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Research report

Spatial learning, contextual fear conditioning and conditioned emotional response in *Fmr1* knockout mice

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Abstract

Fmr1 knockout mice are an animal model for fragile X syndrome, the most common form of heritable mental retardation in humans. *Fmr1* knockout mice exhibit macro-orchidism and cognitive and behavioural deficits reminiscent of the human phenotype. In the present study additional behavioural and cognitive testing was performed. Knockouts and control littermates were subjected to a spatial learning test using a plus-shaped water maze. Animals had to learn the position of a hidden escape platform during training trials. The position of this platform was changed during subsequent reversal trials. Previously reported deficits in reversal learning were replicated, but we also observed significant differences during the acquisition trials. A plus-shaped water maze experiment with daily changing platform positions failed to provide clear evidence for a working memory impairment, putatively underlying the spatial learning deficits. Two different test settings were used to examine the reported deficit of *Fmr1* knockout mice in fear conditioning. Conditioned fear responses were observed in a contextual fear test, and the ability to acquire an emotional response was tested by means of response suppression in a conditioned emotional response procedure. Neither protocol revealed significant differences between controls and knockouts.

Keywords: Fragile X syndrome; Knockout mice; Behavioral testing; Cognitive functioning; Spatial memory; Fear conditioning

1. Introduction

With its incidence of 1 in 4000–6000 fragile X syndrome is the most frequent form of hereditary mental retardation in humans (for review see Ref. [17]). Fragile X syndrome is caused by deletion or transcriptional inactivation of the fragile X mental retardation 1 (*FMR1*) gene, leading to the absence of FMRP, the protein derived from the *FMR1* gene. An animal model with inactivation of the *Fmr1* gene was developed by Bakker et al. [1]. *Fmr1* knockouts were shown to be negative for *Fmr1* RNA in testis, and for FMRP in testis, brain, and other organs tested. They show normal reproductive fitness and do not display major neurological dysfunction. Testicular weight was significantly higher in knockouts than controls, a finding which may relate to the macro-orchidism observed in fragile X men [1].

Fragile X patients exhibit a wide range of clinical characteristics, including moderate to severe mental retardation [7,8,13,17,19]. Fragile X males and mentally retarded fragile X-negative males have several behavioural and cognitive characteristics in common (including memory deficits), but fragile X patients have relatively better vocabulary and receptive word knowledge and verbal-expressive skills. However, they display

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inferior visual-motor co-ordination and manual skills, and are less capable of mental reasoning in solving new problems [19]. Since fragile X patients have, indeed, a distinct profile of behavioural and cognitive deficits, several attempts were made to assess these functions in the animal model [1,3,6,9,16,23]. Place learning of *Fmr1* knockouts and normal littermates was compared in different spatial navigation tasks and training protocols. *Fmr1* knockouts showed mildly impaired performance in a Morris-type water maze [1,6,16]. Knockouts especially experienced difficulties in learning to locate the hidden platform when, after a period of intensive acquisition training, the platform's position was changed during the reversal trials [1,6,16]. However, in a recent publication, Paradee et al. [23] were able to replicate only part of these results, which could have been due to strain differences between C57BL/6 and 129Re/J influencing the *Fmr1* knockout phenotype. Also, in a simplified navigation task using an E-shaped water maze, we found no significant differences between *Fmr1* knockouts and normal littermates, either during the initial acquisition training, or during reversal training [16]. On the other hand, a preliminary report has mentioned dramatic differences in acquisition performance between knockouts and controls using massed-trial training in a plus-shaped water maze [3]. However, no further confirmation of these observations has ensued, and they might have been due to retinal degeneration in the background strain (T. Brown, personal communication).

Several authors also examined non-spatial learning abilities in *Fmr1* knockouts. Passive avoidance learning in the step-through box was shown to be normal [1]. Using operant conditioning techniques in a small number of animals, knockouts were found to be similar or even superior to controls in acquiring visual and auditory discriminative responses [9]. Finally, a recent well-performed study by Paradee et al. [23] did demonstrate deficits in conditioned fear responses in *Fmr1* knockouts.

In the present study we have made an effort to replicate previously reported results in *Fmr1* knockout mice using different behavioural test protocols. *Fmr1* knockouts and normal littermates were tested in two independent plus-shaped water maze experiments using the same initial training protocol as Brown et al. [3]. The initial training was followed by an additional series of reversal trial blocks to examine reversal learning in this task. It was expected that knockouts might show a similar reversal deficit as previously reported using the Morris-type water maze. Secondly, we have examined whether the previously demonstrated reversal deficit could have been due to defective working memory functions, rather than to relative inability of knockouts to change a previously learned navigation strategy [1]. To test this, mice were subjected to a plus-shaped water maze learning protocol with changing platform positions. Finally, the putative deficit of *Fmr1* knockouts in fear conditioning was examined. Context-dependent fear conditioning was studied using the same protocol as Paradee et al. [23]. In another series of experiments, the effect was studied of a conditioned fear response component superimposed on a food-reinforced response schedule.

2. Materials and methods

2.1. Transgenic animals

Fmr1 knockout mice and wildtype littermates were derived from our previously described line, backcrossed to the C57BL/6JIco inbred strain for at least ten generations [1]. Genotypes were determined by polymerase chain reaction (PCR) and Southern blotting. Male *Fmr1* knockout mice and control littermates with an average age of 2–3 months were used. Mixed genotype groups of approximately eight littermates were housed in standard mouse cages under conventional laboratory conditions (food and water ad libitum, constant room temperature and humidity, 12/12 h light-dark cycle). Behavioural experimenters were blinded as to the genetic status of the animals. The colony was tested for the presence of the retinal degeneration (Rd) mutation by PCR, but no mutant alleles were identified.

2.2. Plus-shaped water maze learning

The plus-shaped water maze was used in an effort to replicate the previously described reversal deficit in the Morris-type water maze.

The test was executed according to the protocol described by Silva et al. [27]. The maze consisted of a transparent Plexiglass plus-shaped swimming maze (20 cm arm width × 26 cm arm length × 25 cm high) filled with opaque water. An escape platform is placed 1 cm under the water surface in one of the four arms. Mice are subsequently released from the other three arms and are allowed to swim for 1 min. The number of entries in the three arms not containing a platform and in the target arm are counted, and latency to reach the platform is measured. A choice is considered 'correct' when the animal turns directly to the arm containing the platform at the intersection of the maze and, hence, successfully escapes. After reaching the platform the animals have to stay on it for 20 s. Mice that are unable to reach the target after 1 min are also placed on the platform for a period of 20 s. Because the walls of the plus-shaped water maze are transparent, the animals can use distal cues in the environment of the plus-shaped water maze to locate the platform.

During 6 days of acquisition, the platform was placed in the eastern arm, and the mice were subsequently released from the north, west, and south during two trial blocks with an inter-trial interval of 30 min. After this acquisition period the position of the platform was changed to the opposite arm and the animals had to learn this new location during four reversal trial blocks.

2.3. Plus-shaped water maze with changing platform position

This second experiment in the plus-shaped water maze was performed to examine whether the previously demonstrated reversal deficit could have been due to defective working memory functions.

Mice were trained to find the platform during an acquisition period comparable with the plus-shaped water maze experiment described above. After 6 days of training the position of the platform was changed daily in a clockwise manner and mice were released from the three remaining arms, again during two trial blocks with an interval of 30 min. Mice were submitted to 14 trial blocks of this protocol.

2.4. Contextual fear test

Context-dependent fear conditioning was studied using the same protocol as Paradee et al. [23] to investigate the reported deficit in fear conditioning in *Fmr1* knockouts.

The experiment was based on the original protocol by Paylor et al. [24]. During the procedure an aversive, unconditioned stimulus (an electric shock), is paired with a conditioned stimulus (the experimental chamber) to elicit a freezing response, a reliable measure of fear in rodents [2]. On the first day the animals were placed in the testing chamber (22.5 cm wide $\times$ 32.5 cm long $\times$ 33.3 cm high Plexiglass cage with a grid floor) and were allowed to acclimate for 5 min. On day 2 they were first allowed to explore the testing chamber for 2 min (pre-US score). After this exploration, a 30-s tone was delivered with a buzzer (frequency: 2150 $\pm$ 200 Hz, Star Micronics, Piscataway, USA). This auditory cue or conditioned stimulus (CS), was followed by a 2-s, 0.35-mA foot shock, which served as the unconditioned stimulus (US). Again the mice were allowed to explore for 2 min. A second pairing of the CS and US was presented after these 2 min, followed by another 30-s exploration (post-US score). Twenty-four hours later the animals were returned to the testing chamber for 5 min exploration in the same context as the previous day (context score). Ninety minutes later the animals were returned to the test chamber, but now the grid floor was hidden with a Plexiglass plate and sawdust to alter the context of the testing chamber. The animals were observed for 6 min. During the first 3 min no stimulus was delivered (pre-CS score). During the next 3 min phase the auditory cue was delivered (CS score).

Under the different conditions animals were scored for freezing every 10 s, leading to a maximum score of 12 bouts of freezing during baseline trials, 21 during the shock trials, 30 during the context trials, and both 18 for the pre-CS and CS trials. A freezing score was calculated by expressing the number of observed freezing bouts as the percentage of freezing bouts versus the total number of bouts in each of the five trial blocks.

2.5. Conditioned emotional response procedure

The reported deficit in fear conditioning was also investigated in a conditioned emotional response (CER) procedure. A protocol adapted from Hoehn-Saric et al. [14] was used to perform this conditioned emotional response procedure in an operant conditioning chamber. Habitest™ mouse modular test cages (Coulbourn Instruments, Allentown, USA) were equipped with a grid floor connected with an electric shocker, a pellet feeder, and a response lever (minimum actuating weight, 5 g). Auditory stimuli were delivered through a high amplitude tone signalling device. The operant conditioning chambers were placed in isolation cubicles to prevent disturbance by background noises or other interfering stimuli. Different reinforcement schedules were programmed, and data were collected with Winlinc 1.1 experiment control and data acquisition software (Coulbourn Instruments, Allentown, USA). During the complete test period animals were imposed with different reinforcement schedules in daily 30-min trials. They were restricted from food with the exception of 1 h immediately after each trial. Water was present ad libitum.

During a training period animals were first subjected to fixed-ratio (FR) trials, during which they had to press the response lever a fixed number of times to receive a food pellet (FR1: 1 response/reinforcement, FR2: 2 responses/reinforcement, and FR5: 5 responses/reinforcement). These fixed-ratio trials were followed by a variable-interval constant-probability protocol (VI30 or pre-CER trials), during which lever presses produced on the average a food pellet every 30 s. Training trials were performed until a stable response rate was reached. The next seven trials, an average of eight 20-s CER components was randomly superimposed on the VI30 schedule. Each CER component comprised a clearly audible tone terminated by a 200-ms, 0.2-mA electric foot shock. Finally, the animals were presented with the tone, but without the accompanying shock (post-CER schedule).

The number of responses (lever presses/trial) was registered to investigate the effect of the different reinforcement schedules on the response rate. A suppression ratio (SR) was calculated as:

$$SR = \frac{RR_{CER}}{RR_{CER} + RR_{VI30}}$$

with RR_{CER} and RR_{VI30} response rates (# responses/s) during CER and VI30 schedules, respectively.

2.6. Statistics

Two-way repeated measures analysis of variance (RM-ANOVA) with genotype and trial block as sources of variation were used to analyse the learning curves showing the number of errors/trial block, the number of correct trials/trial block, and the escape latency in the plus-shaped water maze task during acquisition trials, reversal trials and under changing platform conditions. Two-way RM-ANOVA was also used to examine the effect of genotype and trial on the freezing score during the conditioning and the actual testing phase of the contextual fear test, and on the response rate and suppression ratio in the conditioned emotional response test. Differences between pairs of means were further assessed using two tailed Student's *t*-tests.

3. Results

3.1. Plus-shaped water maze learning

Both controls and *Fmr1* knockout mice were able to learn the location of the platform as a result of training. For the acquisition trials two-way RM-ANOVA revealed a significant effect of trial block on escape latency ($F_{5,65} = 40.83$; $P < 0.001$; Fig. 1A). The effects of genotype and of genotype × trial block on escape latency were not significant ($P = 0.573$, $P = 0.929$, respectively). Two-way RM-ANOVA revealed a significant effect of trial block on the number of correct trials/trial block ($F_{5,65} = 31.87$; $P < 0.001$; Fig. 1B). The number of correct trials/trial block was significantly affected by genotype (controls performed better than knockouts) and by genotype × trial block during acquisition ($F_{1,65} = 18.99$; $P < 0.001$ and $F_{5,65} = 2.923$; $P = 0.019$, respectively).

During reversal trials two-way RM-ANOVA showed a significant effect of trial block on escape latency ($F_{3,39} = 27.38$; $P < 0.001$; Fig. 1A), and a statistical trend for the effect of genotype on the same parameter ($F_{1,39} = 3.22$; $P = 0.096$). Knockout mice needed more time to find the escape platform than controls. The effect of genotype × trial block on escape latency was not significant ($P = 0.393$). The number of correct trials/trial block significantly increased during reversal trials for both groups ($F_{3,39} = 35.33$; $P < 0.001$; Fig. 1B). The number of correct trials/trial block was significantly affected by genotype — knockouts showed less correct trials than controls ($F_{1,39} = 19.50$; $P < 0.001$) — and by the interaction genotype × trial block ($F_{3,39} = 2.94$; $P = 0.045$).

3.2. Plus-shaped water maze learning with changing platform position

A second plus-shaped water maze experiment consisted of six acquisition trial blocks followed by 14 days of changing platform condition. After 6 days of acquisition, the animals entered a training period during which the position of the platform was changed daily. All animals improved their search strategy and learned to move much faster to other arms of the plus-shaped water maze in search of the platform when it was not found in the arm where it had been the day before. Two-way RM-ANOVA revealed a significant effect of trial block on escape latency, and on the number of correct trials/trial block ($F_{13,299} = 11.71$ and $F_{13,299} = 6.37$, respectively; $P < 0.001$ for the two parameters; Fig. 2A and B). When considering escape latency there was no significant difference between controls and *Fmr1* knockouts ($P = 0.776$). Two-way RM-ANOVA revealed only a statistical trend for the effect of the interaction genotype × trial block on escape latency ($F_{13,299} = 1.72$; $P = 0.056$). Both the effect of genotype and the effect of the interaction genotype × trial block on the number of correct trials/trial block were not significant ($P = 0.963$ and $P = 0.751$, respectively).

3.3. Contextual fear test

The freezing behaviour of both the controls and the knockouts was affected by introduction of the paired conditioned and unconditioned stimulus. Two-way RM-ANOVA revealed a significant effect of trial on the freezing score during the conditioning phase ($F_{1,32} = 67.44$; $P < 0.001$), but there was no significant effect of genotype ($P = 0.750$), nor of the interaction genotype × trial ($P = 0.997$; Fig. 3A).

During the actual testing phase (Fig. 3B) again a significant effect of trial on freezing score was observed ($F_{2,64} = 39.01$; $P < 0.001$). Re-exposing the animals to the same context as during the second trial of the conditioning phase led to a higher freezing score than baseline freezing (context score). Alterations in the environment (the test chamber) led to a decrease in freezing score (pre-CS). Introduction of the conditioned stimulus — without the unconditioned stimulus this time — resulted in an increase in freezing score (CS). During the testing phase again both groups showed the same amount of freezing since no significant effect of genotype or of the interaction genotype × trial was demonstrated by two-way RM-ANOVA ($P = 0.202$ and $P = 0.220$, respectively). A two tailed Student's *t*-test comparing the CS score between controls and knockouts revealed a statistical trend ($P = 0.081$).

3.4. Conditioned emotional response

During the fixed-ratio and variable-interval reinforcement schedules the animals progressively increased their response level until a stable response rate was reached during pre-CER (Fig. 4A). No significant difference in response rate between controls and *Fmr1* knockouts was observed prior to the introduction of the CER components (two-tailed Student's t-test, $P = 0.743$ on the final day of pre-CER). Introduction of the CER components led to a decrease in response rate for both groups (Fig. 4A), but animals once again increased their response rate over time during CER ($F_{6,108} = 3.90$; $P = 0.001$). Omitting the unconditioned stimulus (shocks) during post-CER resulted in an increase in response rate when compared to the rate under CER conditions (Fig. 4A). Two-way RM-ANOVA revealed no significant effect of trial on response rate under post-CER conditions ($P = 0.298$). There was no significant effect of genotype on response rate during the

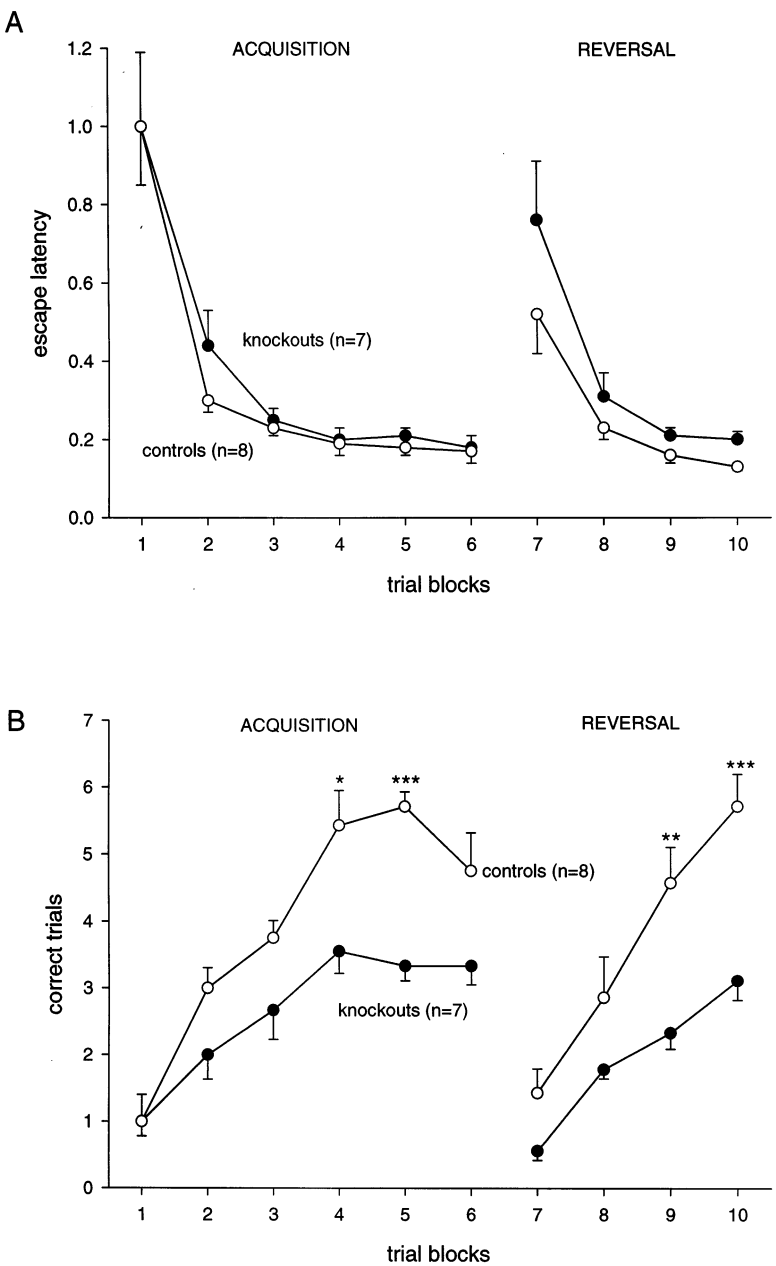

Fig. 1. Escape latency (A) and proportion of correct trials (B) during 6 days of acquisition and four reversal trial blocks. Each data point represents mean summed results of four daily trials ± S.E.M., and is presented as a proportion of the mean on the first day of acquisition. There was no significant effect of genotype on escape latency, but a significant difference between controls and knockouts in proportion of correct trials was observed during both acquisition and reversal trials blocks. Asterisks indicate significant differences by post hoc two tailed Student's t-test (*$P < 0.05$; **$P < 0.01$; ***$P \leq 0.001$).

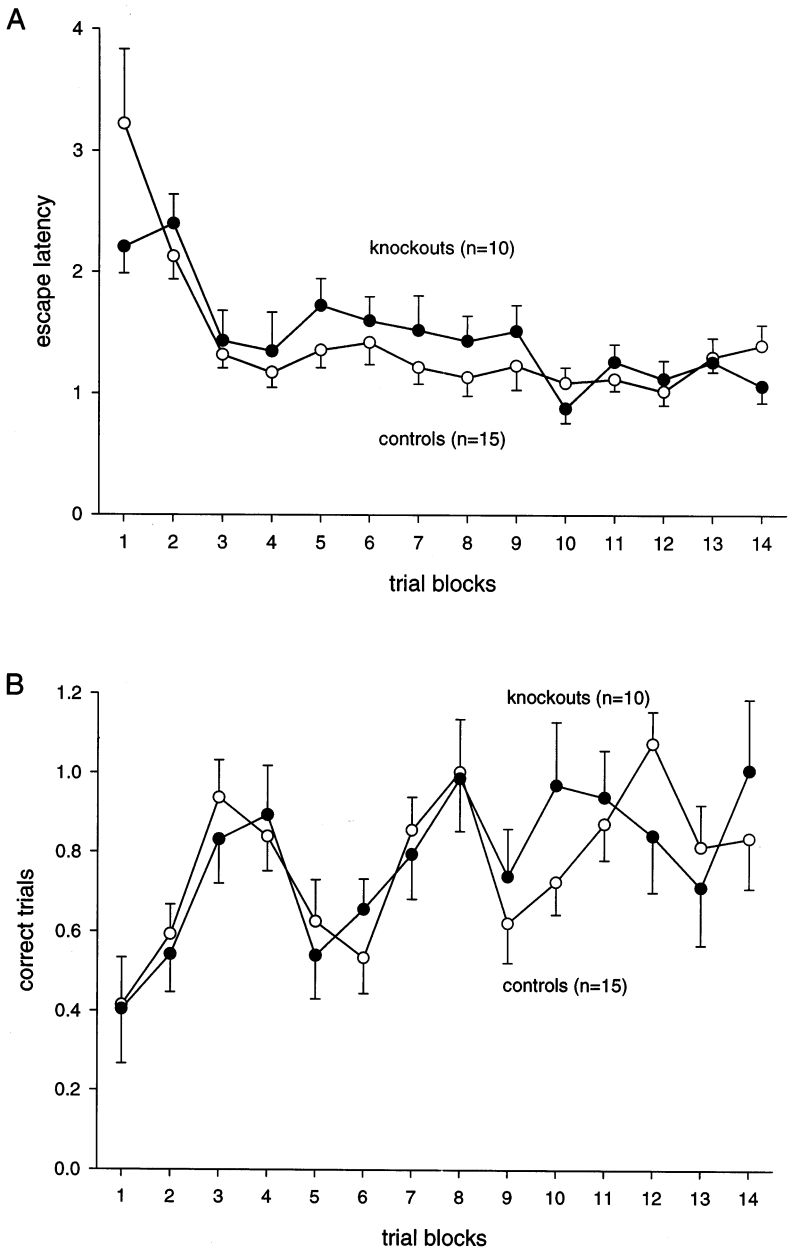

Fig. 2. Escape latency (A) and proportion of correct trials (B) during 14 trial blocks of plus-shaped water maze-training with daily changing platform position. Each data point represents mean summed results of four daily trials ± S.E.M., and is presented as a proportion of the mean of the six acquisition trial blocks. No significant differences between controls and knockouts were observed when considering escape latency, nor for the proportion of correct trials.

CER phase ($P = 0.644$), or under post-CER conditions ($P = 0.630$). The interaction genotype × trial had no significant effect on response rate during CER, or during post-CER conditions ($P = 0.803$ and $P = 0.276$, respectively).

For both groups the suppression ratio shows a striking decline on trial 2 (Fig. 4B). Two-way RM-ANOVA showed no significant effect of genotype on the suppression ratio ($P = 0.763$). Two-way RM-ANOVA revealed a significant effect of trial on suppression ratio ($F_{6,108} = 5.23$; $P < 0.001$). The interaction genotype × trial had no significant effect on this parameter ($P = 0.925$).

4. Discussion

Previous histological and neurocognitive studies identified *Fmr1* knockout mice as a putative model for fragile X syndrome, the most common form of inherited mental retardation in man. In the present study we have examined acquisition and reversal learning of plus-shaped water maze navigation in *Fmr1* knockouts and their wild-type littermates in order to compare these results with earlier findings in the Morris-type water maze [1,6,16]. Plus-shaped water maze training with daily changing platform position was used to

examine whether a deficiency in working memory functions could underlie the reported reversal deficit. Finally, fear conditioning was assessed using the same context-dependent fear conditioning protocol as Paradee et al. [23] and a CER protocol.

The first plus-shaped water maze experiment showed that controls as well as knockouts increased their efficiency and accuracy to find the hidden platform as a result of training. However, knockouts were unable to reach the same level of accuracy as controls at the end of the acquisition period (i.e. knockouts have a lower number of correct trials). The impairment we observed is definitively less severe than the one previously reported by Brown et al. [3], but their observations might be explained by retinal degeneration in their background strain. In the Morris water maze experiments,

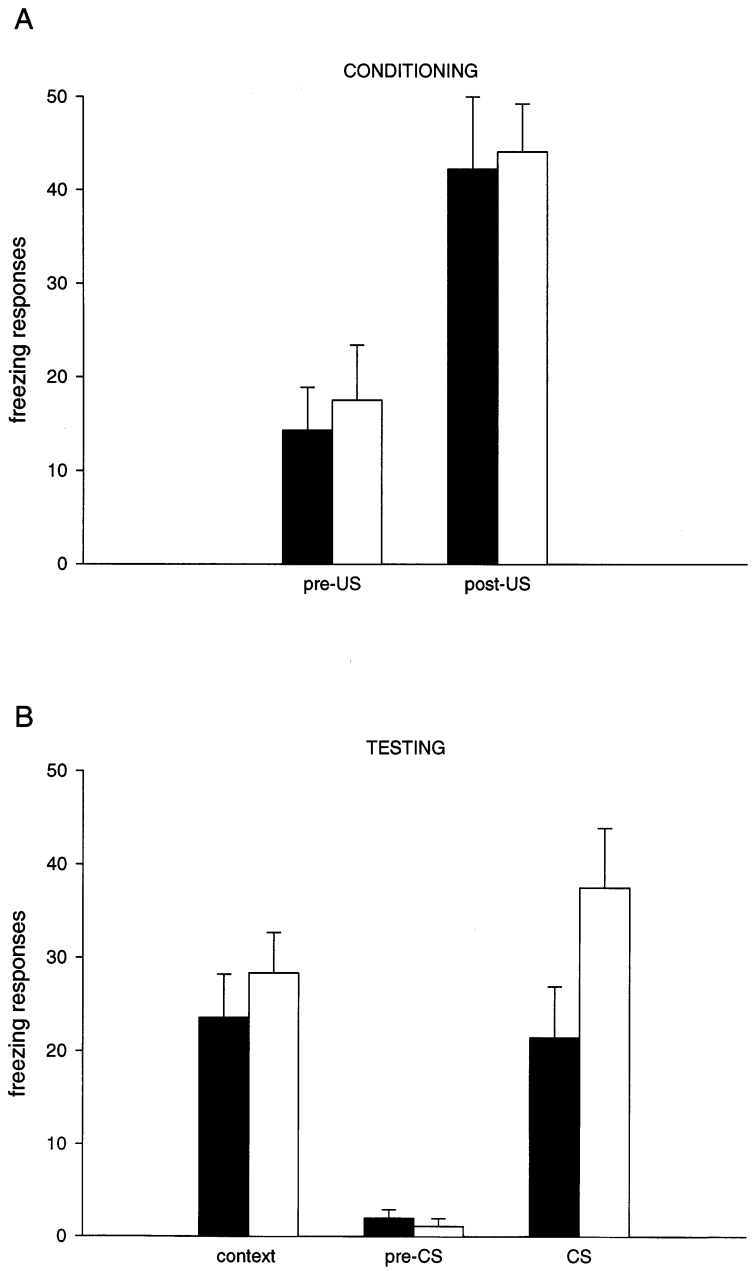

Fig. 3. Freezing responses during the conditioning phase (A) and the actual testing phase (B) of contextual fear conditioning. Data points represent the mean summed freezing score expressed as a percentage of the maximum amount of detectable freezing responses during each observation ± S.E.M. No significant differences between controls (presented by white blocks; $n = 20$) and knockouts (presented by black blocks; $n = 14$) were observed during the conditioning phase, nor during the actual testing phase. There was however a significant effect of testing procedure on the freezing score during the conditioning and the testing phase.

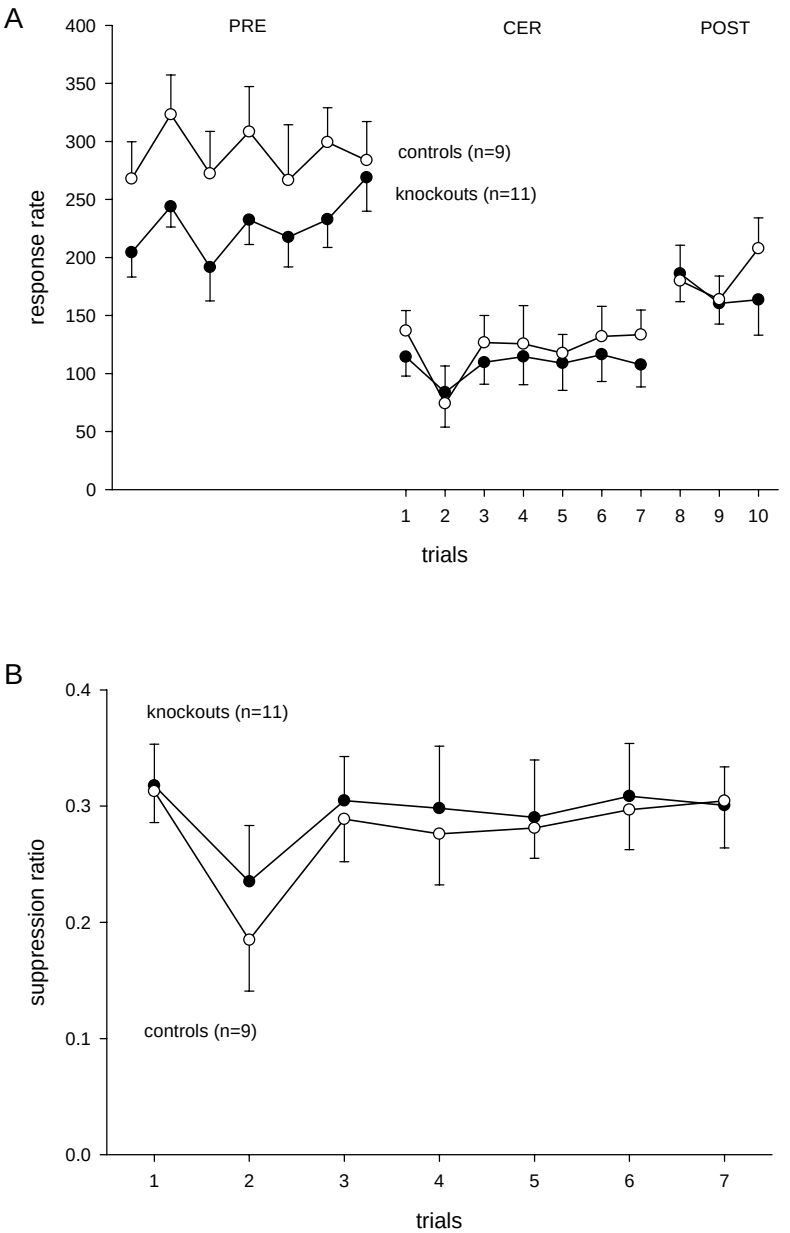

Fig. 4. Response suppression in a conditioned emotional response (CER) procedure. Response rate (A) during the final 7 days of pre-CER, CER and 3 days of post-CER, and suppression ratio (B) during CER. Controls and knockouts reached a stable response rate under pre-CER reinforcement (A). There was no significant difference between both groups prior to the introduction of the CER components. No significant differences were observed during CER and post-CER conditions. Data are presented as mean summed response rate (# responses/30 min) $\pm$ S.E.M. There was no difference in suppression ratio (SR) between controls and knockouts (B). For calculation of SR see text.

on the other hand, no difference was found between knockouts and controls in the acquisition phase of the test, either in escape latency [1,6], or in length of the escape path [6]. However, although both tests require the animal to perceive and use distal environmental cues to locate a hidden escape platform, spatial learning might be fundamentally different between these two spatial learning tests. Amongst other things, the Morris water maze is an open environment in which the animal can freely move around. Finding a hidden platform in such an environment may depend upon the construction and use of a single spatial map based on a set of distal cues. McNaughton et al. [22] argued that restriction of trajectories would make it necessary for the animals to develop multiple spatial maps. The observed difference in their performance in the two spatial tasks

may, therefore, indicate a specific inability of *Fmr1* knockouts to use different spatial maps.

The inability to use different spatial maps could be due to impaired working memory functions. In addition, the previous Morris water maze results had already indicated a reversal deficit in *Fmr1* knockout mice [1,6,16]. This might either be explained by decreased response flexibility (i.e. knockouts may have difficulties in changing a learned spatial navigation strategy) or by a more general working memory problem impairing the acquisition of any new task. The second plus-shaped water maze experiment showed no differences between knockouts and controls in the number of errors, or in the number of correct trials/trial block. When considering escape latency only a statistical trend for the effect of the interaction genotype × trial block was revealed. These results are, therefore, not in favour of a more general working memory impairment underlying the reversal deficit in *Fmr1* knockouts.

A state of social anxiety has been reported in female fragile X patients [10,11]. We have compared the performance of *Fmr1* knockout mice and their control littermates in two fear conditioning experiments. Conditioning of fear responses has been shown to rely upon hippocampus-dependent conditioning to non-specific cues (e.g. the context of the experimental chamber) as well as hippocampus-independent conditioning to specific cues (e.g. a tone) [18,26]. Both aspects of fear conditioning were tested in the contextual fear conditioning test. Paradee et al. [23] recently reported that, compared to their wild-type littermates, *Fmr1* knockouts display significantly less auditory-stimulated freezing behaviour in the context and CS phase of the test. Although using the same conditioning protocol as Paradee et al. [23], we only found slightly but not significantly decreased freezing in knockouts during both phases of the test. Also, our effort to investigate the effects of auditory fear conditioning in a CER procedure failed to show any differences between knockouts and controls. The reason for these conflicting results is not clear but might be due to differences in laboratory environment. Crabbe et al. [5] found that especially results of anxiety-related tests are highly susceptible to laboratory-specific environmental influences. Notably, our findings were recently confirmed by Peier et al. [25]. Using a similar fear conditioning protocol as Paradee et al. [23], they were equally unable to detect significant differences between knockouts and control littermates.

Although no alterations in gross hippocampal histology were found [1], altered dendritic spine morphology in occipital cortex of *Fmr1* knockouts does suggest deficits in cortical synapse maturation [4], which could underlie behavioural changes or other alterations of cortical function in *Fmr1* knockouts. Hippocampal long-term potentiation (LTP) has been suggested as the candidate cellular mechanism of fear conditioning [15,20,21]. However, previous examination of late- and early-phase hippocampal LTP revealed no differences between *Fmr1* knockouts and control littermates [12,23]. The absence of such differences may explain the rather subtle nature of the behavioural alterations observed in *Fmr1* knockouts as well as the failure to replicate the observations of Paradee et al. [23].

In the present study we have made an effort to replicate previously reported results in the *Fmr1* knockout model using different behavioural tests. Extensive behavioural validation of this animal model will benefit the use of the model in pathophysiological and/or therapeutic studies. Our plus-shaped water maze experiments confirmed some of the specific previously described abnormalities in visuo-spatial learning in the knockout model and revealed some additional impairment during acquisition. These deficits are reminiscent of some of the cognitive deficits in fragile X patients. On the other hand we were unable to detect abnormalities in conditioned fear responses in *Fmr1* knockouts. By and large our results illustrate the complexities of the effects of *Fmr1* deficiency on brain function and further emphasize the importance of the *Fmr1* knockout model in research on the fragile X syndrome.

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