow the animal to transition between the compartments. The large chamber was brightly lit (~800 lux), transparent and uncovered (30 cm × 21 cm × 21 cm). The small chamber was made of black Plexiglas and cover with a lid (15 cm × 21 cm × 21 cm). At the start of the experiment the animals were placed in the light chamber and the number of transitions between the chambers (all four paws inside a chamber) over a 10 minute period was recorded. White noise was present throughout testing.

**2.4.3. Marble Burying**—Perseverative behavior was measured using the marble-burying (MB) assay [34]. A standard mouse cage filled with approximately 10 cm of SANI-CHIP bedding was used as the test chamber. Twenty black marbles (15 mm) were arranged in the testing chamber in a 4×5 matrix, equidistant from each other. Each animal was allowed to explore the chamber for 20 minutes and at the end of the testing period the number of marbles buried was determined. We defined buried as more than 50% of the surface being covered by the bedding. White noise (55 dB) was present throughout testing.

**2.4.4. Prepulse Inhibition of the acoustic startle response**—The acoustic startle responses and prepulse inhibition were used to assess sensorimotor gating using the SR-Lab System (San Diego Instruments, San Diego, CA, USA) as previously described [33]. The apparatus comprised of a sound attenuating chamber, which contained a cylindrical tube where the animal was placed during testing. Each animal was acclimated to a background white noise of 70 dB for about 5 minutes prior to the test session. Each test session consisted of 48 trials comprising of 6 blocks of eight trial types each presented in a pseudo random order. Each block had a “No stimulus” trial used to measure baseline movement where no sound was presented, a “startle only” trial comprised of a 40 ms, 120 dB sound burst, a “prepulse only” trials (74, 78 or 82 dB) comprising of three different 20 ms prepulses and finally the “prepulse inhibition” trials composed of the presentation of one of the three prepulse sounds, 100ms prior to the startle stimulus. The inter-trial interval ranged from 10 s– 20 s, and the startle response was recorded every 1 ms for 65 ms following the onset of the startle stimulus. Percent PPI of the startle response was calculated as follows:  $100 - [(response\ to\ acoustic\ prepulse\ plus\ startle\ stimulus\ trials / startle\ response\ alone\ trials) \times 100]$ .

**2.4.5. Rotarod**—Motor coordination and skill learning were investigated by assessing the ability of the animal to walk on a rotating rod (Ugo Basile, Comerio, VA, Italy). We used a 2-day protocol consisting of 4 trials per day. Each trial lasted for 5 minutes with the rod accelerating at a speed of 4–40 rpm in 5 minutes. The time spent walking on the rod was recorded. Intertrial interval was 10–15 minutes.

**2.4.6. Passive Avoidance**—Learning and memory performance was assessed using a passive-avoidance assay. A modular shuttle box (Med Associates Inc., St. Albans, VT, USA) containing two chambers, one illuminated with bright light and the other covered with black felt, separated from each other by a barrier containing a door was used for training and testing. The first day is the training day which began with the animal being placed in the light chamber and allowed to explore the chamber for 10 seconds and then the door to the dark chamber was opened. As soon as the animal entered the dark chamber with all its 4 paws, the door was shut and a mild foot shock (0.75 mA, 2 s) was delivered. Following the foot shock the animal was allowed to stay in the dark chamber for an additional 10 seconds and returned to its' home cage. The latency to enter the dark side was measured. Memory for this aversive event was tested the following day. The test animal was again placed in the light chamber and allowed to explore the chamber for 10 seconds after which the door to the dark chamber is raised and the animal was allowed to enter the dark chamber. The latency to enter the dark chamber was measured and this was used as the index for learning and memory. Maximum time of 300 s was given to each animal and then the animal was returned to the home cage.

**2.4.7. Hot plate**—Analgesic response to a thermal stimulus was measured using a hot plate analgesia meter (Columbus instruments, Columbus, OH, USA). Each animal was placed on the hot plate (55° C) and the latency (maximum 45 s) to first hind limb response (i.e. hind paw shake, jumping and hind paw licking) was measured.

**2.4.8. Audiogenic seizures**—Susceptibility to audiogenic seizure was measured in 19–21 days old mice in a sound attenuated chamber illuminated with bright light using a priming protocol adopted by our lab [35]. Two mini personal alarms (SKU 49-728, RadioShack, Fort Worth, TX, USA) emitting 140 dB sound stimulus were attached to the underside of two clear plexiglass lids used to cover the testing chamber, that comprised of a standard mouse cage. The protocol consists of a one-minute silent period, which was followed by two minutes of loud sound (alarm on), then another minute of silence followed by two minutes of loud sound. Based on previous studies in the lab we did not observe seizures in the KO mice with a single presentation of sound stimulus and hence we have used a priming protocol that involves 2 sound presentations. Both wild running and clonic tonic seizures were recorded and percentage of animals that exhibited seizures were calculated as follows: (total number of animals seized/total number of animals) × 100

## 2.5. Testis weight measurement

Testis weight was measured from animals that had completed the behavior battery. In order to control for the body weight, we calculated testis: body weight ratio.

## 2.6. Statistics

For each behavioral measure except audiogenic seizures, one-way ANOVA were used to analyze the data. Follow-up comparisons were made using Least Significant Difference (LSD) test. For Audiogenic seizures, we used the Fischer exact probability test (one tailed) to analyze the percentage of seizures. The level of significance was at  $p < 0.05$ . Data were analyzed using the SPSS statistical software (SPSS, Chicago, IL).

## 3. Results

The goal of this study was to assess the modulation of *Fmr1*KO behaviors by reducing the amount of M4 receptor protein by 50%. Here we defined modification by changes in the double mutant (DM; heterozygous for the M<sub>4</sub> receptor gene and hemizygous null for the *Fmr1* gene) in comparison with the *Fmr1*KO phenotype. A rescue was defined by significant



differences between the DM and the *Fmr1*KO and no significant difference between DM and the WT. A partial rescue was when the DM was neither different from the WT nor from the *Fmr1*KO. No modulation was when the DM resembled the *Fmr1*KO phenotype. All the follow-up analyses comparing the genotypes were performed only when the one-way ANOVA revealed a significant overall effect of genotype.

### 3.1. Behavioral modifications

We observed modifications in only select *Fmr1*KO behaviors and hence in this section we present behavioral data from all the assays in which we observed a modification of *Fmr1*KO phenotype followed by assays that showed a lack of modulation of the *Fmr1*KO phenotype.

**3.1.1. Modulation of thermal sensitivity by the M<sub>4</sub> receptors**—M<sub>4</sub> receptors have been implicated in mediating analgesic response [36]. In this study we examined thermal sensitivity using the hot-plate assay as a measure of analgesic response. One-way ANOVA revealed an overall main effect of genotype [F (3,47)= 8.35; P= 0.0001]. The *Fmr1*KO mice showed a decreased sensitivity to the thermal stimulus indicated by an increased latency to respond to the stimulus (p< 0.001) (Figure 1). Previous studies have reported an absence of a hot plate phenotype in the M<sub>4</sub> KO mice under basal conditions [24]. But the M<sub>4</sub> heterozygotes in our study showed an increased latency compared to the WT (p<0.05). However, the M<sub>4</sub> mutation in combination with the *Fmr1* mutation, rescued the increased latency phenotype seen in the *Fmr1*KO mice. The latency to respond to the thermal stimulus in the double mutant mice were comparable to the WT (p=0.09) and significantly different from the *Fmr1*KO (p<0.01) indicating a reversal of the phenotype, suggesting a potential interaction between these pathways for this type of response.

**3.1.2. Modulation of the acoustic startle response by the M<sub>4</sub> receptors**—The prepulse inhibition (PPI) test was used to assess sensorimotor gating impairments. Previous studies have reported a decreased startle response and increased PPI in the *Fmr1*KO mice [3, 8, 28, 33]. Similarly, the M<sub>4</sub> receptor has also been shown to be a key player in controlling acoustic startle response [22]. In this latter study, the M<sub>4</sub> KO mice showed increased startle response, but normal PPI compared to the WT mice [22]. Analysis on the startle response data revealed an overall effect of genotype [F (3,47)= 4.8; P= 0.005]. The M<sub>4</sub> heterozygotes displayed a normal startle response (p= 0.653). Also, previous studies have shown that the *Fmr1*KO mice showed a decreased startle response phenotype [3, 8, 28, 33]. Consistent with the literature the *Fmr1*KO mice in our study also showed decreased startle response compared to the WT mice (p=0.012). The startle response in the DM mice was not significantly different from the WT (P= 0.28), and trending to be significantly different from the *Fmr1*KO (P=0.072), suggesting a partial rescue of the startle response phenotype (Fig. 2a). Thus the M<sub>4</sub> mutation appears to act as a dominant modifier modulating the acoustic startle response phenotype in the *Fmr1*KO mice.

Interestingly, the PPI analysis also showed an overall effect of genotype [F (3,47)= 6.6, P=0.001] (Fig.2b) with the *Fmr1*KO having an increased PPI (p= 0.01), which is consistent with the literature [28,33]. Similarly, the DM mice had increased PPI (p= 0.01) that was not different from the *Fmr1*KO response (p=0.958). PPI measurements in the M<sub>4</sub> heterozygotes were comparable to the data obtained with WT mice (p= 0.838). These results demonstrate that although M<sub>4</sub> receptors regulate startle in *Fmr1*KO mice, the reduction in M<sub>4</sub> receptor expression in the M<sub>4</sub> heterozygotes might not be sufficient to alter sensorimotor gating.

**3.1.3. Modulation of activity by the M<sub>4</sub> receptors**—Figure 3 illustrates total distance travelled in an open field as a measure of activity in the four genotypes of animals. There was an overall effect of genotype [F(3,47)=4.5; p= 0.007]. Follow-up analysis revealed that

neither *Fmr1*KO mice ( $p=0.126$ ) nor M4 heterozygotes ( $p=0.621$ ) were differentially active relative to the WT littermates. However the DM mice showed a significant increase ( $p=0.003$ ) in the total distance travelled compared to the WT suggesting a synergistic effect on activity between M<sub>4</sub> and *Fmr1*. Also, habituation to the novel environment was assessed by measuring total distance travelled in the same open field on the following day. All the genotypes showed a decrease in activity on the open field suggesting similar habituation to the environment (data not shown).

**3.1.4. Absence of modulation of the learning and memory phenotype**—The passive avoidance test was used to assess learning and memory performance. The passive avoidance phenotype in the *Fmr1*KO mice has been inconsistent with some reports suggesting an impairment [26,30] while others reporting no effect [2,29]. Figure 4a and 4b illustrate the latency to enter the dark in the passive avoidance test across all the genotypes on day 1 and day 2 respectively. One-way ANOVA revealed no overall effect of genotype on the latency to enter the dark on day 1 [ $F(3,47)=0.948$   $p=0.425$ ] or day 2 [ $F(3,47)=0.992$ ;  $p=0.405$ ]. The day 2 data clearly indicate a lack of interaction between the M<sub>4</sub> receptor and *Fmr1*.

**3.1.5. Absence of modulation of perseverative behavior, anxiety-like responses, motor behavior, or audiogenic seizures**—Figure 4b illustrates marble burying across the different genotypes. One-way ANOVA revealed an overall effect of genotype [ $F(3,47)=30.96$ ;  $p=0.013$ ] and follow-up tests showed that the *Fmr1*KO and DM mice buried significantly more marbles compared to the WT ( $p<0.01$ ). The M<sub>4</sub> heterozygotes buried similar number of marbles as the WT ( $p=0.426$ ).

Anxiety-like traits were measured by the center: total distance ratio in an open field and number of transitions in the light dark assay (Fig 5a). One-way ANOVA revealed an absence of an overall effect of genotype [ $F(3,47)=1.967$ ;  $p=0.132$ ] in the center: total distance ratio. However, the number of transitions in the LD assay revealed an overall effect of genotype [ $F(3,47)=4.551$ ;  $p=0.007$ ]. The *Fmr1*KO ( $p<0.05$ ) and the DM ( $p<0.05$ ) mice were both significantly different from the WT mice (Fig 5b) indicating a lack of modulation of the *Fmr1*KO phenotype by the M<sub>4</sub> receptors.

Motor coordination was assessed using the rotarod assay. Figure 6 illustrates the average time spend over a period of 2 days on a rotating rod. One-way ANOVA revealed no overall effect of genotype [ $F(3,47)=0.822$ ;  $P=0.488$ ]. The lack of a motor coordination phenotype in the *Fmr1*KO mice is consistent with a previous study from our lab [28].

Figure 7 illustrates the percentage of audiogenic seizures across various genotypes. *Fmr1*KO mice have been shown to be susceptible to audiogenic seizures [3, 7, 10, 30, 37]. Consistent with the literature, the *Fmr1*KO mice showed a significantly increased percentage of seizures compared to the WT mice ( $P<0.05$ ). However, the DM mice were similar to the *Fmr1*KO mice ( $p=0.5$ ) and did not modulate the *Fmr1*KO phenotype.

### 3.2. Absence of modulation of testis weight in the *Fmr1*KO mice

One of the most consistent morphologic phenotypes in the *Fmr1*KO mice is machroorchidism [2], which has been shown to be caused by increased proliferation of the sertoli cells [38]. Muscarinic receptors are also expressed in the testis and have been shown to regulate sertoli cell proliferation [39]. Hence we investigated the effect of reducing the M<sub>4</sub> receptor levels by 50% on testis weight in the *Fmr1*KO mice. Testis weights were measured from animals that completed the behavior battery (10–12 weeks). In order to control for variations in body weight, a ratio of testis weight: body weight was analyzed. One-way ANOVA revealed an overall main effect of genotype [ $F(3,33)=13.9$ ;  $p<0.001$ ],

with the *Fmr1* KO showing an increased testis weight compared to the WT mice ( $p < 0.01$ ) (Figure 8). The  $M_4$  heterozygotes were not significantly different from the WT mice ( $p = 0.375$ ). In the double mutants, reduction in  $M_4$  receptor expression did not alter the *Fmr1*KO phenotype. The double mutants were significantly different from the WT ( $p = 0.001$ ) but comparable to the KO ( $p = 0.475$ ) mice suggesting that 50% reduction of  $M_4$  receptor expression did not modulate the testicular phenotype in the *Fmr1*KO mice.

## 4. Discussion

The main objective of this study was to determine whether reducing the muscarinic  $M_4$  receptor levels by creating a double mutant heterozygous for  $M_4$  and hemizygous null for the *Fmr1* gene, modulates fragile  $\times$  phenotypes in *Fmr1*KO mice. In the double mutants, we observed modulation of select behaviors including the hotplate-analgesia response and the acoustic startle response in the *Fmr1*KO mice, suggesting that the  $M_4$  receptor positively modulates some of the *Fmr1* phenotypes. However, we did not observe any modulation of a number of other behaviors including PPI, anxiety-related responses, learning and memory and audiogenic seizures suggesting that other factors are involved in modulating these processes.

### 4.1. $M_4$ receptors mediated modulation of analgesic response and sensorimotor gating in the *Fmr1*KO mice

Overall, the analgesic response in the *Fmr1*KO mice assessed by the hot-plate assay is clearly influenced by the genetic background. Spencer et al. [28] have shown that in the C57BL/6J background there was a trend towards a significant analgesic response in the hot plate assay, whereas in the D2.B6-F1 hybrid background there seems to be a robust analgesic effect seen by an increase in the latency to respond to the thermal stimulus [28]. However, in general, studies involving the B6 mice have shown an absence of the pain-sensitivity phenotype in the *Fmr1*KO mice, using the hot plate and tail flick assays [40, 27]. In F $\times$ S patients, self-injurious behavior has been reported which is thought to be due to altered pain sensitivity [41–43]. Also, FMRP is highly expressed in the dorsal root ganglion, a region that is involved in pain processing [27]. In our study the *Fmr1*KO mice showed a robust analgesic response seen by a significant increase in the latency to respond in the hot-plate assay. Muscarinic receptors have also been implicated in analgesic response in rodents (reviewed by [36]). Similar to FMRP, the  $M_4$  receptors have also been found to be present in the dorsal root ganglia of the rat spinal cord, which is essential for pain transmission [44]. Tropicamide, an  $M_4$  receptor-preferring antagonist, reduces the antinociceptive effects of LXM-10, an acetylcholine receptor agonist [45]. Also, the analgesic responses mediated by the muscarinic agonists CMI-936 and CMI-1145 were reduced in  $M_4$  KO mice, indicating a role for the  $M_4$  receptors in pain sensitivity [24]. In this study, the  $M_4$  heterozygous mice also showed an increased latency to respond compared to the WT. Interestingly, reducing  $M_4$  receptor expression in combination with the *Fmr1* mutation reversed the analgesic response seen in the *Fmr1*KO mice, suggesting an interaction between the pathways. These findings are intriguing and other types of pain sensitivity assessments in these mutants will be necessary in order to thoroughly understand modulation of pain processing in the *Fmr1*KO mice by the  $M_4$  receptors.

Prepulse inhibition is one of the most robust phenotypes observed in the *Fmr1*KO mice. Several studies have reported an increased prepulse inhibition response and decreased startle in the *Fmr1*KO mice suggesting a role for FMRP in sensorimotor gating [3,6,8,28,33]. Interestingly, cholinergic receptors also play an important role in sensorimotor gating with evidence from lesion studies involving the cholinergic brain stem nuclei (pedunculopontine and laterodorsal tegmental nuclei) showing defects in PPI [46, 47]. However, despite having increased startle the  $M_4$  KO mice have normal prepulse inhibition [22]. Also,



pharmacological antagonism using the M<sub>4</sub> preferring antagonist tropicamide resulted in decreased PPI in mice [48, 30] and an increased startle response [48]. In the current study, the *Fmr1*KO mice showed decreased startle response consistent with the literature. In the current study the startle response of M<sub>4</sub> heterozygotes was comparable to WT mice; however, the reduction in M<sub>4</sub> receptor expression in the presence of *Fmr1* mutation partially modulated the reduced startle in the *Fmr1* KO mice. These data demonstrate that M<sub>4</sub> acts as a dominant modifier of the *Fmr1* startle response phenotype. We do not observe any modulation of the increased PPI response in *Fmr1*KO mice by the presence of a M<sub>4</sub> heterozygous mutation, suggesting that the mechanism underlying the PPI phenotype in the *Fmr1*KO mice might not involve M<sub>4</sub> receptors.

#### 4.2. M<sub>4</sub> receptor mediated modulation of learning and memory in the *Fmr1*KO mice

Several studies have implicated muscarinic receptors in learning and memory processes [see reviews 49,50,51,52]. However, previous studies have reported no impairment in the passive avoidance assay in the M<sub>4</sub> KO mice [23]. In our lab we have shown that the M<sub>4</sub> receptor-preferring tropicamide enhances learning and memory in the passive avoidance assay in the WT and in the *Fmr1*KO mice [29]. *Fmr1*KO mice exhibit learning and memory defects [2,5,25], but the passive avoidance phenotype in the *Fmr1*KO mice has been inconsistent with some reports suggesting an impairment [26,30] while others reporting no effect [2,29]. In our current study, we did not observe an *Fmr1*KO phenotype, and the DM mice also did not show any modulation. Since we have shown that genetic background significantly impacts the behavior of *Fmr1*KO mice [28], it is possible that the lack of an impairment seen in the present study resulted in part from the fact that the genetic background of the present mice was an F1 between B6J (*Fmr1* female heterozygotes) and B6NTac (M<sub>4</sub> male heterozygotes) instead of the pure B6J that we used previously. It is also possible that in this present study the decrease in pain sensitivity seen in the *Fmr1*KO mice might contribute in part to the lack of phenotype in the passive avoidance assay. Investigating other types of learning and memory assays might help us better understand the potential for modulation of learning and memory process in the *Fmr1*KO by changes in M<sub>4</sub> receptors.

#### 4.3. Synergistic effect of the M<sub>4</sub> receptors on locomotor activity in the *Fmr1*KO mice

The *Fmr1*KO mice have been shown to exhibit a hyperactivity phenotype [2,25,26,28]. Similarly, the M<sub>4</sub> KO mice showed hyperactivity in an open field [19,23]. Tropicamide, an M<sub>4</sub> receptor-preferring antagonist, has been shown to increase activity in the WT and in the *Fmr1*KO mice [30]. In the present study, neither the *Fmr1* mutation nor the M<sub>4</sub> mutation led to significant hyperactivity, but when combined in the DM mice a synergistic effect was observed, suggesting that the M<sub>4</sub> receptors and the FMRP pathway either directly or indirectly interact with each other to increase locomotor activity. Along with other factors, the balance between the cholinergic and the dopaminergic neurotransmission is essential for maintaining normal motor activity [53]. In the M<sub>4</sub> KO mice, activation of the D1 receptors by a D1 agonist further enhanced the locomotor responses compared to the WT mice, suggesting that the M<sub>4</sub> receptor acts as an inhibitor of the D1 mediated locomotor response in the striatum [19]. Also, FMRP has been shown to be important to mediate dopamine-induced locomotor response in the *Fmr1*KO mice [54]. Hence, the interaction between the M<sub>4</sub> receptor and FMRP might be mediated through the dopaminergic system, which could play an important role in this synergistic enhancement in hyperactivity. However further investigations are required to understand the mechanism.

#### 4.4. Absence of modulation of fragile x behaviors

Decreased anxiety-like responses have been reported in the *Fmr1*KO mice, however this has been shown to be dependent on the genetic background [28]. Little is known about the role of M<sub>4</sub> receptors in mediating anxiety-related responses. In the current study, we observed an

absence of an anxiety-like phenotype in the *Fmr1*KO mice in an open field. However, in the light-dark assay we observed an *Fmr1*KO phenotype that was not modulated in the DM mice suggesting that M<sub>4</sub> is not an important modifier in regulating anxiety-like responses in the *Fmr1*KO mice. Similarly, perseverative behavior in *Fmr1* KO mice measured by number of marbles buried has been shown to be strain dependent [28]. Recent work from our lab has shown that the M<sub>4</sub> receptor-preferring antagonist tropicamide reduced marble burying in WT and in *Fmr1*KO mice [29]. In the present study, we observed an *Fmr1*KO phenotype with the KO mice burying more marbles than the WT. However the DM mice failed to modulate the *Fmr1*KO phenotype, suggesting that reducing M<sub>4</sub> receptor expression by 50% is not sufficient in modulating perseverative behavior in *Fmr1*KO mice. Muscarinic M<sub>4</sub> receptors are highly expressed in the striatum and have been shown to be important for coordinating motor responses [19,55]. However, in our study we failed to observe an overall effect of genotype suggesting that the M<sub>4</sub> receptor might not play a role in modulating motor coordination. Similarly, we failed to observe a modulation of the AGS phenotype in the *Fmr1*KO mice suggesting that 50% reduction in M<sub>4</sub> receptor expression does not modulate the seizure phenotype.

#### 4.5. Absence of modulation of macroorchidism by the M<sub>4</sub> receptors in the *Fmr1*KO mice

One of the most robust anatomical phenotypes in the *Fmr1*KO is macroorchidism [2], which has been shown to be due to the increased proliferation of sertoli cells in the *Fmr1*KO mice [38]. Interestingly, muscarinic receptors have been shown to be important for sertoli cell proliferation [39,56]. All five mAChR subtypes (M<sub>1</sub>–M<sub>5</sub>) are expressed in the rat testis [57,58]. In our study, we observed an increase in the testis: body weight ratio in *Fmr1* KO mice, but reducing M<sub>4</sub> receptor expression in the DM mice failed to rescue this macroorchidism phenotype.

Previously, we had used a pharmacological approach to reduce M<sub>4</sub> receptor activity in the *Fmr1*KO mice and observed modulation of perseverative behavior, learning and memory and AGS [29]. Both the genetic and pharmacological approaches have advantages and disadvantages. While the M<sub>4</sub> antagonist tropicamide showed five fold selective affinity to M<sub>4</sub> receptors, studies have shown that the antagonist binds other muscarinic receptors with lower affinity relative to M<sub>4</sub> [59]. In contrast, the major advantage of the genetic approach is that it offers receptor selectivity by targeting M<sub>4</sub> receptor and investigates specific roles for M<sub>4</sub> receptor in modulating fragile × behaviors. Our recent study evaluating the effect of tropicamide on *Fmr1* KO behavior [29] utilized a single injection of the antagonist; therefore the observed modulation of *Fmr1* KO and WT behavior was due to the acute effect of the antagonist at the time of testing. However, the genetic mutation reflects a change that is present throughout lifetime and the modulation observed reflects a chronic change in the receptor level and activity. Although the M<sub>4</sub> knockout mice do not exhibit embryonic lethality, the M<sub>4</sub> receptors are expressed during embryonic development and absence of M<sub>4</sub> receptors throughout development, might result in developmental changes in the double mutants (M<sub>4</sub>.Het.*Fmr1*KO) that could also account for the discrepancy between the genetic and pharmacological approaches. Also, the stress associated with the injection itself could add variability to the behaviors assessed by the pharmacological study. Regardless of the approach the reduction of M<sub>4</sub> receptor clearly modulates select fragile × behaviors.

In summary, the present study has shown that a reduction in M<sub>4</sub> receptor expression modified select behaviors in the *Fmr1*KO mice. However, in a number of behaviors reduced M<sub>4</sub> receptor expression failed to modulate *Fmr1*KO phenotypes. Some of the possible reasons for this lack of modification are as follows: 1. The M<sub>4</sub> receptors might not be important in mediating these particular behavioral responses 2. Immunoprecipitation studies using the M<sub>4</sub> subtype specific antisera showed a significant reduction in the expression of functional M<sub>4</sub> receptors in the M<sub>4</sub> heterozygotes mice (Gomez et al., 1999). However,

reduction of M<sub>4</sub> protein levels in the heterozygotes in the DM mice might not be sufficient to produce the modulation. As mentioned above, one aspect of the current study was to determine if the M<sub>4</sub> gene was a dominant modifier of *Fmr1*KO phenotypes, therefore, we limited our present study to examining DM mice carrying only one copy of the M<sub>4</sub> receptor gene. Thus, future studies will be necessary to determine if M<sub>4</sub> null mutants have a different impact on an *Fmr1*KO background. 3. There might be compensation through other muscarinic receptors including the M<sub>1</sub> subtype, which is highly expressed in the brain.

In conclusion, our study suggests that reducing M<sub>4</sub> receptor expression positively modulates select behaviors in *Fmr1* KO mice including analgesic response and acoustic startle response, supporting our previous findings that the M<sub>4</sub> receptor represents a potential therapeutic target. More studies involving other behaviors and electrophysiological and biochemical studies will help to understand the mechanism through which the M<sub>4</sub> receptor modulates the behavior of the *Fmr1*KO mice. Nevertheless this study provides further evidence for the involvement of the cholinergic system in F×S.

### Research Highlights

- M4 receptor modulates select fragile × behaviors
- Genetic reduction of M4 rescued the analgesic response
- Genetic reduction of M4 partly rescued the acoustic startle response
- M4 receptors are a potential therapeutic target for Fragile × Syndrome

## Acknowledgments

This research was supported by the Baylor Fragile × Center and the Baylor College of Medicine Intellectual and Developmental Disabilities Research Center

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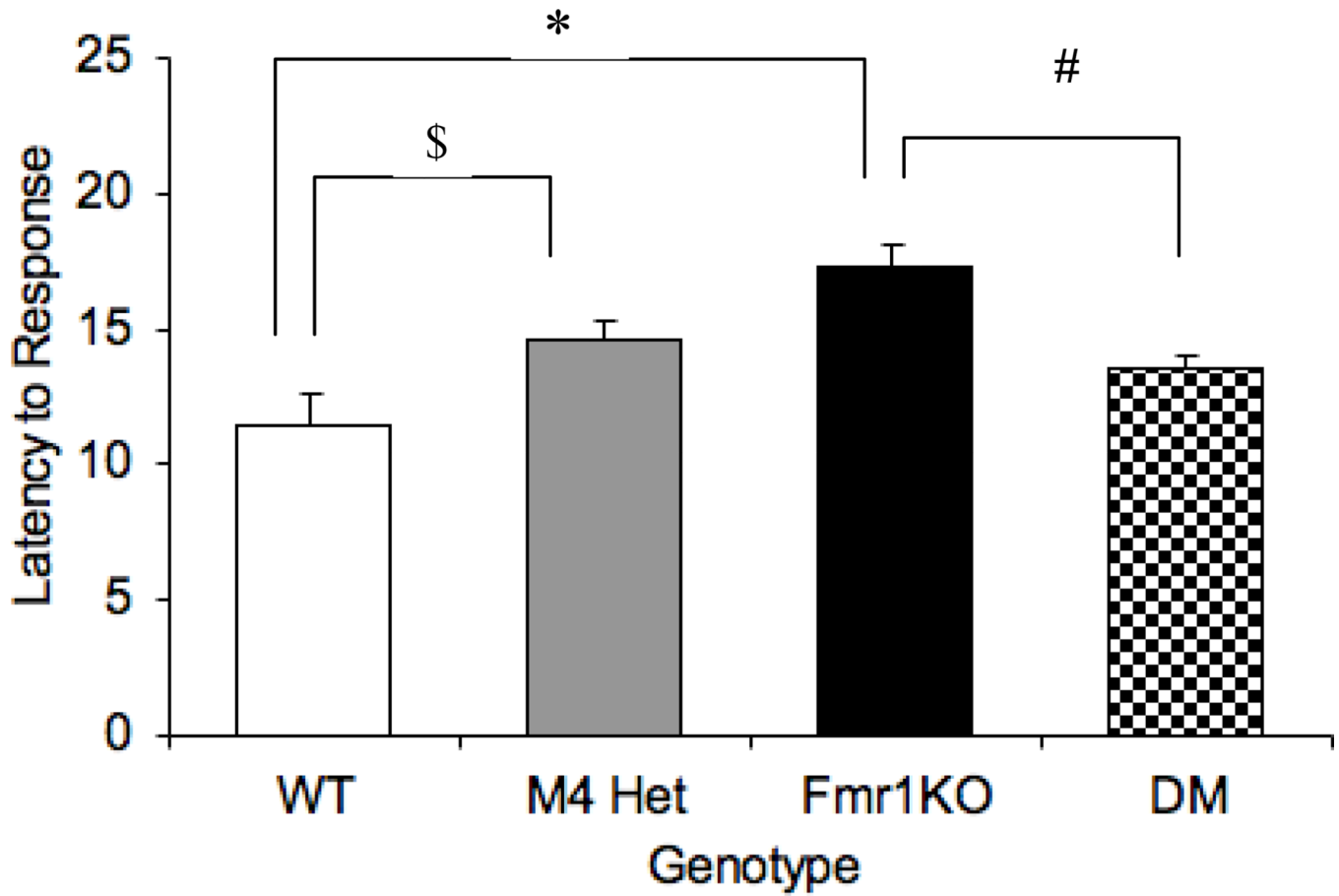
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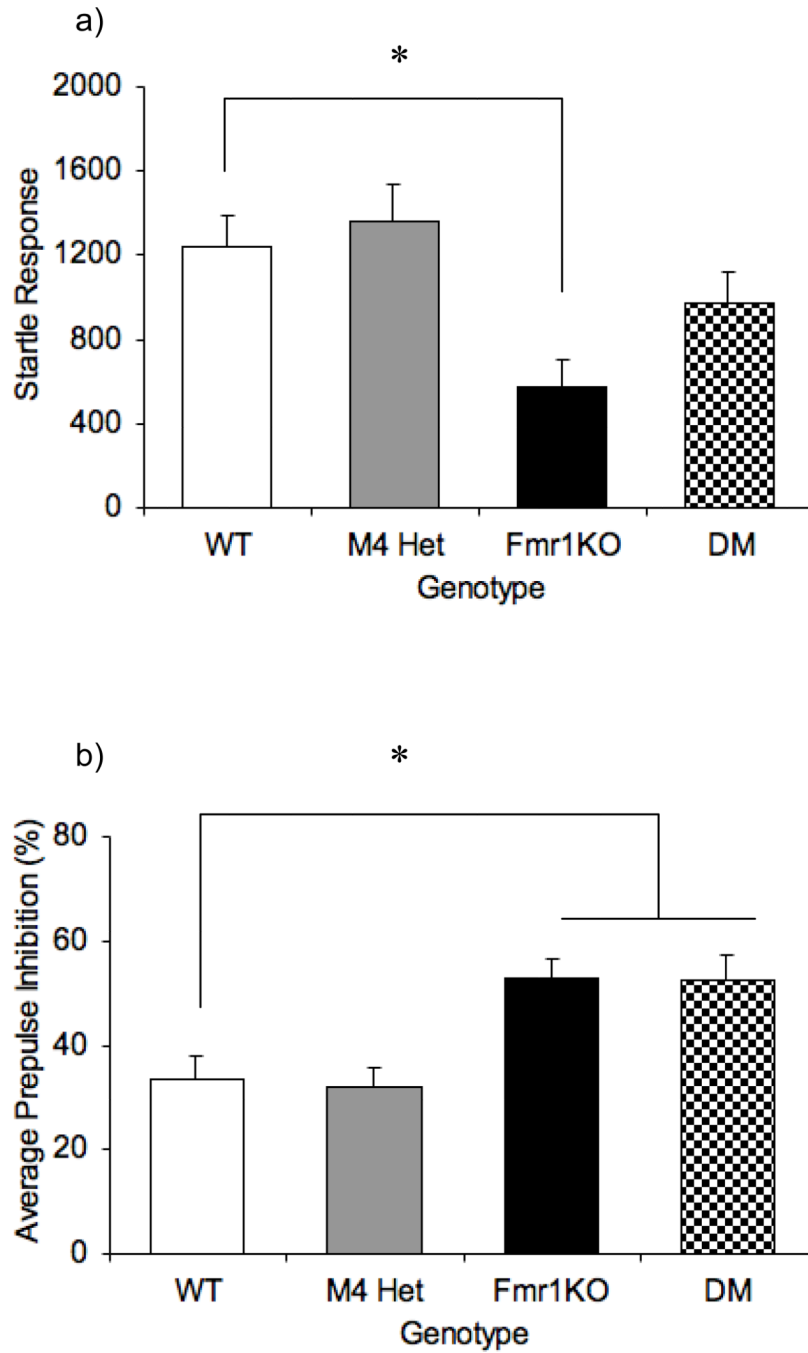
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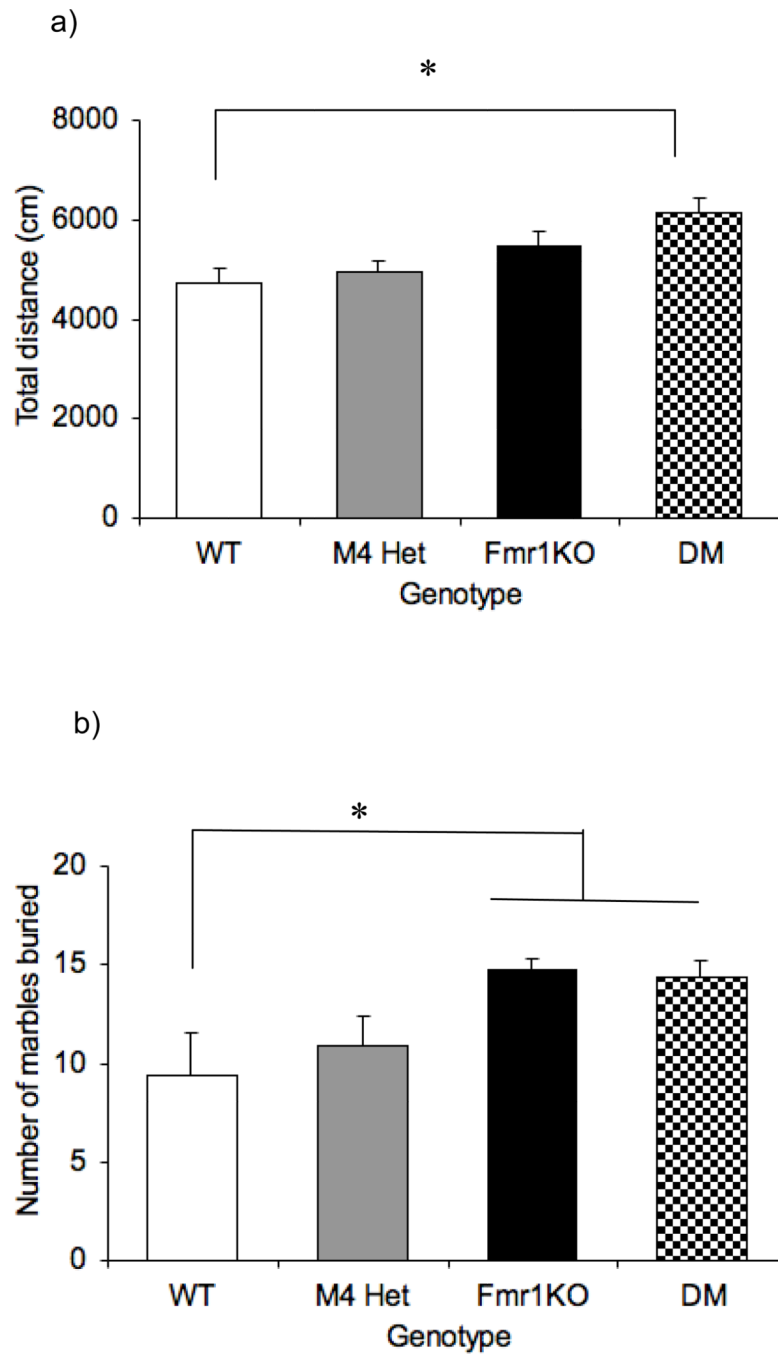
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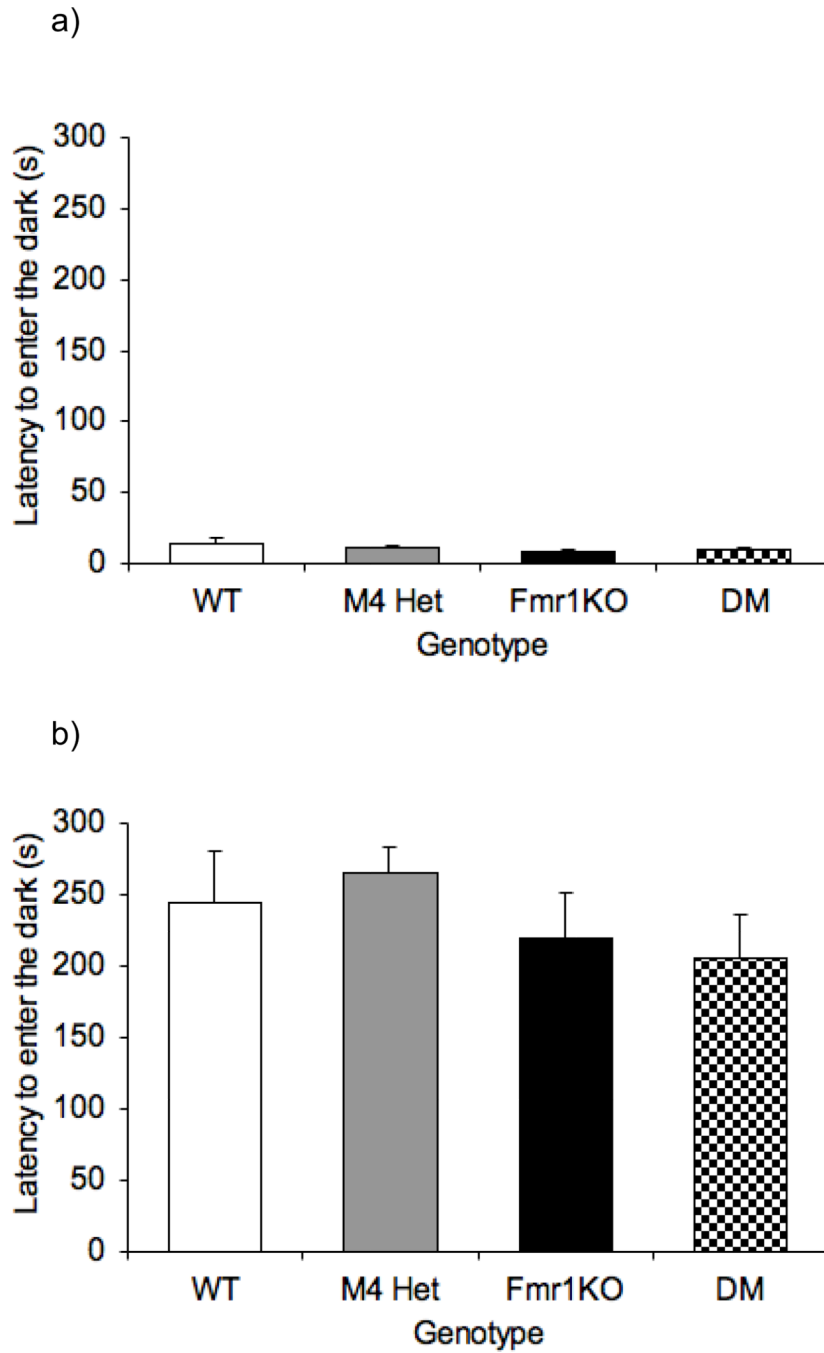
**Fig 1.** Modification of analgesic response by the  $M_4$  receptor in the *Fmr1*KO mice. Latency to hind limb response in the hot plate assay across different genotypes. #  $p < 0.01$  compared to the *Fmr1*KO and \$  $p < 0.05$  compared to the WT (n = 8–14).



**Fig 2.** Modification of acoustic startle response by the M<sub>4</sub> receptor in the *Fmr1KO* mice. Bars indicate mean ± SEM for acoustic startle response to the 120dB startle stimulus (a), average percentage inhibition of the startle response (b) across the four genotypes. \* p<0.05 compared to the WT (n=8–14).

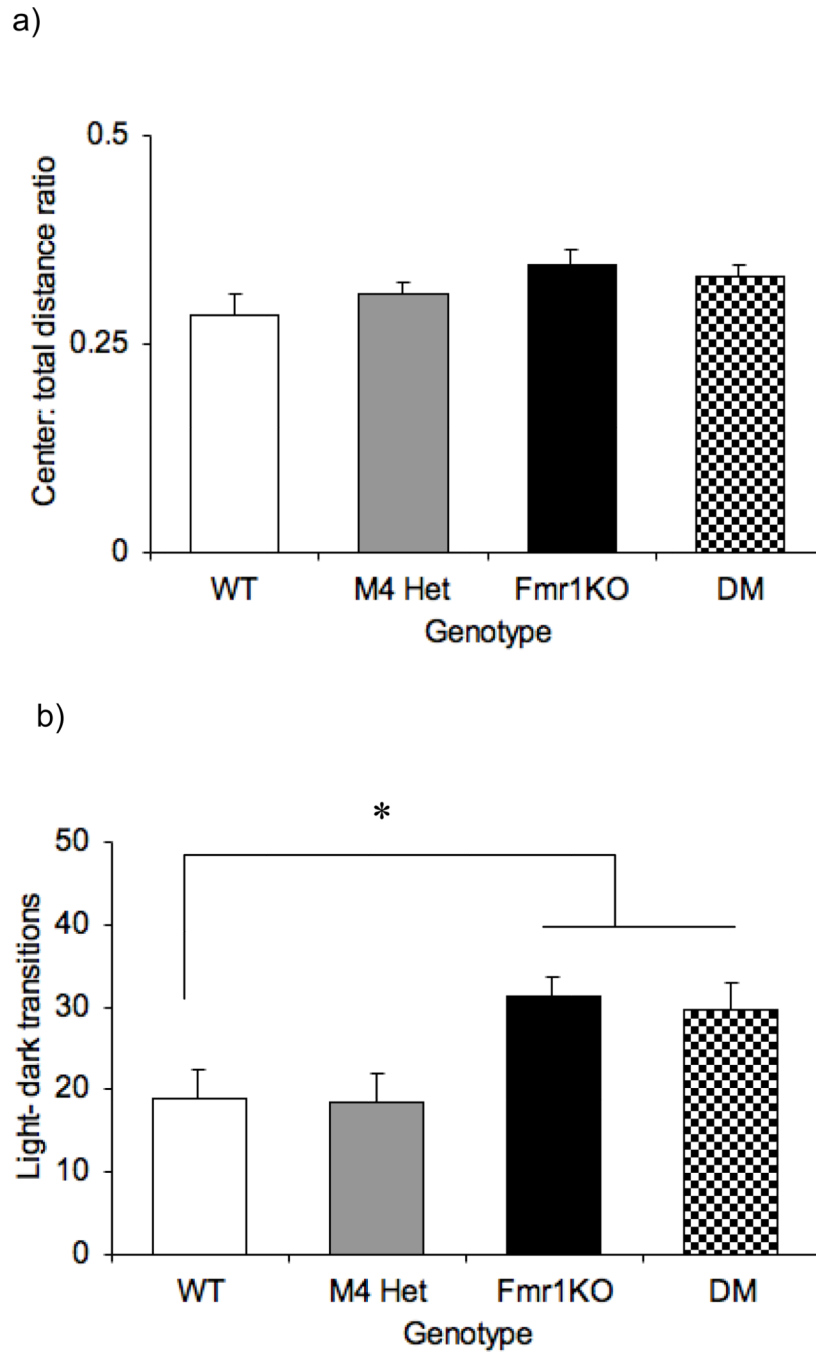


**Fig 3.** Effect of reducing  $M_4$  protein levels on activity and perseverative behavior. Bars represent  $\pm$  SEM for total distance in the open field as a measure of activity (a) and number of marbles buried as a measure of perseverative behavior (b) across the four genotypes. \* $p < 0.01$  compared to the WT ( $n = 8-14$ ).

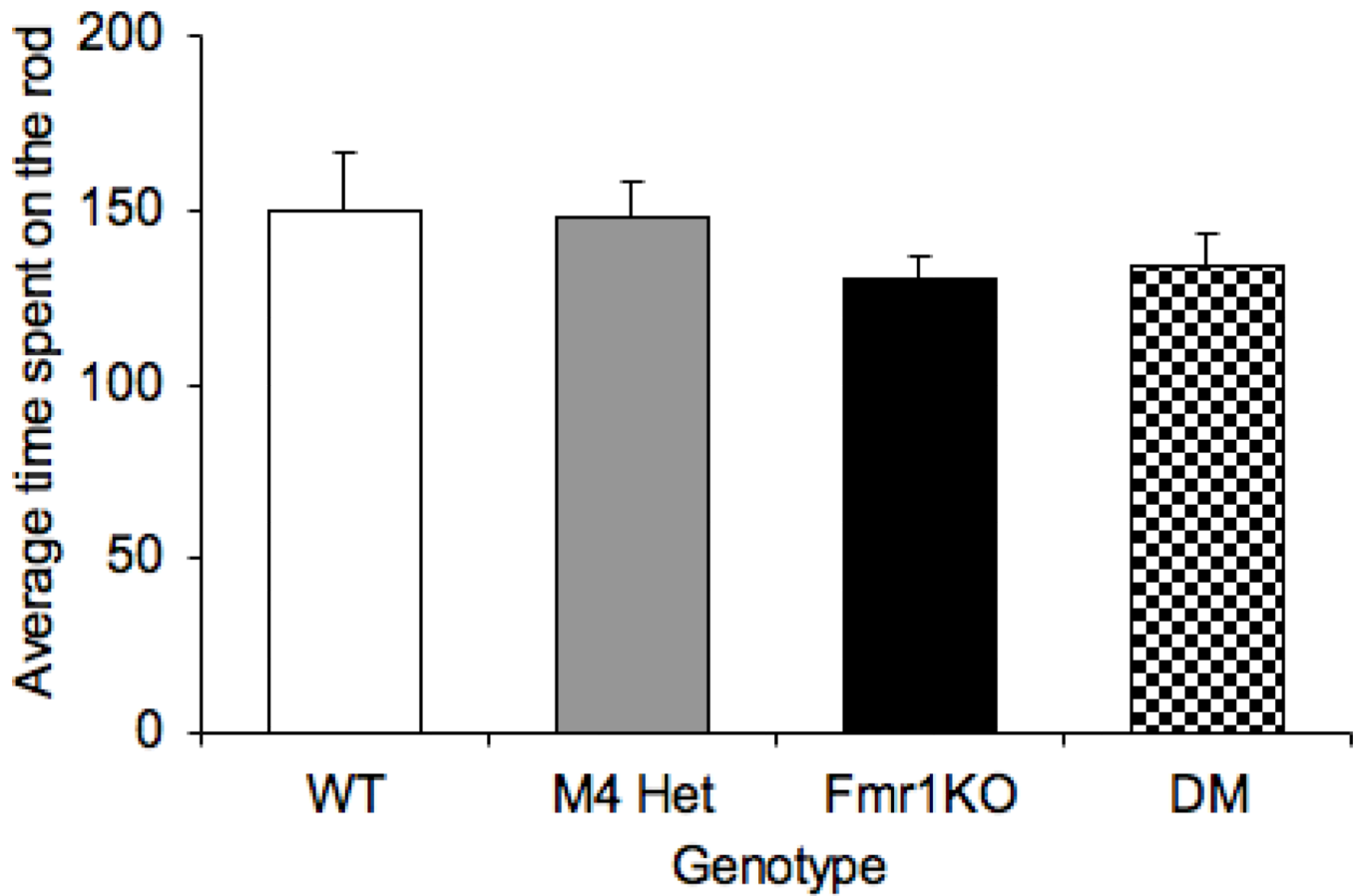


**Fig 4.** Absence of modification of the learning and memory phenotype in the *Fmr1KO* mice. Bars indicate mean  $\pm$  SEM for latency to enter the dark chamber in the passive avoidance assay on day 1 (a) and day 2(b) across four genotypes (n=8–14)

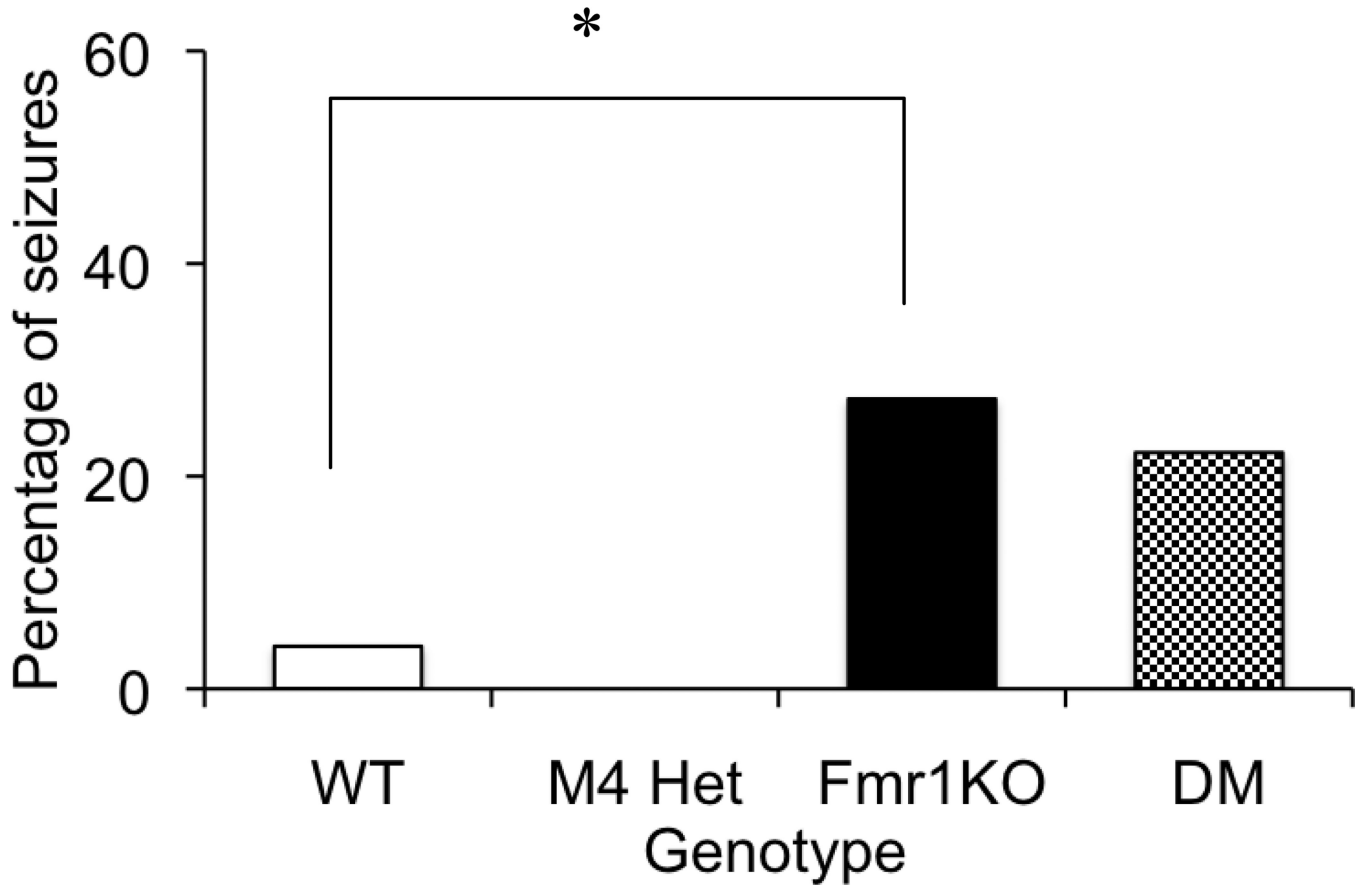




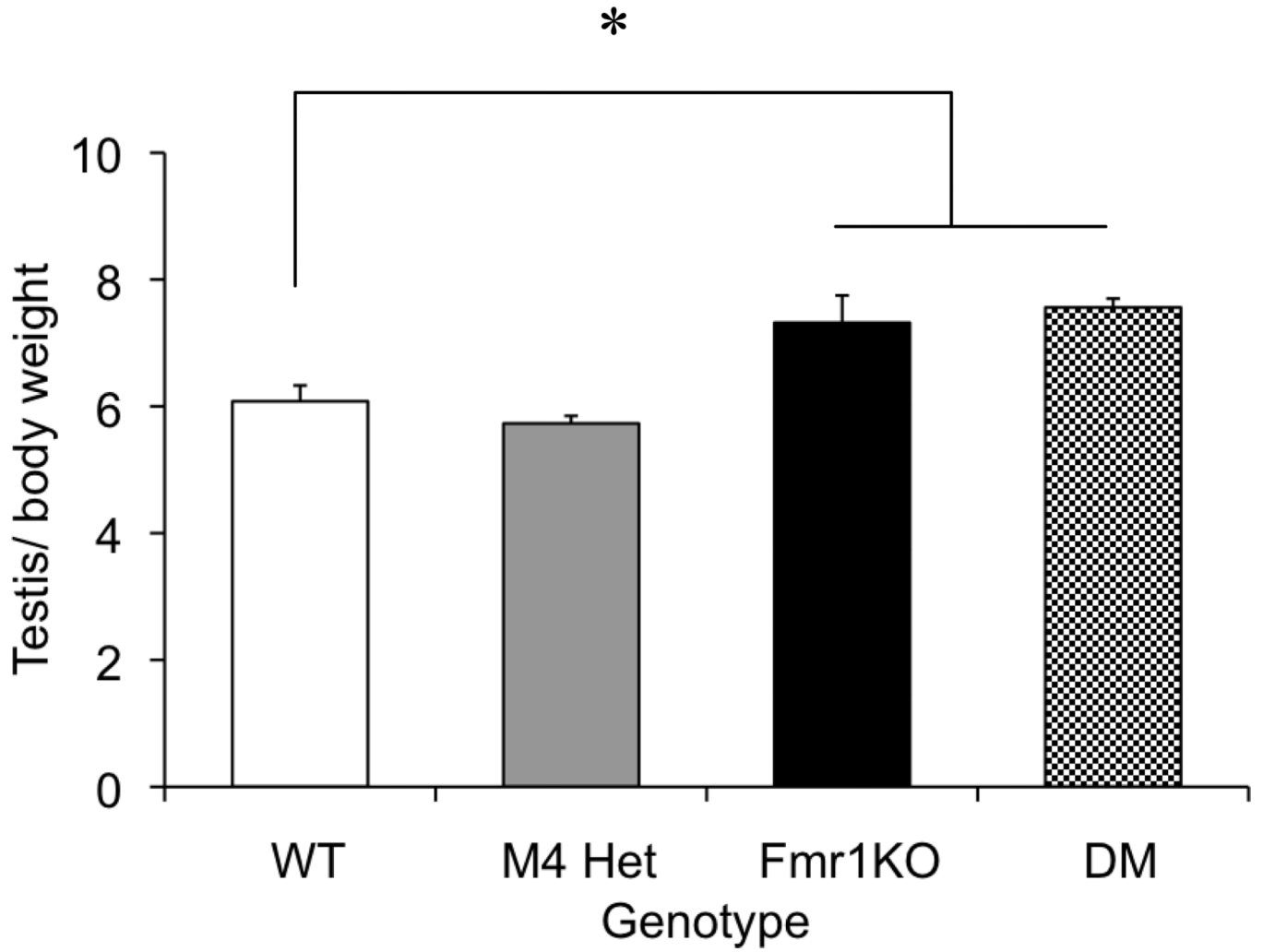
**Fig 5.** No modulation of anxiety like responses by the  $M_4$  receptor. Bars represent  $\pm$  SEM for the center: total distance traveled in an open field, an index of anxiety response (a) and number of light-dark transitions in the light-dark assay (b). \*  $p < 0.05$  compared to the WT (n=8-14)



**Fig 6.** Effect of reducing  $M_4$  protein levels on motor coordination assessed by rotarod assay. Bars indicate  $\pm$  SEM for the average time spent on the rotating rod across the four genotypes (n=8–14).



**Fig 7.** No modulation of AGS by the M<sub>4</sub> receptors. Bars represent value  $\pm$  SEM for percentage of seizures across genotypes. \*p<0.05 compared to the WT



**Fig 8.** No modulation of the macroorchidism phenotype in the *Fmr1KO* mice. Bars represent values  $\pm$  SEM for testis: body weight measurements across four genotypes. \* $p < 0.05$  compared to the WT (n=8–14)