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Olfactory discrimination learning in mice lacking the fragile X mental retardation protein

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Abstract

An automated training system was used to compare the behavior of knockout (KO) mice lacking the Fragile X Mental Retardation Protein with that of wild-type (WT) mice (C57Bl/6 strain) in the acquisition and retention of olfactory discriminations. KO and WT mice did not differ in the acquisition of a four-stage nose poke shaping procedure. In two separate experiments, mutant mice required substantially more training to acquire a series of novel olfactory discrimination problems than did control mice. The KO mice required significantly more sessions to reach criterion performance, made significantly more errors during training, and more often failed to acquire discriminations. Both KO and WT mice showed similar error patterns when learning novel discriminations and both groups showed evidence of more rapid learning of later discriminations in the problem series. Both groups showed significant long-term memory two or four weeks after training but WT and KO mice did not differ in this regard. A group of well-trained mice were given training on novel odors in sessions limited to 20–80 trials. Memory of these problems at two day delays did not differ between WT and KO mice. Tests using ethyl acetate demonstrated that WT and KO mice had similar odor detection thresholds.

Keywords

Fmr1; FMRP; odor discrimination; olfaction; olfactory learning; memory

1. Introduction

The most prevalent inherited form of mental retardation is the fragile X syndrome (FXS), a disorder caused by mutations of the fragile X mental retardation 1 (*FMR1*) gene (O'Donnell & Warren, 2002). In nearly all cases, the mutations involve a trinucleotide (CGG) repeat expansion in the 5' untranslated region of the gene that leads to DNA methylation and transcriptional silencing. As a result, the *FMR1*-encoded protein, the fragile X mental retardation protein (FMRP) is absent in individuals affected by FXS. In some rare cases, point mutations within the protein-coding sequence or deletions of *FMR1* also result in FXS, indicating that the syndrome is indeed caused by absence of functional FMRP (Hoogeveen & Oostra, 1997). A mouse model for FXS has been developed by targeted mutation of the

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Fmr1 gene; these *Fmr1* knockout (KO) mice lack expression of functionally intact FMRP (Bakker et al., 1994).

FMRP is an RNA binding protein that in brain is localized to neurons and is found in dendrites; the protein appears to regulate translation by binding to mRNAs in large messenger ribonucleoprotein particles (O'Donnell et al., 2002). An increasingly large body of evidence implicates FMRP in synaptic function (Antar & Bassell, 2003; Antar, Afroz, Dichtenberg, Carroll, & Bassell, 2004; Antar, Li, Zhang, Carroll, & Bassell, 2006; Muddashetty, Kelic, Gross, Xu, & Bassell, 2007; Nakamoto et al., 2007; Greenough et al., 2001). Dendritic spines on neurons in brains from FXS patients exhibit immature morphology, suggesting that the protein is necessary for normal spine development and/or adult spine plasticity (Irwin, Galvez, & Greenough, 2000); these spine abnormalities are also seen in *Fmr1* KO mice (Irwin et al., 2002; Comery et al., 1997). In isolated synaptoneurosome preparations, stimulation of metabotropic glutamate receptors (mGluRs) increases FMRP synthesis by activating its local translation (Weiler et al., 1997; Muddashetty et al., 2007; Todd, Mack, & Malter, 2003). Similar effects have been observed in cultured neurons (Todd et al., 2003) where mGluR stimulation also induces translocation of FMRP and *Fmr1* mRNA to dendrites and FMRP away from synapses (Antar et al., 2004) and induces the synthesis of PSD-95, a postsynaptic scaffolding protein, via an FMRP-dependent mechanism (Todd et al., 2003). Finally, mice lacking FMRP show enhanced long-term depression (LTD) in hippocampus (Huber, Gallagher, Warren, & Bear, 2002) and impaired long-term potentiation (LTP) in several cortical areas (Li, Pelletier, Perez Velazquez, & Carlen, 2002; Wilson & Cox, 2007; Larson, Jessen, Kim, Fine, & du Hoffmann, 2005; Zhao et al., 2005). However, LTP in hippocampus appears to be normal in the KO mice (Godfraind et al., 1996; Larson et al., 2005; Li et al., 2002; Paradee et al., 1999; Lauterborn et al., 2007).

Fmr1 KO mice are important tools for understanding how synaptic dysfunction in the absence of FMRP impairs cognitive function and cognitive development. Behavioral studies of these mice have shown deficits in aversively-motivated spatial learning and fear conditioning tasks (D'Hooge et al., 1997; Brennan, Albeck, & Paylor, 2006; Dobkin et al., 2000; Fisch, Hao, Bakker, & Oostra, 1999; Van Dam et al., 2000; Mineur, Sluyter, de Wit, Oostra, & Crusio, 2002; Paradee et al., 1999). However, the mild nature of the deficits observed in most cases suggest that either mice do not serve as ideal models of human cognition or that the tasks that have been used are not sensitive to the functional impairments caused by the absence of FMRP. It has been suggested that olfactory-guided tasks may be more appropriate tests of cognitive capacity in rodents (Otto & Eichenbaum, 1992; Slotnick, 1994; Staubli, Fraser, Faraday, & Lynch, 1987) and mutant mice in particular (Larson & Sieprawaska, 2002; Larson, Hoffman, Guidotti, & Costa, 2003; Bodyak & Slotnick, 1999; Mihalick, Langlois, Krienke, & Dube, 2000). Therefore, the present study used an olfactory discrimination learning paradigm to compare learning and memory abilities in normal mice and mice lacking FMRP.

The present experiments were designed to address two main issues. First, can an appetitively-motivated olfactory discrimination paradigm be used to investigate cognitive and memory disabilities in mice lacking FMRP? With a few exceptions (Fisch et al., 1999; Yan, Asafo-Adjei, Arnold, Brown, & Bauchwitz, 2004; Moon et al., 2006), behavioral studies of cognition and learning in *Fmr1* knockout mice have used aversively-motivated tasks such as water mazes (Bakker et al., 1994; Van Dam et al., 2000; Peier et al., 2000; Dobkin et al., 2000; Kooy et al., 1996; D'Hooge et al., 1997; Paradee et al., 1999); or fear conditioning/shock avoidance (Bakker et al., 1994; Brennan et al., 2006; Paradee et al., 1999; Dobkin et al., 2000; Van Dam et al., 2000; Peier et al., 2000). The deficits that have been observed in these tasks have been subtle and some have not been readily reproducible. We show that it is possible to train *Fmr1* knockout mice on olfactory discriminations using an automated method. Second, do mice lacking FMRP differ from wild-type mice in learning olfactory discrimination problems? We find that

Fmr1 KO mice learn novel olfactory discrimination problems significantly more slowly than WT control mice. Long-term memory for the discriminations appears normal in the KO mice and their sensitivity to odors also appears similar to that of WT mice.

2. Materials and methods

2.1 Subjects

Subjects were male, *Fmr1* knockout (KO) mice (C57BL/6J background) born in our colony from stock originally obtained from Jackson Laboratories (Bar Harbor, ME) and male, age-matched, wild-type (WT; C57BL/6J) mice obtained from Jackson or born from breeders in our laboratory, all at least 2.5 months old at the onset of training. The KO mice were backcrossed at least 10 times into the C57BL/6J background. They were housed in groups of three or four in plastic cages in a climate-controlled animal colony on a normal 14:10 light:dark cycle. The mice were maintained on a water deprivation schedule with access to 1.0–2.0 mL water once per day for at least five days prior to and throughout training. This schedule reduced body weight by about 20% in the first few days but maintained the mice at a stable weight throughout the study. All testing was done during the light phase. All experiments were conducted blind with respect to genotype.

2.2 Apparatus

As described previously (Larson et al., 2002; Patel & Larson, 2007; Larson et al., 2003), the testing chamber was made of black acrylic and consisted of a straight alley 60 cm long and 10 cm wide. The two side (long) walls sloped upward and outward at an angle of 15° off vertical and were 30 cm high. The end walls were vertical. At each end (“East” and “West”) of the alley were two cylindrical “sniff ports” (1.5 cm i.d.) for nose poke responses (2 cm from the floor and centered 5 cm apart) and a single small cup in the floor for water delivery. The two sniff ports at the West end of the alley were connected to individual air-dilution olfactometers for odor stimulus delivery; all of the sniff ports were equipped for photobeam detection of nose pokes. Odor and water delivery were controlled by electrically-driven, teflon-body solenoid valves (General Valve Co., Fairfield, NJ); a microcomputer (PC) detected infrared photobeam breaks and activated the valves under custom software control. The whole chamber was enclosed and the ceiling was equipped with an exhaust fan to remove odorants.

An air dilution system described previously (Larson et al., 2002) was used to generate odorants. Bottles containing odorants (diluted to 25% in solvent) were located downstream of computer-operated control valves and flowmeters in order to minimize odorant contamination of these elements. The clean air supply (bottled zero air, AGA Gas Co., Lansing, IL) in each channel was run at 1.8 L/min; odorized air was injected into this stream at 0.2 L/min for an air dilution of 10%. The bottles and all common tubing in the system were made of teflon or glass to facilitate cleaning. The odorant bottles and tubing elements exposed to odorants were replaced as a unit when odor pairs were changed. Odorants used in these experiments were selected from a large stock of chemicals obtained from International Flavors and Fragrances (Union Beach, NJ), Aldrich Chemical Co. (Milwaukee, WI), and McCormick and Co. (Baltimore, MD).

2.3 Procedures

All procedures were fully automated and controlled by computer within each training session.

2.4 Shaping

A shaping procedure was used to familiarize the mice to the training procedures and to reinforce nose poke responses prior to the introduction of odor cues. This proceeded in four stages. The first stage consisted of 20-trial, daily sessions in which the mice were reinforced with a small

drop of water (12.5 μ L) for a nose poke in either sniff port at either end of the alley. Each trial was a maximum of 120 sec. long and was followed by a 10 sec. inter-trial interval (ITI). After each reinforced response, the next reinforcement was contingent on the mouse making a nose poke in one of the sniff ports at the end of the alley opposite the last correct response, i.e., the mouse was required to traverse the alley repeatedly. If a correct response did not occur within the 120 sec. period, that trial was scored as incorrect and the identical contingency was in effect on the following trial. A lamp at each end of the alley was lit during the ITI and was extinguished over the ports at which reinforcement was available during the trials. Each mouse was trained in this way until it had made 90% reinforced responses in one 20-trial session. The second stage of shaping was identical to the first except that each session had 40 trials and mice were trained to criterion of 90% reinforced trials in one 40-trial session. The third stage of shaping consisted of 20-trial sessions in which each trial began with the extinguishing of the lamp at the East end of the alley. A nose poke in either of the East sniff ports (a "trial initiation" nose poke) within 120 sec. of the trial onset was reinforced with a drop of water, turned on the lamp, and extinguished the lamp at the West end of the alley. No response within the 120 sec. period terminated the trial and was followed by a 10 sec. ITI. A nose poke at either West sniff port within 60 sec. after the trial initiation nose poke was rewarded with water and followed by the ITI. Mice were trained to a criterion of 90% of trials rewarded at both ends in one 20-trial session. The fourth stage of shaping was identical to stage three except that trial initiation nose pokes were not rewarded with water, each session was 40 trials, and mice were trained to a criterion of three sessions in which 90% of trials were rewarded.

2.5 Olfactory discrimination

Olfactory discrimination training used a simultaneous-cue, two-odor, forced-choice paradigm. The trial procedures and timing were similar to those of shaping stage four except that a trial initiation nose poke at the East end also activated the delivery of the two discriminative odors to the West sniff ports. The spatial position of the two odors (S+ and S-) on any given trial was randomly determined except that no more than three identical trials could occur in succession. A nose poke in the port containing the S+ stimulus was rewarded with a drop of water (12.5 μ L) and scored as a correct trial; a nose poke in the S- port was not rewarded and was scored as an error. The inter-trial interval was 60 sec. No response within 60 sec. after trial initiation also terminated the trial but no-response trials were not scored as errors and were followed by a 60 sec. ITI.

All mice were trained on a series of eight different two-odor discrimination problems. Each mouse was given a single session of 40 training trials per day. Training on a given discrimination problem continued until the mouse achieved a performance criterion of 90% correct responses or better in the last 20 trials of a session. Training on the next problem then commenced with the following session. If a mouse failed to reach criterion on a discrimination problem after eight training sessions, it was determined to have "failed" that problem and advanced to the next. The mice were trained successively on eight different two-odor discrimination problems. The odor pairs and valences were as follows: strawberry (S+) and banana (S-), propionic acid (S+) and hexyl octanoate (S-), ethyl lactate (S+) and methyl salicylate (S-), terpinyl acetate (S+) and anisole (S-), pineapple (S+) and cherry (S-), maple (S+) and cycloprop (S-), dihydrojasnone (S+) and cis-3-hexen-1-ol (S-), walnut (S+) and butter (S-). One subset of each group learned the discriminations in the order listed; the other subsets learned the discriminations in the reverse order. In a previous study, these odor discriminations were acquired with comparable facility by mice (Larson et al., 2002). The number of errors committed before learning criterion was made for each discrimination was tabulated for each mouse.

2.6 Memory test

After each mouse reached criterion performance on the eighth (final) discrimination, it was not tested for a four-week period. After this delay, it was given a series of ten probe trials, each of which was identical to a training trial in the final discrimination problem except that no differential reinforcement was provided. Responses at the port containing the odor trained as S+ were scored as correct and responses to the trained S- were scored as incorrect. However, no water was given on either correct or incorrect trials. The ITI was 60 sec.

2.7 Memory after limited training in well-trained mice

An additional set of male *Fmr1* KO mice and male WT littermates mice raised in our colony were trained to nose poke and to acquire a series of eight olfactory discriminations as above except for two modifications: First, during olfactory discrimination training the ITI after S+ responses was 10 sec and that after S- responses was 30 sec. Second, criterion performance for olfactory discrimination training was set to be 80% correct in the final 20-trial block of a session. Mice were tested for retention of the eighth discrimination two weeks after the last training session. Mice were then trained on four additional discrimination problems. In the first, mice were trained to discriminate between brandy and lemon odors in one 20-trial session. The odors used as S+ and S- were counterbalanced across mice in each group in this and all subsequent discriminations. Mice were tested for retention of the discrimination two days later. In the second discrimination, mice were trained in one 40-trial session to discriminate almond and root beer, with retention tested two days later. The third test used a training session with 60 trials and isoamyl nonanoate and phenethylamine as the discriminative odors. The fourth test used an 80-trial session with geranium bourbon oliffac (IFF) and Diola (IFF) as the odors.

2.8 Olfactory sensitivity

Tests for threshold detection of ethyl acetate used six male *Fmr1* KO and seven littermate WT mice raised in our colony and maintained as described above. The apparatus was the same except that the odor generator was modified for olfactory sensitivity testing as in Patel and Larson (Patel et al., 2007).

The procedure used to assess olfactory sensitivity was as described previously (Patel et al., 2007). Mice were initially trained to nose poke using the shaping procedure described above. They were then trained in 40-trial sessions to discriminate between a 0.001% (10^{-3} %) ethyl acetate stimulus (S+) and a clean air stimulus (S-). The S+ odor tube contained a 50 ml solution of 0.02% ethyl acetate in ultrapure water; the S- odor tube contained ultrapure water alone. A final air dilution in the odor generator diluted the vapor in each S+ and S- odor tube by 20-fold (0.1 lpm through the odor tubes and 1.9 lpm in the main airstream). Lower concentrations of ethyl acetate in subsequent tests were generated by further dilution of ethyl acetate in water in the odor tubes. The final (and only) air dilution was always 20-fold and was the same for the ethyl acetate and clean air stimuli. The odor tubes and odor-exposed tubing for each concentration of ethyl acetate (S+) and water (S-) tested were prepared as separate odor cartridges that were inserted in the odor generator as required.

Mice were trained in two 20-trial sessions each day. In the second session each day, the connections between the odor tubes and the control valves for each channel in the olfactometer were reversed and the software controls for the valves were also reversed in order to ensure that mice could not use the sounds of the valves as cues in the task. (This maneuver switched the valves controlling S+ and S- but did not alter the reward contingencies.) Mice were trained on the "high intensity" ethyl acetate (10^{-3} %): water discrimination until all had performed at least two sessions at criterion (80% correct) on one day. They were then trained in two daily 20-trial sessions on a different concentration selected from a series consisting of one-half log-unit steps between 10^{-4} % and 10^{-8} % ethyl acetate. One day training on the high concentration

(10^{-3} %) was interleaved between each pair of lower concentration days. S+ was always ethyl acetate and S- was always ultrapure water (no odor). The percentage of correct trials was calculated for each session. Response latencies were also computed for each correct trial and averaged across trials within a session.

3. Results

3.1 Shaping

WT mice required 6–11 sessions (mean = 7.54 ± 0.23 , $n = 24$) to satisfy shaping criteria; KO mice required 6–10 sessions (7.68 ± 0.25 , $n = 25$), a statistically insignificant difference ($t_{47} = 0.40$, $p > .5$). The mean number of sessions required to reach criterion at each stage of shaping are provided in Table 1. The mean latencies to perform nose pokes as required at the different stages of shaping are also shown in Table 1. These measures also did not discriminate between the *Fmr1* KO and WT mice (sessions to criterion: $F_{1,5} = 0.16$, $p > .5$; latency: $F_{1,5} = 0.36$, $p > .5$).

3.2 Olfactory discrimination learning

Two KO mice were excluded from further analysis due to procedural mistakes in the odor assignments. One WT mouse failed to reach criterion on six of the eight discriminations; the data from this mouse was excluded since it was clearly an outlier (MNR statistic) (Snedecor & Cochran, 1989). The behavioral data for the remaining 23 WT and 23 KO mice are described here. Most of the mice were littermate ($n=15$ WT and 16 KO) progeny of heterozygous KO females; however, some of the KO mice ($n=7$) were offspring of homozygous mutants ($n=7$) and some of the WT mice ($n=8$) were age-matched controls obtained from Jackson Laboratories.

From one to the maximum of eight training sessions were required for WT mice to either reach the performance criterion or fail to acquire the first odor discrimination problem (mean = 4.65 ± 0.50). KO mice required from two to eight sessions (mean = 5.30 ± 0.43). The mean values were not significantly different ($t_{44} = 0.98$, $p > .30$). Three WT (13%) and six KO (26%) mice failed to make criterion in the maximum of eight training sessions on the first discrimination problem. The mean errors to criterion averaged $58.96 (\pm 7.99)$ for WT mice and $68.83 (\pm 8.21)$ for KO mice ($t_{13} = 0.86$, $p > .35$).

For each of the eight discrimination problems, the number of training sessions required to either reach criterion performance or fail (eight sessions) was recorded for each mouse. The number of errors made during training on each discrimination was also recorded. The total errors made during training on the eight olfactory discriminations in WT and KO mice are displayed in Figure 1A. The *Fmr1* KO mice made significantly more errors than the WT mice ($t_{44} = 3.17$, $p < .01$). The difference in total errors was nearly 50%. The eight discrimination problem series was divided into blocks of four problems to assess improvement of performance across problems. The mean number of errors committed during training on discriminations 1–4 and 5–8 are shown in Figure 1B. Analysis of variance indicated that there was a significant main effect of genotype on errors ($F_{1,44} = 10.04$, $p < .01$), a significant main effect of problem block ($F_{1,44} = 44.63$, $p < .0001$), but no interaction between genotype and block ($F_{1,44} = 0.26$, $p > .60$). The KO mice made more errors than WT mice in both problem blocks 1–4. Both groups of mice made significantly fewer errors in problems 5–8 than in problems 1–4.

Response accuracy in the first training session for each discrimination was analyzed to determine if the KO mice were specifically impaired early in training on novel discriminations. Figure 1C shows the percent correct responses in the first session of training in blocks of five trials, averaged across problems 1–4. Both WT and KO mice demonstrated an increase in

choice accuracy during the first session of training for these discrimination problems. Analysis of variance indicated no main effect of genotype ($F_{1,44} = 0.44, p > .50$). There was a highly significant effect of trial block ($F_{6,264} = 10.90, p < .0001$) but no interaction between genotype and block ($F_{6,264} = 0.41, p > .85$). Similar results were obtained in problems 5–8 (Figure 1D). The main effect of genotype was not significant ($F_{1,44} = 1.77, p > .15$), the block effect was significant ($F_{6,264} = 20.01, p < .0001$), and the interaction was not significant ($F_{6,264} = 0.53, p > .75$). These results suggest that KO mice were not selectively impaired in the early trials of novel discriminations; however, over the entire course of training, they commit significantly more errors.

Mice occasionally failed to make an odor response within the 60 sec. choice period of a trial, especially during training on the first discrimination problem. The mean total number of “no response” trials over the eight discrimination problem series was $9.4 (\pm 3.1)$ for WT mice and $14.1 (\pm 3.0)$ for KO mice, a difference that did not reach statistical significance ($t_{44} = 1.09, p > .25$).

A two-way analysis of variance was conducted to determine if the differences between WT and KO mice in errors committed were specific to certain of the odor pairs used in training. There was the expected main effect of genotype ($F_{1,44} = 10.04, p < .01$) and a main effect of odor pair ($F_{7,308} = 6.11, p < .0001$), indicating that the odor pairs differed in difficulty. However, the interaction between genotype and odor pair was not significant ($F_{7,308} = 1.21, p > .25$). Thus, the impairment of KO mice in olfactory discrimination learning was not specific to certain odor pairs.

3.3 Error patterns

Training sessions in which 15 or more errors were committed were designated as “high-error” sessions (Larson et al., 2002). There were 109 such sessions for WT mice (21/23 mice) and 161 for KO mice (23/23 mice). The average number of high-error sessions per mouse was $4.74 (\pm 0.73)$ for WT and $7.04 (\pm 0.92)$ for KO mice, a difference that only approached statistical significance ($t_{44} = 1.96, p = .056$). However, the average number of different discrimination problems on which each mouse had at least one high error session was significantly larger for KO (3.47 ± 0.29) than for WT (2.48 ± 0.27) mice ($t_{44} = 2.52, p < .02$).

High error sessions were analyzed for error patterns. Errors were classified into four types, based on spatial position of the error trial and the spatial position and odor response on the preceding trial. Spatial bias was manifest in sessions in which the number of errors preceded by same-side responses (stay-side errors) was greater than that predicted by the binomial distribution for chance responding. This occurred in 87 high-error sessions (79.8%) for WT mice and 118 sessions (73.3%) for KO mice. As can be seen in Figure 2, the distributions of stay-side errors were clearly skewed to the right, for both WT and KO mice; the two groups did not differ in this regard (χ^2 test, $p > .05$). Most high-error sessions resulted from mice adopting a spatial bias for a large number of trials. Perseverative responding was usually consistently to the right or left sniff port within a high-error session but the numbers of right-side and left-side sessions were about equal. Some mice preferred the right side sniff port in all of their high-error sessions, some the left side, and some were inconsistent; there was no indication that genotype had any effect on side preference.

There were no sessions in which mice made significant numbers of errors at the opposite spatial port location of the preceding response, indicating that spatial alternation was not responsible for any high error sessions. Nor were there any instances in which error trials were preceded by error trials more often than would occur by chance alone, suggesting that high error sessions were not due to perseverative responding to the incorrect odor. There were several instances of sessions (WT: $n = 9$, KO: $n = 19$) in which errors were preceded by errors less often than

should occur by chance, possibly indicating odor alternation. However the majority of these sessions (WT: 6/9; KO: 16/19) also had significant spatial bias in responding, suggesting that the seeming odor alternation was probably a spurious consequence of the trial sequences in those few sessions.

The total number of training sessions required for all eight discrimination problems (Figure 3A) was significantly greater for the KO than for the WT mice ($t_{44} = 3.53, p < .002$). The average number of high-error sessions in which mice showed spatial bias (Figure 3B) was greater for KO than for WT mice, but this difference was not statistically significant ($t_{44} = 1.01$, ns). Subtracting the high-error sessions with spatial bias from the total training sessions (Figure 3C) did not eliminate the WT-KO difference ($t_{44} = 4.10, p < .001$).

3.4 Failure to learn

Fourteen KO mice and five WT mice failed to reach criterion on at least one discrimination problem. The average number of failures per mouse (Figure 3D) was significantly greater for KO than for WT mice (Mann-Whitney U-test, $p < .01$). On the other hand, failure to reach criterion does not necessarily mean failure to learn anything about the odors. Of 24 “failed” discriminations by KO mice, all but three (88%) had at least one session in which 70% correct responses were made, a level of performance that would be reached by chance less than 1% of the time. For the WT mice, four of the five “failed” discriminations (80%) had sessions with above-chance (70% correct) responding. Thus despite failure to reach criterion performance on these discriminations, the mice did show significant discrimination ability and learning of the odor-reward association. However, the KO mice took significantly more sessions to reach the first 70% correct session (summed across all eight discriminations: KO mean = 15.43 ± 0.80 sessions; WT mean = 12.87 ± 0.63 ; $t_{44} = 2.52, p < .05$) and had more sessions at this level of performance without reaching the 90% correct criterion (KO: 9.87 ± 1.01 sessions; WT: 6.43 ± 0.66 sessions; $t_{44} = 2.85, p < .01$).

3.5 “Performance” Variables

The mean latency from the “trial initiate” nose poke to the “odor-choice” nose poke was calculated for the correct trials in the last block of 20 trials for each discrimination problem for each mouse. Since this was the criterion run for each problem, performance accuracy was very high in these trials (>90% correct, except for the cases in which mice failed to acquire the discrimination). This latency includes the time needed to run the length of the alley, sample the odors, and make a choice; it thus should be sensitive to these “performance” variables. Analysis of variance indicated that there was no main effect of genotype on response latency ($F_{1,44} = 0.15, p > .05$). There was a highly significant effect of discrimination number ($F_{7,308} = 34.30, p < .0001$); mice in both groups became faster with training (Fig. 4). There was no interaction between genotype and problem number ($F_{7,308} = 0.43, p > .05$).

3.6 Long-term odor memory

Retention of the eighth discrimination problem was tested four weeks after the last training session in a session of ten unreinforced probe trials. Both WT and KO mice demonstrated memory for the discrimination problem, choosing the previously rewarded odor more frequently than expected by chance (WT: $59.00\% \pm 2.86, p < .01$; KO: $60.87\% \pm 3.55, p < .01$); however the two groups did not differ in retention scores.

3.7 Memory after limited training in well-trained mice

A second group of 24 WT and 26 KO mice were trained to nose poke and to learn a series of eight two-odor discrimination problems as above prior to being tested for acquisition and retention of novel discrimination problems with variable numbers of training trials. All of these

mice were littermate progeny of heterozygous KO females. The first part of the experiment serves as a partial replication of the original study with shorter inter-trial intervals (10 sec after correct trials, 30 sec. after incorrect trials) and a lower performance criterion for learning (80% correct in the last 20 trials of a session). These changes were made in order to decrease the number of sessions needed for training in this phase.

As in the first experiment, there was no significant effect of genotype on the number of sessions required to reach criterion for each shaping stage (main effect: $F_{1,48} = 3.47, p > .05$; interaction: $F_{3,144} = 0.47, p > .70$) or mean latency to nose poke in the final session at each stage (main effect: $F_{1,48} = 1.11, p > .25$; interaction: $F_{5,240} = 1.29, p > .25$). WT mice required $6.96 (\pm 0.14)$ and KO mice required $7.42 (\pm 0.20)$ total shaping sessions.

With the relaxed learning criterion and reduced inter-trial intervals, instances of failure to learn in the series of eight olfactory discrimination problems were infrequent. Only one WT mouse failed to reach criterion on a single discrimination problem (the first) and three KO mice failed to learn one to three discriminations problems. Across the entire eight-problem discrimination series, total errors made (Figure 5A) were significantly higher in the KO than in the WT mice ($t_{47} = 2.02, p < .05$). As before, the discrimination series was divided into problems 1–4 and problems 5–8 (Figure 5B). Analysis of variance indicated that there was a significant main effect of genotype on average error number ($F_{1,47} = 4.09, p < .05$), a significant effect of problem block ($F_{1,47} = 101.31, p < .0001$), and no interaction ($F_{1,47} = 0.33, p > .55$). Both groups had significantly fewer errors on problems 5–8 than on problems 1–4; KO mice made significantly more errors WT mice. Trial blocks analysis of the first 35 training trials averaged across problems 1–4 (Figure 5C) indicated no significant effect of genotype on choice accuracy ($F_{1,47} = 0.15, p > .70$), a significant effect of trial block ($F_{6,282} = 13.15, p < .0001$), and insignificant interaction between genotype and trial block ($F_{6,282} = 0.93, p > .45$). In problems 5–8 (Figure 5D), there was a significant main effect of genotype on accuracy in early trials ($F_{1,47} = 4.89, p < .05$) as well as a significant trial block effect ($F_{6,282} = 26.44, p < .0001$) but no interaction ($F_{6,282} = 0.60, p > .70$). However, the block by block comparison of WT and KO mice was only significant for trials 31–35 (block 7, $p < .05$). In the 20-trial block at which mice reached criterion, the percentage of correct responses (averaged across all eight discrimination problems) was significantly higher in WT ($89.7\% \pm 0.7$) than in KO ($87.2\% \pm 0.7$) mice ($t_{47} = 2.60, p < .05$), although the absolute difference was quite small.

Error distributions and response latencies were similar to those of the first experiment and, again, did not differentiate the WT and KO mice. Mice were tested for retention of the eighth discrimination two weeks after the last training session. Retention scores for both WT ($61.3\% \pm 4.1$) and KO ($59.2\% \pm 3.3$) mice were significantly above chance (one-sample *t*-tests, $p < .05$) but not different between the two groups ($t_{47} = 0.41, p > .65$).

These mice were subsequently trained with four novel odor pairs, each in a single session consisting of 20, 40, 60, or 80 trials. Two days after each training session, mice were given retention tests for the previously-trained odor pair. Figure 6 shows the number of correct choices made during the training sessions for WT and KO mice. There were no apparent differences between the two groups in choice accuracy in any of the training sessions. The retention data are shown in Figure 7. Analysis of variance indicated no significant effect of genotype on retention scores ($F_{1,47} = 0.03, p > .85$). There was a significant effect of number of training trials on retention score ($F_{3,141} = 3.17, p < .05$); as expected, more training trials led to improved memory. The interaction between genotype and training trials was not significant ($F_{3,141} = 0.33, p > .80$).

3.8 Olfactory sensitivity

An odor detection task was used to assess olfactory sensitivity in WT and *Fmr1* KO littermate mice. Mice were first trained to discriminate ethyl acetate (S+, 0.001%) from clean air (S−). They were then tested on the same discrimination using decreasing concentrations of ethyl acetate. Tests with the original ethyl acetate concentration (0.001%) were interleaved with lower ones to maintain behavioral performance. The results are shown in Figure 8. Both groups showed excellent performance at the highest concentrations of ethyl acetate; performance declined slowly over a broad concentration range, until chance levels were reached at an ethyl acetate concentration of 0.0000001%. Although WT and KO mice had slightly different performance scores at several different concentrations, the results suggest that threshold detection of ethyl acetate odor is equivalent in both groups.

4. Discussion

Although the *Fmr1* knockout mouse does not exactly reproduce the genetic abnormality of human fragile X syndrome at the molecular level, the mouse model and human syndrome share the fundamental common end-point of a lack of expression of FMRP throughout the lifespan. Since the definitive phenotypic characteristic of fragile X patients is mental retardation, understanding the cognitive consequences of silencing the *FMR1* gene in mice is of paramount importance for exploiting the mouse model for testing therapeutic strategies. This problem has three distinct elements: First, what are the cognitive consequences of a lack of FMRP in mice? Second, how do any observed cognitive deficits relate to the cognitive disabilities exhibited by patients with fragile X syndrome? Third, what neurobiological mechanisms are responsible for the cognitive effects? Behavioral studies can only address the first two issues; electrophysiological and molecular studies are necessary to illuminate the third.

Previous behavioral studies of *Fmr1* knockout mice have primarily relied on aversively-motivated tasks to assess cognitive performance and the results have not been entirely consistent. In the circular water (Morris) maze, some studies have found behavioral impairments during learning trials (Kooy et al., 1996) while others have not (Bakker et al., 1994; D'Hooze et al., 1997; Paradee et al., 1999). In none of the studies have probe trials revealed spatial memory deficits in *Fmr1* knockout mice. Reversal learning has been found to be impaired in the mutants in several (Bakker et al., 1994; Kooy et al., 1996; D'Hooze et al., 1997), but not all (Paradee et al., 1999; Peier et al., 2000) studies. Some of the variability may be attributable to interactions between background strain and the mutation (Paradee et al., 1999). Importantly, in cases where learning to find the hidden platform (i.e. spatial learning) was impaired, there were similar deficits in swimming to a visible (i.e., non-spatial learning) platform (D'Hooze et al., 1997). Since these are the two conditions of the water maze task that differ in their reliance on spatial representations and that differentiate intact animals from hippocampal animals (Morris, Garrud, Rawlins, & O'Keefe, 1982), these findings do not suggest that the spatial component of the hippocampal memory system is specifically impaired in the *Fmr1* mice. Van Dam, *et al.* (Van Dam et al., 2000) found knockouts to make more errors in spatial learning in a plus-shaped water maze; however, such deficits may be dependent on the background strain (Dobkin et al., 2000). Fear conditioning experiments have also produced mixed results (Dobkin et al., 2000; Paradee et al., 1999; Van Dam et al., 2000; Peier et al., 2000).

We were led to investigate olfactory learning in the FMRP-deficient mouse for two reasons: First, *Fmr1* mRNA and FMRP expression is high in olfactory structures, notably the olfactory bulb (Cohen, Smith, Weiler, & Greenough, 1997) and olfactory (piriform and entorhinal) cortex (Hinds et al., 1993) as well as hippocampus (Hinds et al., 1993; Feng et al., 1997; Abitbol et al., 1993), a memory-related structure that receives direct projections from the entorhinal segment of primary olfactory cortex. Second, a variety of diverse learning and memory

phenomena are easily demonstrated in rodents using olfactory cues (Eichenbaum, 1998; Slotnick, 2001); we (Larson et al., 2002) and others (Bodyak et al., 1999) have recently developed automated olfactory testing procedures for mice.

Fmr1 knockout (KO) and wild-type (WT) mice were first shaped to perform trial-structured nose pokes using operant techniques. There were no evident differences between KO and WT mice in the number of sessions required to acquire operant responses, in agreement with prior studies in which *Fmr1* knockouts were trained to acquire lever-pressing responses (Van Dam et al., 2000; Fisch et al., 1999; Frankland et al., 2004). Measures of response latency also did not reveal any differences between the fragile X mice and the controls. These results indicate that the KO mice were adequately motivated and were unimpaired in acquiring the operant nose poke response.

Olfactory discriminations were learned more slowly in *Fmr1* KO mice than in WT controls. This was manifested as increases in the number of sessions required to reach criterion performance, increases in the number of errors made before reaching criterion, and increases in the number of discriminations in which criterion performance was never achieved. Nevertheless, all of the animals improved learning rate across discrimination problems and both groups showed above-chance responding on the first few trials of later discrimination problems.

Analysis of training sessions in which more than 15 errors were committed did not reveal any differences between KO and WT in error patterns. In the large majority of these high-error sessions, mice exhibited a spatial bias by responding at the same sniff port on most trials. This spatial bias appears to be the default strategy adopted before the mice learn the odor-reward association or have difficulty distinguishing the odors (Larson et al., 2002). Most of the high-error sessions occurred on the first two odor discriminations, although they occasionally occurred later in the problem series as well. Subtracting the sessions in which spatial bias led to high error rates from the total number of sessions required for training somewhat accentuated the difference between WT and KO mice; it does not appear that the KO mice are more likely than WT mice to adopt a default spatial strategy rather than learning the odor-reward associations.

The animals were tested for long-term memory of the last discrimination problem four weeks after training. Both KO and WT mice showed significant memory at this delay and the two groups did not differ in retention scores. In a second experiment, memory was tested two weeks after the last training session. Retention scores were again equivalent for WT and KO mice at this delay.

In the second experiment, mice were also tested for memory two days after a single training session consisting of 20, 40, 60, or 80 trials with a novel odor pair. The number of correct responses during training did not differ between the two groups. Retention scores increased with the number of training trials but memory was equivalent for WT and KO mice. These results do not support the hypothesis that long-term memory after suboptimal training is weaker in *Fmr1* KO mice. The fragile X mice are only weakly impaired early in training on novel odor pairs; however they take more training to achieve high levels of discrimination accuracy.

In the only other investigation of sensory discrimination learning in FMRP-deficient mice, Fisch and colleagues (Fisch et al., 1999) initially trained KO and WT mice to lever press for sucrose reward and subsequently only rewarded the mice for lever presses in the presence of a light or sound stimulus. The KO mice learned to respond only in the presence of the stimulus more rapidly than the controls. In this context it is interesting to note that Hagerman's laboratory has reported that sensory responses in all modalities are greater in humans affected by fragile X syndrome (Miller et al., 1999). On the other hand, sensory gating appears to be

impaired in fragile X children (Frankland et al., 2004). Although we obtained no evidence of more rapid learning of an odor-reward association in the *Fmr1* knockout mice in the present study, the presence of both positive and negative cues simultaneously on each trial might have confounded such an effect. Whether or not the learning deficit observed in the present study has a sensory component is difficult to evaluate. We used odor pairs that are easily discriminable by both humans and mice, and mice in both groups were able to achieve high levels of discrimination performance (90% correct) on most of the discrimination problems. The KO mice simply required more training to achieve criterion performance. Measurements of the time required to traverse the alley and make an odor choice (response latency) did not reveal any differences between KO and WT mice. It is notable also that *Fmr1* KO mice were not found to be impaired in an olfactory-based working memory task (Yan et al., 2004). Finally, threshold discrimination of ethyl acetate from clean air appeared to be equivalent in WT and KO mice.

Further work will be required to determine how absence of FMRP results in impairments of olfactory discrimination learning. It seems likely that synaptic plasticity is impaired at some stage of encoding of an olfactory representation or an odor-reward association. This could be either because synapses develop abnormally in the absence of FMRP or because FMRP is a necessary component of the plasticity process. Several lines of evidence implicate the primary olfactory cortex, including the piriform cortex (PC) and lateral entorhinal cortex (LEC) among other regions, in encoding the significance of novel odors (Lynch, 1986). Olfactory cortex receives convergent inputs from the olfactory bulb, the first olfactory processing stage; since bulb neurons appear to respond to odor features rather than to particular smells (Mori, Nagao, & Yoshihara, 1999), the cortex is the first stage at which combinatorial representations of smells can occur (Haberly, 2001). Evidence suggests that representations for novel smells are not entirely hard-wired but are subject to experience-dependent plasticity and that this plasticity is necessary for odor discrimination (Wilson & Stevenson, 2003). Synapses in PC (Jung, Larson, & Lynch, 1990; Kanter & Haberly, 1990; Racine, Milgram, & Hafner, 1983) and LEC (Racine et al., 1983) exhibit LTP, and simulations of piriform cortex indicate that LTP is a suitable mechanism for odor discrimination learning (Granger & Lynch, 1991; Hasselmo, Anderson, & Bower, 1992). We have found that LTP in piriform cortex is impaired in *Fmr1* KO mice (Larson et al., 2005). The deficit, however, only appeared in slices from mice greater than six months of age; most of the mice in the present studies were younger than this, so it is unclear whether or not impairment of LTP in PC can account for the learning impairment in FMRP-deficient mice.

On the other hand, the finding that mGluR-dependent LTD is enhanced in the *Fmr1* KO mouse (Huber et al., 2002) has, along with other evidence, led to the hypothesis that many of the symptoms of fragile X syndrome may be due to abnormalities in mGluR-dependent signaling (Bear, Huber, & Warren, 2004). The role of mGluRs and/or LTD in olfactory discrimination learning requires further investigation. It will also be of interest to know whether or not the spine shape abnormalities observed in visual cortex of *Fmr1* KO mice (Irwin et al., 2002) also can be seen in other areas such as olfactory cortex and hippocampus.

Finally, in addition to simple discrimination learning, olfactory paradigms have been used to investigate other aspects of memory, including fear conditioning, paired-associate learning, transitive inference, and gestalt encoding of complex cues (Eichenbaum, 1998; Staubli et al., 1987; Schettino & Otto, 2001). Paradigms like these may prove useful in characterizing the extent and nature of learning impairments caused by a deficiency of FMRP.

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Figure 1.

Olfactory discrimination learning is impaired in *Fmr1* KO mice. A) The mean ($\pm$ s.e.m.) number of errors made during acquisition of a series of eight discrimination problems was significantly higher for KO than for WT mice. B) Both WT and KO mice had significantly fewer errors per problem in the second block of four problems (5–8) than in the first block (1–4). KO mice made more errors than WT mice in both blocks. C) Average percent correct responses (mean $\pm$ s.e.m.) in five-trial blocks during the first training session, averaged across the first four discrimination problems. The first training trial in each new discrimination was omitted as an information trial. Mice occasionally did not make a choice on one to a few trials in a session; these no-response trials were not scored, so only the first 35 response trials in the session were used in the figure. D) Average percent correct responding (mean $\pm$ s.e.m.) in five-trial blocks during the first training session on problems 5–8. **: $p < .01$.

Figure 2.

Spatial bias is the dominant error pattern in both WT and KO mice. Frequency histograms show distribution of sessions (as a percent of the total) for the KO group and the WT group in which the indicated percentage of errors were errors that were preceded by a response in the same spatial port (stay-side errors). Both groups showed a similar skewed distribution, indicating that most sessions with high error rates were dominated by stay-side errors.

Figure 3.

Slowed acquisition and failure to learn in KO mice. A) Total training sessions (mean + s.e.m.) required for the eight two-odor discriminations were greater in KO than in WT mice. B) The average number of sessions (mean + s.e.m.) with high error rates due to spatial bias were not significantly different between KO and WT mice. C) Subtracting the spatial bias sessions from the total does not eliminate the difference between KO and WT mice (mean + s.e.m.). D) Mean (+ s.e.m.) number of discriminations not learned to criterion was greater in KO than in WT mice. **: $p < .01$, ***: $p < .001$.

Figure 4. Performance latencies on olfactory discriminations by WT and KO mice. Graph shows the mean ($\pm$ s.e.m.) latency from the trial-initiate nose poke to the odor-choice nose poke for the last block of 20 trials of training on each discrimination problem. Latencies for WT (open circles) and KO (filled circles) mice did not differ.

Figure 5.

Replication of impairment in olfactory discrimination learning in *Fmr1* KO mice. A) The mean ($\pm$ s.e.m.) number of errors made during acquisition of a series of eight discrimination problems was significantly higher for KO than for WT mice. B) Both WT and KO mice had significantly fewer errors per problem in the second block of four problems (5–8) than in the first block (1–4). KO mice made more errors than WT mice only in problems 1–4. C) Average percent correct responses (mean $\pm$ s.e.m.) in five-trial blocks during the first training session, averaged across the first four discrimination problems. D) Average percent correct responding (mean $\pm$ s.e.m.) in five-trial blocks during the first training session on problems 5–8. *: $p < .05$, **: $p < .01$.

Figure 6. Acquisition scores (number of correct responses, mean + s.e.m.) for WT and KO mice given limited training on novel two-odor discrimination problems. There were no differences between scores for WT and KO mice in sessions consisting of 20 (A), 40 (B), 60 (C), or 80 (D) training trials.

Figure 7.

Retention scores (percent correct responses, mean + s.e.m.) for WT and KO mice two days after limited training on novel two-odor discrimination problems. There were no differences between scores of WT and KO mice after training sessions consisting of 20 (A), 40 (B), 60 (C), or 80 (D) training trials.

Figure 8.

Detection thresholds for ethyl acetate vapor in WT and *Fmr1* KO mice. Graph shows percent correct responding (mean $\pm$ s.e.m.) as a function of ethyl acetate concentration in sessions involving discrimination of ethyl acetate (S+) from clean air (S-). Both groups of mice showed very similar effects of concentration on discrimination accuracy.

Table 1
Performance of *Fmr1* KO and WT mice in shaping sessions.

	Sessions to Criterion			
	Stage 1	Stage 2	Stage 3	Stage 4
WT	2.00±0.19	1.17±0.10	1.25±0.85	3.13±0.09
KO	2.16±0.16	1.04±0.04	1.24±0.12	3.24±0.13
	Mean Latency in Last Session			
	Stage 1	Stage 2	Stage 3 (I)	Stage 4 (I)
WT	39.75±2.21	20.85±9.64	12.30±1.66	21.27±1.06
KO	35.81±1.70	23.11±1.60	12.20±1.04	21.90±0.93
				24.74±2.12
				6.25±0.38
				6.36±0.52

Latencies were recorded for the final training session at each stage when performance was at criterion. In stages 3 and 4, separate latencies were recorded for trial initiation (I) nose pokes and trial conclusion (C) nose pokes.