© 2000 Oxford University Press Human Molecular Genetics, 2000, Vol. 9, No. 8 1145–1159

ARTICLE

(Over)correction of FMR1 deficiency with YAC transgenics: behavioral and physical features

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Received 22 December 1999; Revised and Accepted 7 March 2000

Fragile X syndrome is a common cause of mental retardation involving loss of expression of the *FMR1* gene. The role of FMR1 remains undetermined but the protein appears to be involved in RNA metabolism. *Fmr1* knockout mice exhibit a phenotype with some similarities to humans, such as macroorchidism and behavioral abnormalities. As a step toward understanding the function of *FMR1* and the determination of the potential for therapeutic approaches to fragile X syndrome, yeast artificial chromosome (YAC) transgenic mice were generated in order to determine whether the *Fmr1* knockout mouse phenotype could be rescued. Several transgenic lines were generated that carried the entire *FMR1* locus with extensive amounts of flanking sequence. We observed that the YAC transgene supported production of the human protein (FMRP) which was present at levels 10 to 15 times that of endogenous protein and was expressed in a cell- and tissue-specific manner. Macroorchidism was absent in knockout mice carrying the YAC transgene indicating functional rescue by the human protein. Given the complex behavioral phenotype in fragile X patients and the mild phenotype previously reported for the *Fmr1* knockout mouse, we performed a more thorough evaluation of the *Fmr1* knockout phenotype using additional behavioral assays that had not previously been reported for this animal model. The mouse displayed reduced anxiety-related responses with increased exploratory behavior. *FMR1* YAC transgenic mice overexpressing the human protein did produce opposing behavioral responses and additional abnormal behaviors were also observed. These findings have significant implications for gene therapy for fragile X syndrome since overexpression of the gene may harbor its own phenotype.

INTRODUCTION

Fragile X syndrome is the most common form of inherited mental retardation affecting one in 3000 to one in 4000 males (1–3). This disorder is most commonly a result of an expanded CGG trinucleotide repeat in the 5′ untranslated region (UTR) of the *FMR1* gene (4–6). The expanded repeat results in abnormal methylation, loss of gene expression and the disease phenotype. Additional mutations have been identified that confirm that this is a single gene disorder (7–9).

Mental retardation and developmental delay are the most significant clinical features of fragile X syndrome. Prepubescent males have delayed developmental milestones (both motor skills and speech) and some may display autistic-like behaviors in addition to hyperactivity and attention deficit (10). In adult males, mental retardation ranges from profound to borderline with an average IQ in the moderately retarded range (10). Affected individuals exhibit a long and narrow face with moderately increased head circumference (>50th percentile), prominent jaw and protruding ears. Macroorchidism is a common finding in post-pubescent affected males with nearly 90% of such males exhibiting testicular volumes in excess of 25 ml (10).

In situ hybridization studies of *FMR1* human mRNA expression during early development demonstrate high levels of expression in the neural tube and a strong widespread labeling of the whole embryo (11). In adults, the highest expression of *FMR1* protein (FMRP) is found in testis and brain, the two tissues that lead to a major defect in fragile X syndrome patients. In brain, the protein is expressed in neurons, particularly those of the hippocampus, as well as in the Purkinje cells of the cerebellum (11,12). Expression in testis is limited to the primary spermatagonia and the earlier stages of spermatogenesis (13). *FMR1* is highly conserved among vertebrates. The murine homolog *Fmr1* is 97% identical in amino acid sequence and exhibits an expression pattern very similar to that observed in humans (14–16). A variety of alternatively spliced transcripts have been observed in human and mouse (6,14)

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suggesting a number of potential protein isoforms; several isoforms have been characterized by protein studies (17,18).

The precise role(s) of *FMR1* remains undetermined but the protein appears to be involved in RNA metabolism. FMRP contains conserved sequence motifs, two KH domains and an RGG box (19) that are present in many RNA-binding proteins. FMRP has also been found to be associated with ribosomes (20,21) and to be present in neuronal dendrites (13,22). Given the presence of a nuclear localization signal (NLS) (23) and a nuclear export signal (NES) (24), FMRP has been proposed to shuttle between the nucleus and cytoplasm carrying a subset of RNAs as a mRNP particle (21).

Bakker *et al.* (25) generated *Fmr1* knockout mice that exhibit a phenotype with some similarities to humans, such as macroorchidism and behavioral abnormalities. Mutant animals displayed hyperactivity and a mild spatial learning impairment in the Morris water maze. No gross pathological abnormalities were identified in the brains of these mice. Abnormalities of the dendritic spine have been observed in fragile X syndrome patients (26–28) and more recently to a lesser extent in the *Fmr1* knockout mice (29). Therefore, the knockout mouse model appears to reproduce some of the features of the human disorder, particularly the macroorchidism phenotype. However, the absence of clear behavioral and pathological phenotypes has limited the utility of these mice in determining the function of *FMR1*.

Recent advances have allowed the generation of transgenic mice by inserting yeast artificial chromosomes (YACs) carrying large fragments of exogenous DNA (reviewed by Huxley, 30). The large size capacity of YACs, up to 1 Mb, allows the inclusion of all possible splice isoforms as well as distant regulatory and intragenic sequences that may be critical for proper expression. Numerous studies have demonstrated that genes present on YACs in transgenic mice are indeed appropriately expressed, replicating endogenous expression (31–33).

As a step toward understanding *FMR1* and determining the potential for therapeutic approaches to fragile X syndrome, we initiated experiments to determine whether the *Fmr1* knockout mouse phenotype could be rescued. Our goal was to generate transgenic mice expressing all forms of human FMRP in a cell- and tissue-specific manner. Such mice would help to determine whether the human protein would be functional in a murine background. To this end, a YAC containing 450 kb of the human Xq27.3 region and the full length of the *FMR1* gene was used to generate transgenic mice.

We investigated whether human FMRP can abolish the macroorchidism phenotype by breeding the YAC transgenic lines to the *Fmr1* knockout mouse. We generated the following four genotypes as littermates: wild type (WT), wild type with the YAC (WT/YAC), *Fmr1* knockout (KO) and knockout mice harboring the YAC (KO/YAC). Testicular weights were restored to within the normal range for the *Fmr1* knockout mice carrying the YAC transgene, indicating functional rescue by the human protein.

We also assessed whether the human *FMR1* gene can compensate for loss of the murine protein by performing extensive behavioral studies on the *Fmr1* knockout mice with and without the YAC transgene. Some of our behavioral assays were similar to those previously used with *Fmr1* knockout mice (e.g. Morris water maze and open-field activity) (25,34,35), but we also included several other tests to further characterize the *Fmr1* knockout mouse model. In addition, since this study sought to evaluate the functional impact of an *FMR1* YAC transgene on behavior, it was important to measure multiple domains of CNS function using a battery of behavioral tests. Our findings demonstrate that the behavioral differences identified in the *Fmr1* knockout mice could be ameliorated in mutant mice carrying the YAC transgene. This finding strongly suggests that the behavioral abnormalities found in the knockout model are specific to *FMR1*. We also provide evidence suggesting that not just the loss of protein expression contributes to behavioral defects in the mouse, but that overexpression of FMRP harbors its own phenotype. These results have important implications in developing therapeutic approaches towards fragile X syndrome individuals.

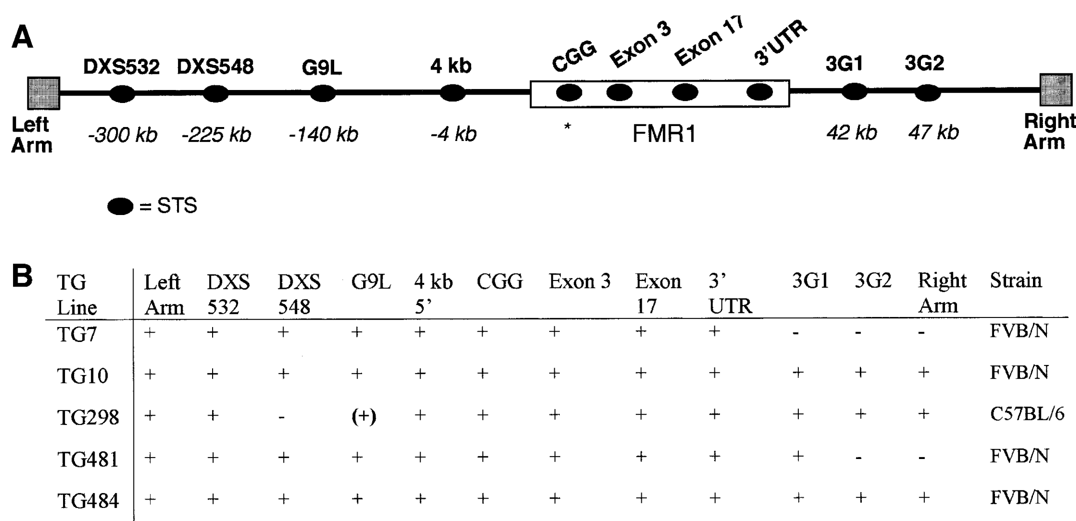
RESULTS

YAC transgenic mice

Transgenic mice were created with the previously characterized YAC 209g4 (5,6). This YAC contains the entire human *FMR1* gene with 20 CGG repeats and ~300 kb upstream of the *FMR1* start site and 100 kb downstream (Fig. 1A). The YAC was retrofitted with pRV1 (36) replacing the *URA3* locus on the YAC vector with the auxotrophic marker *LYS2* and a neomycin resistance gene for cell culture studies. This modified YAC was designated YapRV.2. We also injected a YAC (YapRV-EX) that was modified by homologous recombination to contain an expanded CGG repeat for instability studies (A.M. Peier and D.L. Nelson, manuscript in preparation). Purified YapRV.2 and YapRV-EX YAC DNA were microinjected into fertilized C57BL/6 (B6) and FVB/N (FVB) mouse oocytes, respectively, and transplanted into foster mothers. Potential founders were initially analyzed by the polymerase chain reaction (PCR) using primers specific for the CGG repeat locus in humans. Nine of the 24 FVB founders and five of the 26 mice obtained from microinjections into the B6 strain were positive for this locus. Additional analyses employing primers designed to detect sequence tagged sites (STSs) from the human and YAC vector sequences that span the entire length of the YAC were used to assess the extent of the YAC DNA present in these founders (Fig. 1A). PCR results for potential founders positive for the markers used to detect the *FMR1* gene are shown in Figure 1B. Four FVB (16%) founders (TG7, TG10, TG481 and TG484) and one B6 animal (TG298) (4%) appeared to carry the entire *FMR1* gene. Line TG10 failed to generate offspring and line TG7 had difficulty breeding, as evidenced by small litter sizes and stillborn pups (see below).

Assessment of YAC integrity and copy number

The majority of STS markers in the transgene positive progeny segregated as a single unit in subsequent generations (Fig. 1B). Neither the G9L nor the DXS548 markers were present in the founder and/or subsequent offspring for the TG298 line, indicating that this line appeared to harbor an internal deletion within the YAC ~140–225 kb upstream from the FMR1 start site. This is presumably due to shearing and fragmentation of the DNA during preparation and microinjection of the YAC.



TG Line	Left Arm	DXS 532	DXS 548	G9L	4 kb 5'	CGG	Exon 3	Exon 17	3' UTR	3G1	3G2	Right Arm	Strain
TG7	+	+	+	+	+	+	+	+	+	-	-	-	FVB/N
TG10	+	+	+	+	+	+	+	+	+	+	+	+	FVB/N
TG298	+	+	-	**(+)**	+	+	+	+	+	+	+	+	C57BL/6
TG481	+	+	+	+	+	+	+	+	+	+	-	-	FVB/N
TG484	+	+	+	+	+	+	+	+	+	+	+	+	FVB/N

Figure 1. (**A**) Diagram of YAC209g4c containing the entire *FMR1* gene. The order and relative location of STSs spanning the length of the YAC used for screening potential founders are included and designated by a closed circle. (**B**) STS content analysis of the *FMR1* YAC transgenic mice. For each of the transgenic lines results of the PCR screening are given as follows: +, present; –, absent. Positive results in bold designate markers that did not co-segregate in transgenic progeny in subsequent generations. The mouse genetic background is also listed

To further assess the integrity of the YAC DNA in our transgenic lines, we performed fluorescence *in situ* hybridization (FISH) analysis on cultured mouse tail fibroblasts using the YAC DNA as a probe. One hybridization signal was detected for lines TG298, TG481 and TG484 while two signals were observed for line TG7 (data not shown). We consistently observed two signals in multiple animals and generations for the TG7 YAC transgenic mice. Coupled with the breeding problems, these findings in the TG7 line indicated that a translocation between two murine chromosomes had occurred during the integration of the transgene. The requirement for both chromosomes carrying the YAC transgene to segregate together maintaining a balanced chromosomal set, explains the low fertility in this transgenic line. Given the STS content mapping and the FISH analysis, our data suggest that the human YAC DNA integrated at a single site in the mouse genome in the majority of our transgenic lines.

Southern blotting was also performed to assess the integrity of the *FMR1* locus as well as to determine the number of copies of the transgenic DNA present at the integration site. Southern analysis was done on *Eco*RI and *Bam*HI digested human and mouse genomic DNA using probes encompassing part of intron 1 and also the 3′ genomic sequence of the human *FMR1* gene. All lines tested showed the expected hybridization pattern for the human locus (data not shown). We compared the intensity of the Southern blot bands observed in the transgenic lines with those observed for normal human male and female by densitometry. Results were consistent with the presence of approximately two to three copies of the YAC in most of the transgenic lines tested.

mRNA expression from YAC transgene

Since our goal was to determine whether the human YAC could support production of the *FMRP* in the murine background, we checked for human mRNA expression using reverse transcription–PCR (RT–PCR). RNA was extracted from mouse tails, and following the initial first strand cDNA synthesis, human-specific primers spanning from exon 16 to the 3′UTR were used to amplify human FMR1 message derived from the YAC transgene. All lines that were positive for the *FMR1* gene (TG298, TG7, TG10, TG481 and TG484) were found to be positive by RT–PCR using these primers, demonstrating that human message was being transcribed from the YAC (data not shown). Additionally, northern blot analyses were performed using poly(A)$^+$ RNA prepared from testes of wild type and TG298 transgenic animals. We observed that FMR1 message levels were on average 2–3-fold higher in the transgenic tissues when compared with wild type (data not shown). These findings further support the presence of multiple copies of the human gene in this transgenic line.

Human FMRP expression analysis

In order to determine whether the YAC can support production of FMRP, protein expression was examined by western blot. Since the monoclonal anti-human FMR1 antibody currently available (mAb1a) also recognizes the murine protein (12), we evaluated YAC protein expression in the *Fmr1* knockout background for our transgenic lines TG298 and TG7. Male transgenic wild type mice (WT/YAC) were crossed with *Fmr1* homozygote mutant females. All male progeny from this cross carried the knockout allele and were tested for the YAC transgene. Protein expression from the YAC was analyzed from F1 male knockout littermates that were positive for the human sequence (KO/YAC). Protein from total brain and testis of F1 transgenic, knockout and wild type mice was analyzed by western blotting using the monoclonal antibody, mAb1a. Western blot analysis showed expression of the 69–80 kDa human protein in brain and testis in the transgenic tissues (Fig. 2A). A longer exposure revealed expression in wild type (Fig. 2B). Expression was also observed in all tissues tested in a broad study of transgenic tissues (data not shown).

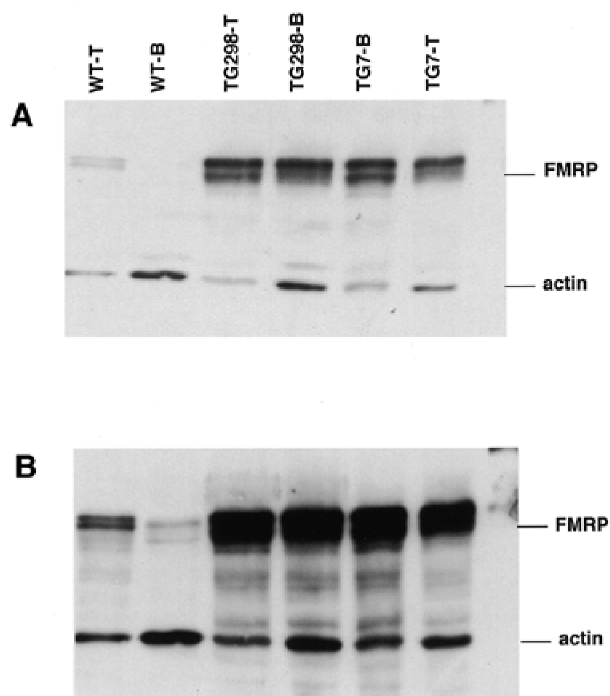


Figure 2. Western blot analysis of wild type (WT) and *Fmr1* knockout mouse carrying the YAC transgene (KO/YAC) for the TG298 and TG7 lines for total brain and testis (**A**). 100 µg of protein was loaded in each lane, incubated with the anti-FMRP monoclonal antibody mAB1a, and detected indirectly with chemiluminescence. An actin antibody was also used to detect the 43 kDa actin protein, which was used as an internal control for loading. A very intense signal is observed in both transgenic lines for both brain and testis. (**B**) A longer exposure of the same blot showing the signal for wild-type mouse protein.

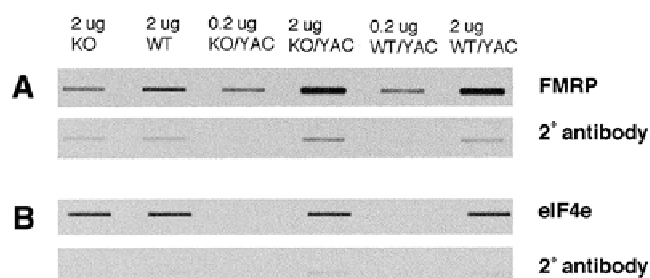


Figure 3. Quantification of FMRP levels in *FMR1* YAC transgenic mice. FMRP levels were calculated for two sets of animals using total brain extracts of KO, WT, KO/YAC and WT/YAC animals. (**A**) Measurement of FMRP levels. Row 1: brain extracts from KO (2 µg), WT (2 µg), KO/YAC (0.2 and 2 µg) and WT/YAC (0.2 and 2 µg). Row 2 was treated with secondary antibody only. There is no secondary signal from purified protein (not shown). To control for loading, the FMRP levels were normalized to eIF4e, the rate-limiting factor in translation. (**B**) Quantification of eIF4e levels. Row 3: brain extracts from KO (2 µg), WT (2 µg), KO/YAC (0.2 and 2 µg), and WT/YAC (0.2 and 2 µg). Row 4 was treated with secondary antibody only. There is no secondary signal from purified protein (not shown). Since the monoclonal antibody cross-reacts with the FXRPs (unpublished data), the FMRP signal in the KO mouse was subtracted from the WT mouse signal to give the endogenous FMRP level. These data reveal that there is a 13- to 17-fold increase in FMRP expression in the FMR1 YAC transgenic mice.

Transgenic mouse tissues consistently demonstrated a more intense signal on the western blots when compared with wild type in all tissues analyzed. This may be due to extra copies of the human *FMR1* gene (more than one YAC present at the integration site) resulting in higher levels of expression or perhaps to the monoclonal antibody having a higher affinity for the human protein. Alternatively, the human protein may be ectopically expressed (i.e. in glial cells where endogenous FMRP is not expressed). To better address this finding, experiments to measure FMRP levels and immunohistochemistry were performed.

Quantification of human FMRP

Since our western analysis suggested that the transgenic protein was overexpressed, we compared levels of expression between human FMRP in line TG298 and the endogenous murine protein using quantitative western blots. Equal amounts of protein from total brain of adult KO, WT, KO/YAC and WT/YAC were used in our analysis. Additionally, we used total human brain protein in our quantitative westerns to determine if the monoclonal antibody had a higher affinity for the human protein. Blots were detected with mAb1a as the primary antibody and protein expression was measured using a biotinylated secondary antibody, followed by detection with ^{35}S radiolabeled streptavidin. Phosphorimager analysis revealed that human FMRP levels expressed from the YAC transgene were estimated to be 10 to 15 times that of endogenous protein and 10 times greater than human protein levels (data not shown). Furthermore, assuming that FMRP is expressed at comparable levels in human and mouse brain, we did not detect significant differences between the human and mouse total brain protein lysates suggesting that the monoclonal antibody does not have a higher affinity for the human protein.

Additionally, we utilized a slot blot assay to confirm our western blot data (Fig. 3) (A. Kenneson, F. Zhang, C. Hagedorn and S.T. Warren, manuscript in preparation). A standard curve was generated for both purified recombinant murine FMRP (37) and eIf4e, the cap-binding factor in translation (38). This factor is also the rate-limiting step in eukaryotic translation (39–41) and as such, appears to be tightly regulated in cells and consequently does not vary between samples that are evenly loaded for total protein. The molar ratio of FMRP to eIF4e was calculated for each sample for two sets of animals of all four genotypes: (KO, WT, KO/YAC and WT/YAC). Mice carrying the transgene were found to express FMRP on average 13–17-fold higher than non-transgenic littermates.

Analysis by both quantitative western and slot blot assay was consistent with >10-fold overexpression of human FMRP in the TG298 transgenic line. The high level of transgene expression observed in this transgenic line cannot be attributed solely to transgene copy number or to the level of steady-state mRNA, suggesting differences in regulation at the translational level.

Immunohistochemistry

Cell-specific localization of transgenic FMRP was compared with endogenous expression in brain and testis from WT, KO and KO/YAC animals. Paraffin embedded sections were prepared from 12 week-old TG298 mice. Experiments on whole brain sections revealed that human FMRP was confined to neurons and expressed at highest levels in the cerebellum

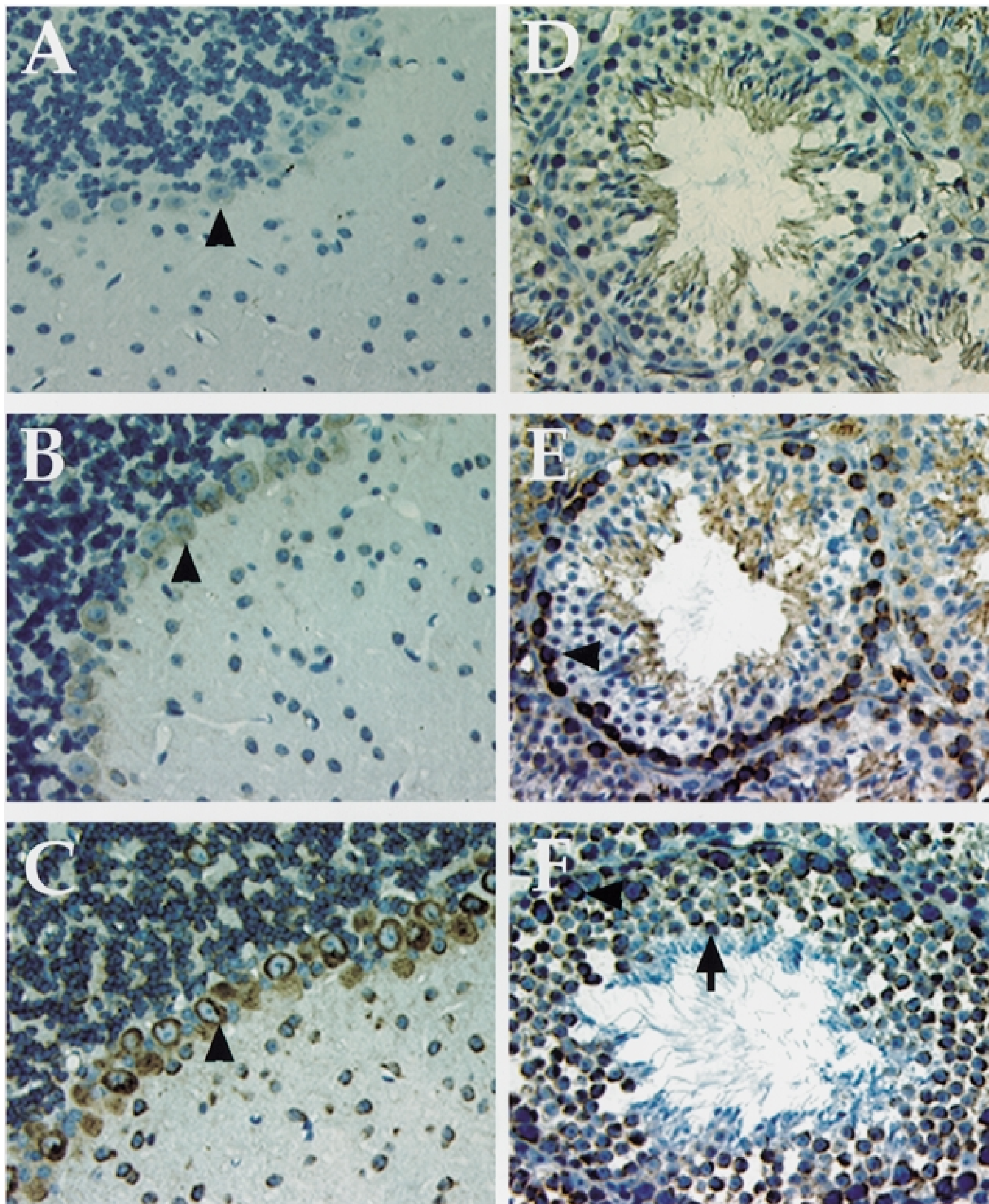


Figure 4. FMRP expression in adult brain and testis. Light-microscopic micrographs of paraffin embedded sections from adult cerebellum (**A**–**C**) and testis (**D**–**F**), and from *Fmr1* knockout (A and D), wild type (B and E) and *Fmr1* knockout mice harboring the YAC (TG298 line) (C and F). Arrowheads designate cytoplasmic expression in Purkinje cells (A–C) and in the primary spermatagonia (D–F). The arrow in (D) indicates FMRP expression in more mature stages of spermatogenesis. Sections were immunostained for FMRP with the monoclonal antibody mAb1a. A positive signal with the indirect immunoperoxidase technique results in a brown precipitate. Nuclei are counterstained with hematoxylin, which yields a blue color.

and hippocampus mirroring the expression pattern of the endogenous protein (Fig. 4). In testis, the transgenic protein was absent in Sertoli cells and present in the primary spermatagonia (Fig. 4). Additionally, YAC FMRP was observed in later stages of spermatogenesis. We also observed faint staining in the testis of knockout animals. This is likely due to cross reactivity of the primary antibody to the *Fxr1* protein, which is highly expressed in maturing spermatogenic cells (13). Immunohistochemistry indicated that the transgenic protein was expressed in a cell- and tissue-specific pattern similar to endogenous protein. Normal morphology was present in light microscopic examination of brain sections prepared from all four genotypes (KO, WT, KO/YAC and WT/YAC).

Rescue of the macroorchidism phenotype

Macroorchidism is one of the key features of fragile X syndrome males. *Fmr1* knockout mice also exhibit enlarged testes, which becomes more significant over time (25). To determine whether the YAC transgene could function in the murine background and rescue this aspect of the phenotype,

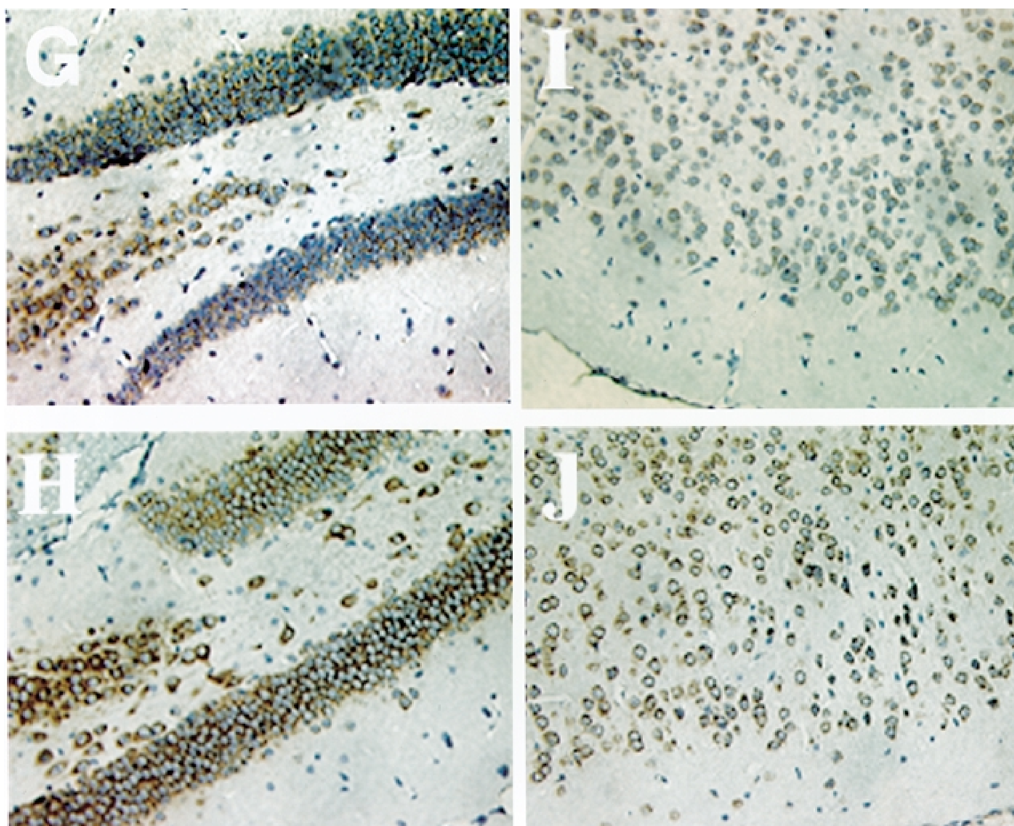


Figure 4. FMRP expression in adult brain and testis. Light-microscopic micrographs of paraffin embedded sections from hippocampus (**G** and **H**) and cortex (**I** and **J**) and from wild type (G and I) and *Fmr1* knockout mice harboring the YAC (TG298 line) (H and J). Sections were immunostained for FMRP with the monoclonal antibody mAb1a. A positive signal with the indirect immunoperoxidase technique results in a brown precipitate. Nuclei are counterstained with hematoxylin, which yields a blue color.

Fmr1 heterozygote knockout females were bred with WT/YAC transgenic males. All four possible genotype classes were represented in the male progeny from these crosses: KO, KO/YAC, WT and WT/YAC. Both total body weight and testicular weight were measured at necropsy in 189 animals, ranging in age from 8 to 20 weeks. A ratio of testis weight to body weight was calculated for each animal and the averages are presented in Figure 5. No significant difference in body weight was observed in all four genotypes. However, testicular weights collected from knockout mice were found to be significantly different from the other three groups ($P < 0.00001$). The testicular weights of KO/YAC animals were not significantly different from WT mice ($P > 0.05$), whereas the WT/YAC mice actually had testicular weights significantly smaller than wild type ($P = 0.0113$). These results indicate that the *FMR1* YAC transgene in the knockout background rescues the macroorchidism phenotype, restoring the testicular weights to within the normal range, and that overexpression of FMRP in wild type animals may reduce testis weight.

Reproductive fitness

Fmr1 knockout males, heterozygote females and homozygote knockout females have been reported to have normal fertility compared with wild type control groups (25). We assessed the reproductive fitness of the YAC transgenic mice in order to ascertain whether there were differences in genotype frequencies between littermates harboring the YAC transgene. Heterozygote transgenic males and females bred with wild type mice exhibited normal litter sizes with the expected distribution of offspring positive for the transgene. However, when transgenic wild type males were bred with heterozygote *Fmr1* knockout females a significant difference was detected in the observed genotype frequencies: 24.7%, KO; 29.8%, KO/YAC; 26.6%, WT; and 18.8%, WT/YAC. The WT/YAC class was under-represented in the 218 progeny genotyped for this study (2×2 $\chi^2 = 5.596$, $P = 0.018$).

Behavioral characterization of *Fmr1* knockout and transgenic mice

Our behavioral analysis of the YAC transgenic mice served a multi-fold purpose. First, we sought to re-examine the *Fmr1* knockout phenotype by subjecting mice to a battery of behavioral assays, testing both for learning and memory performance and extending the analysis to test for anxiety-related responses, motor coordination, skill learning and sensorimotor adaptation. Secondly, the YAC transgenic mice in the knockout background could be tested in order to determine whether the YAC modifies any phenotype observed in the knockout animals. Finally, this line of experiments was also designed to ascertain whether the presence of the YAC has a phenotypic effect in a

Table 1. Summary of the behavioral analysis of *Fmr1* knockout, wild type and *FMR1* YAC transgenic mice

Planned comparisons	Open-field			Light–Dark		Startle	Rotorod	Contitioned fear[a]		Morris
	Total distance	Rearing	Ctr/Total distance ratio	Number of transitions	Total dark time			Context	Auditory cue (CS)	Escape latency
KO versus WT	**0.017**	0.979	**0.009**	**0.003**	0.142	0.237	0.063	0.701	0.482	0.440
KO versus KO/YAC	[b]	0.076	[b]	[b]	**0.002**	**0.002**	0.095	0.0.57	0.378	0.385
WT versus WT/YAC	**0.038**	0.176	0.88	**0.024**	0.331	**0.017**	0.138	0.862	0.582	0.635
YAC versus no-YAC	[b]	**0.04**	[b]	[b]	**0.004**	[b]	**0.027**	0.613	0.318	0.350

The present experimental design enabled the use of *a priori* planned comparisons to further analyze any main effect of genotype. *P*-values are presented for the behavioral tests performed in this study. Bold indicates a significant *P*-value ($p < 0.05$).
[a]*P*-values for the conditioned fear paradigm.
[b]*P*-values ≤ 0.0002.

wild type background. *Fmr1* heterozygote knockout females and wild type transgenic TG298 males were crossed to generate male littermates harboring all four possible genotypes: KO, WT, KO/YAC and WT/YAC. Behavioral testing was performed on 106 animals aged between 3 and 4 months. Two independent batches of mice were evaluated. Table 1 displays the *a priori* planned comparisons that were calculated and their corresponding *P*-values for the behavioral tests used in this study.

Neurologic screen. Fmr1 knockout, wild type and transgenic mice were healthy as assessed by body weight and body temperature. They showed no abnormal physical features. All genotypes displayed normal neurological reflexes and responses (data not shown).

Locomotor activity. The open-field test can be used to assess locomotor activity and anxiety-related responses (42,43). Locomotor activity was evaluated by placing a single mouse in an open-field arena and recording its activity over a period of 30 min. Total distance, vertical activity (rearing) and the center distance ratio—which is the proportion of the total distance that is spent in the center of the area—were determined. Knockout mice traveled a greater distance in the open-field compared with their wild type littermates (P = 0.017) (Fig. 6A). In contrast, mice carrying the YAC transgene were significantly less active than their non-transgenic littermates (P = 0.000004). Moreover, WT/TG animals were less active than their non-transgenic WT littermates (P = 0.038). These data suggest that levels of FMRP affect exploratory activity in the open-field. Rearing was not different between KO and WT littermates (Fig. 6B). However, mice with the transgene reared less often compared with KO and WT mice without the transgene (P = 0.04). The difference in rearing appeared to be limited, however, to the first portion of the test period.

The center distance ratio can be used as an indicator for anxiety-related responses (44). In general, wild type mice will prefer to stay along the perimeter of an area when introduced to a new environment. We found that the *Fmr1* knockout mice traveled a significantly greater proportion of their distance in the center of the open-field compared with WT littermates (P = 0.009). In contrast, KO/YAC and WT/YAC animals were more likely to travel along the perimeter compared with KO and WT mice (Fig. 7A). These findings indicate that levels of *FMRP* may influence anxiety-related behaviors in the open-

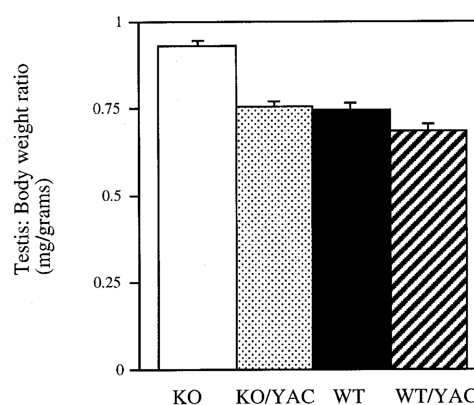


Figure 5. Rescue of macroorchidism phenotype. A ratio of the testicular weight (in milligrams) to body weight (in grams) was calculated for 189 animals ranging in age from 8 to 20 weeks from littermates harboring all four genotypes: KO, KO/YAC, WT and WT/YAC. Average (±SEM) ratios are shown for all four genotypes.

field; no protein produces low anxiety-like responses and higher protein levels result in elevated anxiety responses.

Light–dark exploration test. In this assay, mice are allowed to move freely between two chambers. One chamber is large, open and brightly illuminated, while the other is small, closed and dark. Like the open-field assay, the light–dark test assesses anxiety-related responses. Several behavioral measures are recorded including latency to enter the dark chamber and the total time in the dark, but the number of transitions may be the best indicator of anxiety-like responses because this is known to be sensitive to anxolytic drugs (45). *Fmr1* knockout mice exhibited a greater number of light–dark transitions than their wild type littermates (P = 0.00286), indicating that the KO mice have lower levels of anxiety-related responses (Fig. 7B). Mice carrying the YAC transgene had fewer transitions compared with mice without the YAC (P = 0.0000045). Additionally, WT/YAC animals were significantly different from WT littermates (P = 0.024). Consistent with the open-field data, KO mice have lower levels of anxiety-related responses in the light–dark test and the presence of the transgene appears to increase anxiety-like behavior. No difference was observed between the KO and WT non-transgenic littermates with respect to total time spent in the dark. However, there was a significant effect of the transgene (P = 0.005) on this measure

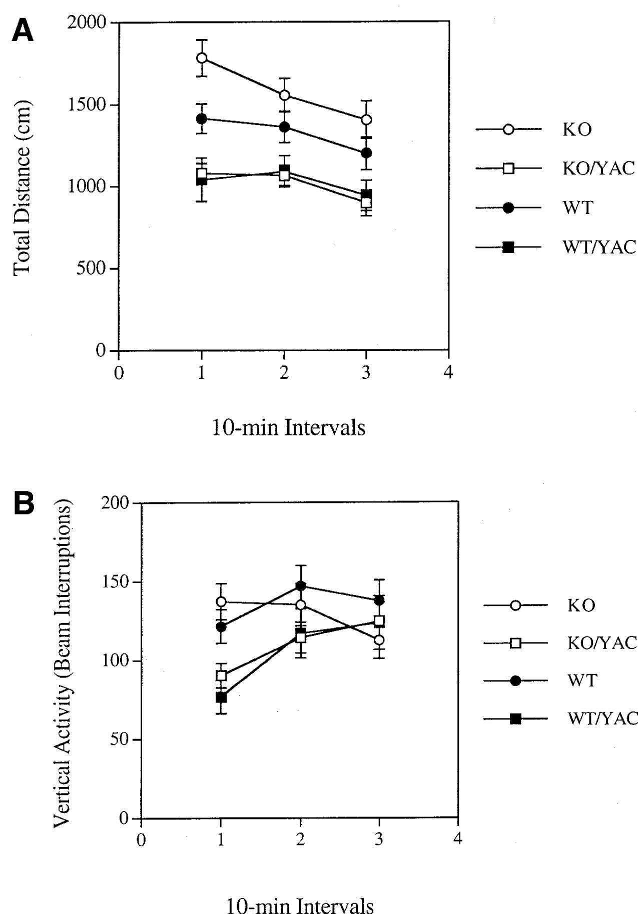


Figure 6. Open-field activity test. (**A**) The total distance (in centimeters) and (**B**) vertical activity for KO, KO/YAC, WT and WT/YAC genotypes. The means (±SEM) are presented.

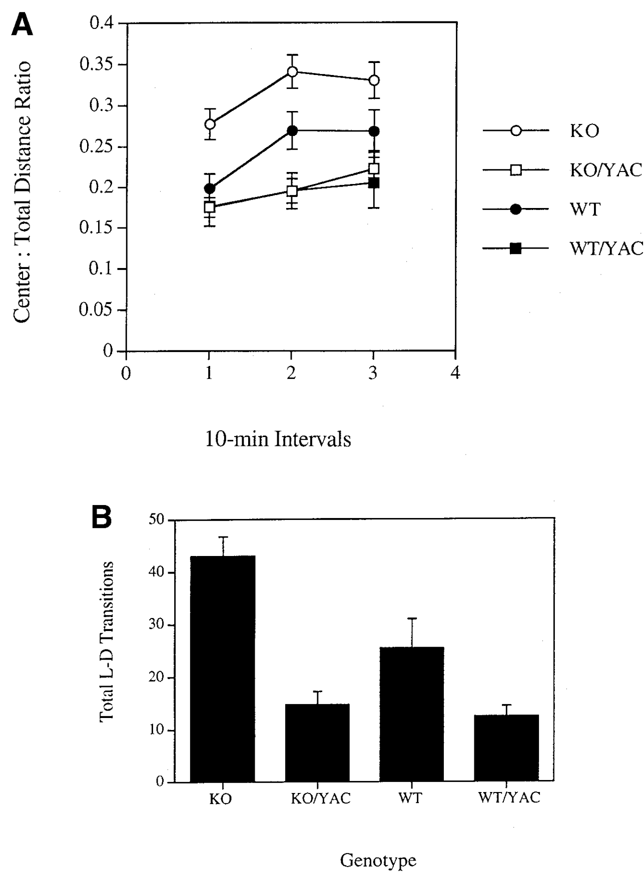


Figure 7. Anxiety-related responses in the open-field and light–dark exploration test. (**A**) The center distance/total distance ratio calculated for the open-field and (**B**) the number of transitions between the light and dark chamber are shown for the four genotype classes. The means (±SEM) are presented.

with transgenic animals spending more time in the dark chamber than non-transgenic littermates (data not shown).

Rotorod. Motor coordination and skill learning were tested with an accelerating rotorod, which measures the ability of a mouse to maintain balance on a rotating rod (46). For this test, mice were given four trials each day for three consecutive days. Initially, the performance of KO and WT mice was similar during the early trials (Fig. 8A). However, with further training, *Fmr1* knockout mice became mildly impaired and reached a lower plateau of performance compared with WT mice. However, this difference was not statistically significant ($P > 0.05$). In contrast, there was an overall transgenic effect observed in the initial trials, which became less apparent as the number of trials increased. On the first day of testing, both transgenic classes performed worse than non-transgenic littermates. As the trials increased however, the WT/TG mice performed comparably to the non-transgenic WT animals. The KO/TG mice performed poorly throughout the duration of the tests. Therefore, FMRP may play a role in motor coordination and skill learning.

Startle habituation. Sensorimotor adaptation was assessed using an acoustic startle habituation paradigm (47). The startle response amplitude was measured to the presentation of 100 startle stimuli (120 dB, 40 ms sound pulses) (Fig. 8B). In general, the acoustic startle response habituated with repeated presentation of the startle stimulus for all mice. However, KO/YAC and WT/YAC mice displayed a heightened startle response compared with their non-transgenic littermates throughout testing ($P = 0.002$). These findings indicate that the responses to sensory stimulation are abnormal in *FMR1* YAC transgenic mice even though they do display normal habituation to repeated stimuli.

Conditioned fear. To measure conditioned fear, mice were placed in a chamber and presented a white noise, conditioned stimulus (CS), followed by an unconditioned stimulus (US) presented as a mild footshock (48). Freezing behavior to the contextual cues and the white noise (CS) were assessed 24 h later. Mice from all four genotypes displayed similar levels of context and auditory-cued conditioned fear (Fig. 9A). These findings indicate that the *FMRP* does not contribute to fear-based forms of learning and memory.

Morris water maze. Mice were trained to locate a hidden platform in a circular pool of water. The time taken to locate the hidden platform (escape latency) and the distance traveled were determined (49). After the training period, a single probe trial was given in which the platform was removed and each

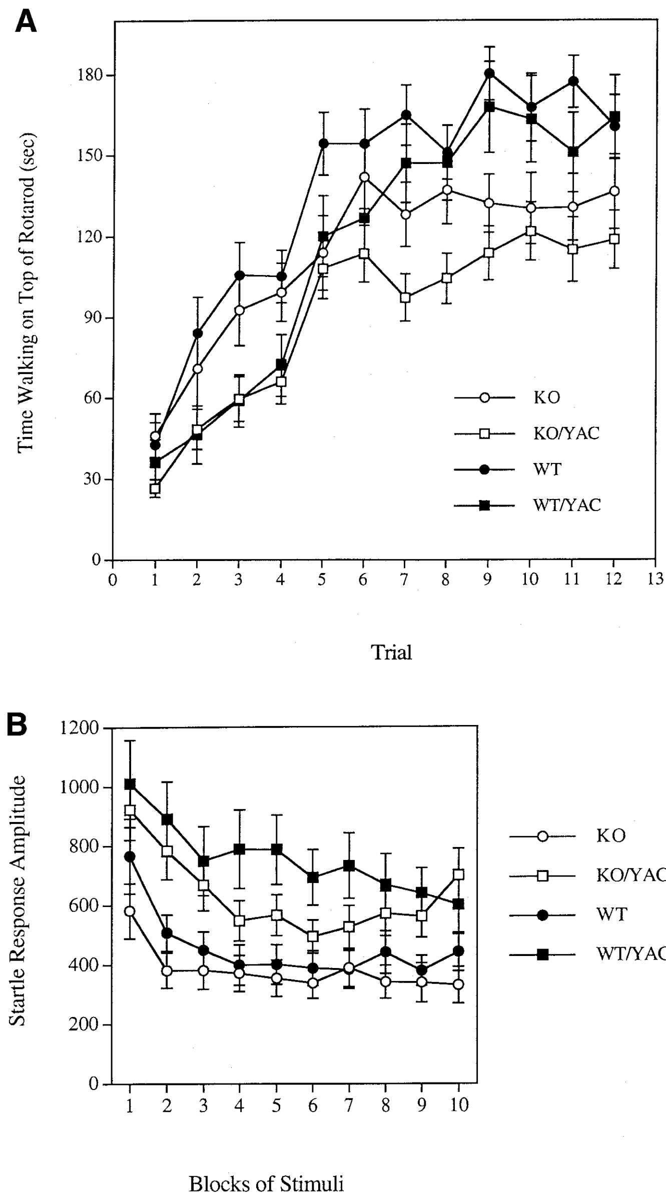


Figure 8. Motor coordination and skill learning was tested in the rotorod assay (**A**). Time spent on the rotorod is presented for KO, KO/YAC, WT and WT/YAC animals. The means (±SEM) are presented. Sensorimotor adaptation was measured in the habituation of the acoustic startle response (**B**). The average (±SEM) startle response to repeated presentations of a 120 dB startle stimulus are given for all four genotypes.

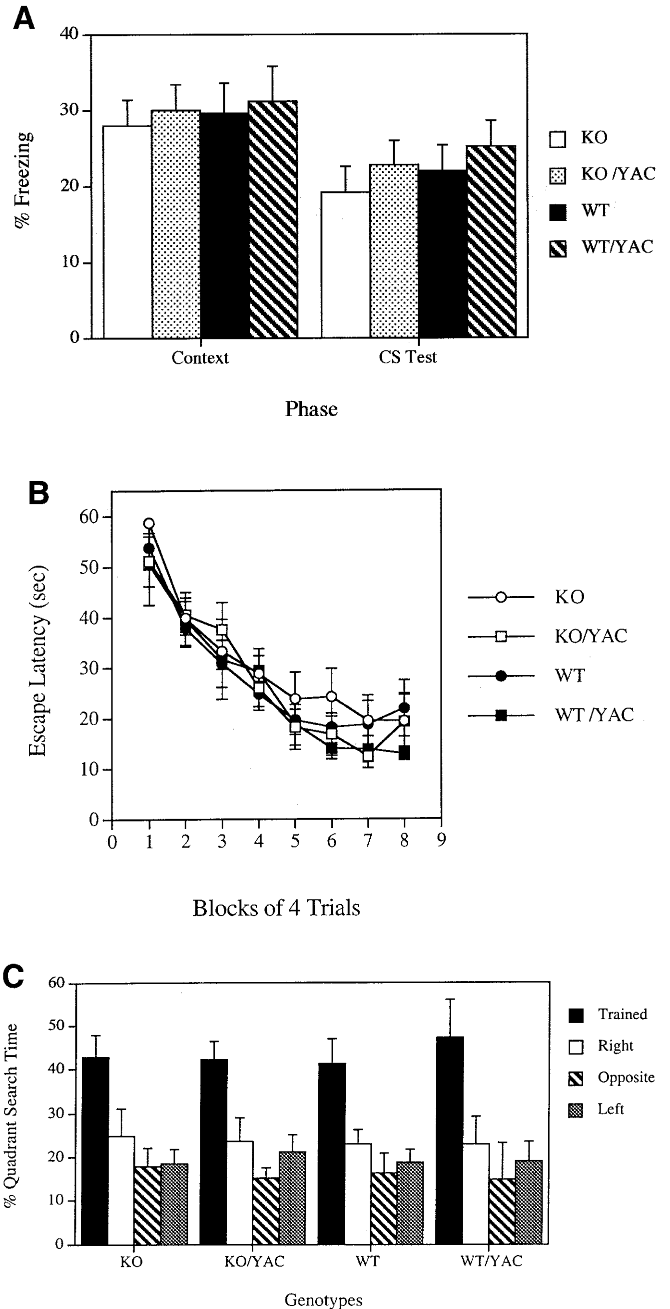


Figure 9. Learning and memory behavior tests. Performance of WT, KO and transgenic mice in the conditioned fear test (**A**); percent freezing assessed during the context and cued tests (CS test) are presented. Morris water maze task: (**B**) time taken to locate the hidden platform and (**C**) QSTs for all four genotypes are given.

animal was allowed to search the pool. The amount of time spent in each quadrant was recorded (quadrant search time, QST). There were no significant differences in performance among the four different groups of mice, as measured by time taken to locate the platform (Fig. 9B) or the QST from the probe trial (Fig. 9C). These findings suggest no difference in spatial learning among all four genotypes.

DISCUSSION

We have successfully generated YAC transgenic mice that carry the entire human *FMR1* gene with extensive amounts of flanking sequence. One line, TG298, was characterized further in this study. FISH and STS content analysis demonstrated a single integration site. Southern analysis and densitometry showed that the *FMR1* locus was present in multiple copies in these YAC transgenic lines. RT–PCR, northern analysis and western blotting indicated that the transgene supported production of the human protein. Western analysis also revealed that the transgenic tissues exhibited a more intense signal when compared with wild type. We therefore performed experiments

to measure FMRP expression in the YAC transgenic mice. A slot blot assay, which compares the molar ratio of Fmrp with the translation factor eIf4e in cells, demonstrated that the transgenic protein was expressed 13–17-fold higher than endogenous Fmrp levels. Quantitative western blots also confirmed that the TG298 line expressed transgenic FMRP at more than 10 times that of endogenous protein. Overexpression was not due to ectopic expression of YAC *FMRP* since immunohistochemical analysis revealed that the transgenic protein was expressed in a cell- and tissue-specific manner similar to endogenous protein in the brain and testis. The macroorchidism phenotype observed in fragile X syndrome males as well as the *Fmr1* knockout mouse was eliminated in knockout mice when the YAC transgene was introduced.

Extensive behavioral analysis was performed on the *Fmr1* knockout and transgenic animals in order to further characterize the consequences of absence of *FMR1* and to determine whether the YAC was able to complement the mutant phenotype. Significant behavioral differences were detected in the open-field and the light–dark exploration tests. Compared with WT mice, *Fmr1* knockout mice were observed to have increased locomotor activity in the open-field test and decreased anxiety-related responses in both the open-field and the light–dark test. There was also a mild impairment on the rotorod test displayed by the *Fmr1* knockout mice, but no effect in the acoustic startle response. The presence of the YAC in both wild type and knockout backgrounds, however, caused an overall decrease in activity and increased anxiety-like responses. In addition, the presence of the YAC also reduced rearing and increased time spent in the dark in the light–dark test. Performance on the rotorod assay in the initial trials was worse for animals carrying the YAC. Presence of the transgene also increased the acoustic startle response. This is consistent with the elevated anxiety-related responses observed for the transgenics in the light–dark and open-field tasks since the startle responses can often be associated with fear and/or anxiety (50,51). Interestingly, there were no behavioral differences in the conditioned fear or spatial learning tests, suggesting that changes in levels of FMRP do not impact particular types of learning and memory.

Our behavioral analysis of the *Fmr1* knockout in the open-field and light–dark activity tests supports previously reported results (25). *Fmr1* knockout mice displayed increased locomotor activity suggesting hyperactivity or increased exploratory behavior. The *FMR1* YAC transgenic mice showed low levels of locomotor activity suggesting decreased exploratory behavior. This observation, coupled with increased levels of protein, is suggestive of FMRP regulating the pathways associated with these behaviors. Additional behavior testing in this area may help to dissect this phenotype.

The *Fmr1* knockout mice did spend a significantly greater proportion of their total distance traveled in the open-field in the center of the arena as compared with their transgenic and wild-type littermates. In addition, the number of light–dark transitions was higher in the knockout mice. An aversion to open spaces and seeking out cover is a rodent behavior believed to be associated with anxiety (44). The present findings suggest that the *Fmr1* knockout mice exhibit reduced anxiety-related responses compared with wild-type behavior. Conversely, the YAC transgenics exhibit a phenotype in the opposite direction indicative of increased anxiety, displaying a significant reduction in exploration of the center area and fewer light–dark transitions remaining near the periphery of the enclosure. Since both the absence of FMRP in the knockout and overexpression of the protein in the transgenics render an anxiety-related phenotype in two different tests, our findings indicate that anxiety-related responses are in some way modulated by *FMR1*. Further testing of these animals in other anxiety tests such as the elevated plus maze (52) and the mirror chamber (53) will aid in better understanding the nature of this phenotype. Additionally, administering pharmacologic agents that act at different receptor systems may assist in elucidating possible receptor-mediated mechanisms involved in this abnormal response in *Fmr1* mutant and transgenic mice.

Somewhat surprisingly, we did not detect differences between the four genotypes in the conditioned fear and the Morris water maze task. Under different training conditions, Paradee *et al.* (54) have recently reported differences in contextual and cued conditioning in the conditioned fear paradigm for the *Fmr1* knockout mouse. The reason for the discrepancy between our present data and that published by Paradee *et al.* (54) is unknown, but clearly indicates that more studies using the fear-conditioning paradigm may be warranted. Moreover, several groups (25,34,35) reported a mild learning impairment in the Morris water maze task but only during the reversal trials. We did not carry out the reverse trials in the Morris task in our analysis since it is difficult to interpret whether the animal has learned the forward trial better or does actually harbor a learning impairment. Paradee *et al.* also reported near normal performance of the *Fmr1* knockout mice in the Morris task for both the forward and reversal trials, and provided data suggesting that strain difference may influence this behavior. These conflicting data indicate the need for further examination of this mutation in learning and memory tests that study other types of learning processes such as working memory (55).

How does the behavioral phenotype observed in the knockout and transgenic mice relate to that reported for fragile X syndrome patients? The increased levels of activity in the *Fmr1* KO mice is consistent with the observation that individuals with fragile X syndrome tend to be hyperactive. In contrast, mice carrying the YAC, and not the *Fmr1* KO mice, displayed increased levels of anxiety-related responses, which is contradictory to the expected result based on observations that individuals with fragile X syndrome exhibit heightened levels of anxiety (10). While this result was not expected, it does demonstrate that FMRP has a role in anxiety-related responses.

Therefore, the locomotor activity and anxiety data are consistent: absence of FMRP produces mice that have increased anxiety (i.e. hyperactive) and have low levels of anxiety-like responses, whereas overexpression of FMRP yields mice that are hypoactive and display high levels of anxiety. Often it is difficult to dissociate differences in exploratory activity from differences in anxiety responses. In the case of the *Fmr1* KO and transgenic mice, we believe that the center distance ratio data from the open-field test support the notion that the behavioral differences in locomotor activity are distinct from the behavioral differences in anxiety. The center distance ratio is calculated in such a manner that differences in overall exploratory activity are accounted. Thus, the center distance ratio should reflect differences in anxiety from differ-

ences in activity. In fact, we have shown that it is possible to dissociate exploratory activity from anxiety-related responses in the open-field using mutant mice (42). We will continue to study the relationship between exploratory activity and anxiety-like responses in the *Fmr1* KO and YAC transgenic mice to determine if these two phenotypes are independent.

Our analysis of testicular weights in KO, WT and transgenic animals indicate that FMRP is involved with regulating testis size. The presence of the transgene not only reduced testicular weights to within the normal range in the KO animals but WT/YAC mice actually had testicular weights that were significantly smaller than WT littermates. Immunohistochemistry demonstrated ectopic expression of the transgenic protein in later stages of spermatogenesis. This observation is most likely due to the autosomal location of the transgene. During spermatogenesis, the X-chromosome becomes inactivated, presumably shutting off expression of FMRP (56). Since this transgene is located on an autosome, its expression is not turned off during maturation of the sperm, thus leading to expression in these cells. Recently, the macroorchidism phenotype in mice was reported to be due to an increase in Sertoli cell proliferation (57). It will be of interest to determine whether the transgenic mice exhibit a reduced number of Sertoli cells comparable to or less than wild type.

Normal fertility was observed in both male and female mice heterozygote for the YAC transgene. However, the transmission of the YAC transgene did not correspond to the expected Mendelian segregation ratio when transgenic mice were bred with *Fmr1* heterozygote knockout females. The WT/YAC class was significantly under-represented. This observation suggests that overexpression of FMRP may contribute to reduced viability. Alternatively, there may be an effect in germ cells that reduces fetal transgene transmission. Furthermore, the generation of homozygotes for the TG298 line has proven to be impossible (unpublished observations). Only progeny carrying one chromosome with the YAC have been obtained to date when crossing two hemizygote transgenics. It is unclear whether this is *FMR1*-related since we have not vigorously pursued this line of breeding.

Our findings raise the question—what is the nature of this behavioral phenotype in the transgenic mice? Given the high levels of transgenic protein in these mice, one possibility is that overexpression of FMRP may manifest pathologic changes in the animal. Cognitive deficits are commonly observed in individuals carrying an imbalance of chromosomal material. Increased expression of genes due to trisomy (Down syndrome) (58), uniparental disomy (Angelman syndrome) (59) and functional disomy of X-linked genes that are expressed inappropriately due to absent X-inactivation has been observed in mentally retarded individuals (60). Similar findings have been reported in mice. Towards the development of a Down syndrome mouse, the overexpression of certain genes has contributed to a behavioral phenotype (61,62). Mice overexpressing *mSim2*, an RNA expressed in the hippocampus and amygdala, exhibited an impairment in learning and memory (61). Mouse models of Angelman syndrome also exhibit behavioral abnormalities (63).

An alternate possibility is that these behavioral observations are due to a position effect associated with the disruption of a mouse gene by insertion of the YAC DNA. Given that most phenotypes for the transgenic were in the opposite direction to those displayed by the knockout and that high levels of protein are expressed in these mice, we favor the hypothesis that the nature of the phenotype in the transgenic model is due to overexpression of FMRP. To directly address this issue we are now testing additional transgenic lines, with different insertion sites.

Overexpression of FMRP may have functional consequences on an organism. We have had difficulty generating stable cell lines that carry *FMR1* constructs. Drug-resistant colonies generated from transfections into COS cells can be obtained, but no *FMRP* is produced from the construct used in the transfections. Spheroplast fusion (64) experiments were performed to determine whether the YAC could support production of FMRP in cell culture prior to microinjection into mice but these experiments failed to yield colonies containing the *FMR1* gene (unpublished observations). Recently, Ceman *et al.* (65) circumvented this problem by generating stable transformants using a cell line that endogenously expresses FMRP at low levels.

How might the overexpression of FMRP cause a behavioral phenotype? Given that FMRP is an RNA binding protein found to associate with ribosomes, high levels of the protein in the cell might alter the metabolism of mRNAs that are normally regulated by FMRP. Recently, Feng *et al.* (66) have shown that a missense mutation in the *FMRP*, associated with an unusually severe fragile X phenotype (7), is capable of forming mRNP particles but they do not associate with polyribosomes. These findings support the idea that regulation of mRNAs by FMRP is functionally important. Secondly, the localization of *FMR1* in the dendrites and its rapid translation observed after metabotrobic glutamate stimulation (67), is suggestive of a role for FMRP in synaptic function. Furthermore, the recent finding by Torre *et al.* (68) of delayed dendritic spine maturation in cultured *Fmr1* knockout hippocampal neurons also supports this role for the protein. Perhaps the levels of FMRP alter synapse activation that in some way contributes to anxiety-related behaviors.

Given the complex behavioral phenotype in fragile X patients and the mild phenotype previously reported for the *Fmr1* knockout mouse, we performed a more thorough evaluation of the *Fmr1* knockout phenotype using additional behavioral assays that had not previously been reported for this animal model. We observed the knockout mouse to display less anxiety-related responses with increased exploratory behavior. *FMR1* YAC transgenic mice overexpressing the human protein did produce opposing behavioral responses and additional abnormal behaviors were also observed. These findings have significant implications for gene therapy for fragile X syndrome since overexpression of the gene may be harmful. Targeting treatment to modify the symptoms with pharmacologic agents may be a more appropriate approach than gene or protein replacement. Finally, animal models with regulated expression of *FMR1* may be useful towards understanding the role of this gene in behavior. The *FMR1* YAC transgenic mice described here may prove to be a useful model to study anxiety-related responses and to aid in understanding the function of *FMR1*.

MATERIALS AND METHODS

Retrofitting YAC 209g4c

YAC 209g4 was retrofitted with pRV1 (36) which carries the neomycin-resistance encoding gene (*neo*), a marker selectable in mammalian cells and the *LYS2* gene, which allows growth of yeast cells on media lacking lysine, inserted within the *URA3* locus. Digestion of plasmid pRV1 with *Hin*dIII released a 9.5 kb fragment containing the *neo* and *LYS2* genes between segments of the *URA3* gene. This fragment was used to transform the yeast strain *AB1380* containing the YAC 209g4. Transformants were plated on media lacking lysine. These colonies were subsequently replica plated onto plates lacking uracil. Growth on lysine but not uracil indicates a recombination event between the pRV1 fragment and the *URA3* gene on the right arm of the YAC. Clones that were Lys^+, Ura^- were then subjected to pulse field electrophoresis, blotted and hybridized with a neomycin gene probe to establish that the YAC had not been grossly rearranged (data not shown). One clone, YapRV.2, appeared to be intact and was used to generate transgenic mice.

Generation of *FMR1* YAC transgenic mice

YAC DNA was prepared for microinjection essentially as previously described (30) with the following modifications. Concentrated YAC DNA was dialyzed in microinjection buffer (10 mM Tris pH 7.4, 0.1 mM EDTA, 100 mM KCl) for several hours prior to microinjection. Initial injections using concentrations at 0.5–1 ng/µl yielded a low number of progeny. We found that a lower concentration of 0.2–0.5 ng/µl was optimal.

Analysis of transgenic founders

DNA was extracted from mouse tails for PCR analysis by standard methods. Primer pairs (previously reported) were used to identify YAC sequences from the right (RA) and left (LA) vector arms (69), the CGG repeat in exon 1 (4,70), DXS548 (71), G9L (72) and 4 kb 5′(73). Primers were also designed to amplify exon 3 with primers Ex3f (5′-GAAATAT-TCCAAAC GGGAGTAGG) and Ex3r (5′-ATTAAC-CCCACTCTTTCAGTGC), the 3′UTR with human specific primers 3utf (5′-CTGTAGATAATTAACCAAGG) and 3utr (5′-GCTAACTTCCTATGACGTGA), 3G1 with primers 3g1f (5′-CATGTAGCCTGGAACATAGA) and 3g1r (5′-ACTGGATTTGCTCACTGGCC) and 3G2 with primers 3g2f (5′-TCAGCTTCTGGCACCATCAG) and 3g2r (5′-GGAGCCATATGGCTAACTCC). PCR was carried out in a 25 µl reaction with 1× PCR buffer (Stratagene, La Jolla, CA), 4 µM of each primer, 200 mM each dNTP and 0.5 U *Taq* polymerase. PCR conditions were: 5 min at 94°C; followed by 32 cycles of 1 min at 94°C, 1 min at 55°C, 2 min at 72°C, with a final extension of 7 min at 72°C. The reaction products were sized on a 1% agarose gel.

Southern blot analysis

For transgene analysis, 10 µg of genomic DNA were digested with *Eco*RI (New England Biolabs, Beverley, MA) and *Bam*HI, size fractionated on a 1% agarose gel in 1× Tris-borate–EDTA. Gels were transferred onto nylon membranes (Genescreen Plus, NEN Research Products, Boston, MA) and hybridized according to standard procedures. A 540 bp PCR product encompassing intron 1 from the human *FMR1* gene and the PCR product generated from the 3g1 and 3g2 primers (described in the above section) were used as a probe. Additionally, a 600 bp PCR product using primers LLK96 (74) and LLK192 (5′-CTCAAGGCACATCTGATG) that span the 3′UTR of the mouse *Fxr1* gene was used as an internal control. Southern blots were scanned by densitometry (Molecular Dynamics, Sunnyvale, CA).

Fluorescence *in situ* hybridization

Mouse fibroblasts were obtained by cutting a small piece of tail from transgenic and non-transgenic littermates. The tail tissue was rinsed in 70% ethanol, minced and seeded on a small Petri dish in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum. Cells were harvested according to standard cytogenetic procedures. YAC 209g4c (500 ng) was labeled with digoxygenin-dUTP (Boehringer Mannheim, Indianapolis, IN) by nick translation as previously described (75). Hybridization and detection were performed essentially as described (75). Hybridization signals were detected with anti-digoxygenin–rhodamine (Boehringer Mannheim) counterstained with DAPI and viewed using a triple band by-pass filter (Zeiss, Thornwood, NY).

RNA analysis

RNA was extracted from mouse tails or cultured mouse tail fibroblasts using Trizol (Gibco BRL, Gaithersburg, MD) following the manufacturer's instructions. Random hexamer primed RNA (5 µg) was reverse transcribed using Superscript (Gibco BRL). An aliquot of prepared cDNA (1 µl) was then amplified by PCR using 4 µM each of human specific primers 16F (5′-CAACAGATGGATCCCTTC) from exon 16 and 16R (5′-GACTTCTATACGAGATTG) from the 3′UTR in a 25 µl reaction volume as described above. $Poly(A)^+$ RNA was extracted from WT and WT/YAC testes using Fastrack 2.0 (Invitrogen, San Diego, CA) following the manufacturer's instructions. Northern blots were hybridized with a PCR product spanning the 3′UTR of the *FMR1* gene and also GAPDH, which was used as an internal control for loading. Blots were exposed to a phosphor screen (Molecular Dynamics), scanned (MDStorm) and quantitated with the ImageQuant software (Molecular Dynamics).

Western blotting

Frozen tissue samples were crushed with a mortar and pestle and homogenized in osmotic lysis buffer (10 mM Tris, pH 7.4 and 0.3% SDS) in the presence of protease inhibitors. Samples were freeze thawed twice, and 1/10 volume of a 10× nuclease stock solution was added and incubated on ice for 15 min. An equal amount of SDS boiling buffer (5% SDS, 10% glycerol and 60 mM Tris, pH 6.8) was added to the sample, boiled for 15 min and then cooled on ice. Unsolubilized debris was pelleted by centrifugation. Protein concentration was determined by a BCA assay (Pierce, Rockford, IL). Fifty micrograms of protein were diluted 2:1 in sample loading buffer and resolved by SDS–PAGE using 7.5% mini gels (Bio-Rad Mini-Protean II Cell system). Proteins were then electro-blotted onto

PVDF membranes (NEN). Immunodetection of FMRP was carried out using the monoclonal antibody (mab1A) (12) diluted (1:2000) and an actin antibody as an internal control for protein loading. The secondary antibody was coupled to peroxidase allowing detection with the chemiluminescence method (ECL KIT, Amersham, Arlington Heights, IL). Quantitative westerns were performed as above except that the secondary antibody was coupled to biotin (Amersham) allowing detection with ^{35}S radiolabeled streptavidin. Blots were exposed to a phosphor screen (Molecular Dynamics), scanned (MDStorm) and quantitated with the ImageQuant software (Molecular Dynamics).

Quantification of human FMRP

A slot blot assay was used to calculate FMRP levels in two sets of animals using total brain extracts of KO, WT, KO/YAC and WT/TG mice. Protein lysates were prepared as for western blot analysis. FMRP was measured using purified FMRP from flag-tagged murine cDNA and detected with mAB1a and HRP-conjugated anti-mouse secondary from KPL. Standard curves were generated from four replicates of purified protein (not shown) and used to calculate FMRP levels in each sample, after subtracting the background due to the secondary only. To control for loading, the FMRP levels were normalized to purified eIF4e (kindly provided by Dr Hagedorn). The elongation factor, eIF4e, was detected with anti-eIF4e antibody from Transduction Laboratory (Lexington, KY), and HRP-conjugated anti-mouse secondary from KPL (Gaithersburg, MD). Standard curves were generated from four replicates of purified protein (not shown) and used to calculate eIF4e levels in each sample, after subtracting the background due to secondary only. Since the monoclonal antibody cross-reacts with the FXRPs, the 'FMRP' signal in the KO mouse was subtracted from the WT mouse to give the endogenous FMRP level.

Immunohistochemistry

Brain tissues obtained from transgenic and non-transgenic knockout mice and also wild type animals were perfusion fixed with 4% v/v paraformaldehyde (PFA)/0.01 M phosphate buffered saline (PBS) under deep anesthesia. Tissues were removed and fixed overnight in 4% PFA. The next day tissue was washed in 0.01 M PBS for several hours and subsequently washed in an ethanol series (20, 50 and 70%) with gentle rocking. Tissues were embedded in paraffin and sections subsequently mounted on poly-lysine coated slides. Sections were deparaffinized in Histoclear (National Diagnostics, Atlanta, GA) and rehydrated to water before being subjected to microwave treatment for 5 min in 10 mM sodium citrate pH 6 for antigen retrieval.

Sections were processed for immunoperoxidase using DAB as the chromogen. Briefly, sections were first blocked in Tris-buffered saline (TBS) with 1% avidin D (Vector Research, Burlingame, CA) and 4% normal goat serum (NGS) for 45 min, rinsed in TBS, followed by incubation in TBS containing 4% NGS and 5% biotin for 45 min to prevent non-specific binding. Slides were then incubated for 48 h at 4°C in TBS containing 2% NGS, and the monoclonal mouse anti-FMRP antibody mAb1a diluted at 1:1000. This incubation was followed by rinses in TBS and an incubation overnight at 4°C with a biotinylated goat anti-mouse secondary antibody at 1:200 (Vectastain Elite ABC Kit: Vector Research). Following rinses in TBS, specifically bound antibodies were visualized by an avidin–biotin complex. Final detection was done with the DAB substrate kit for peroxidase (Vector Research) by incubating the slides in a diaminobenzidine/hydrogen peroxide solution for 10 min, rinsed in water, counterstained with hematoxylin (Vector Research) for 2 min and mounted.

Behavioral analysis

Test animals. Subjects were derived from crosses between wild type C57BL/6 transgenic males and heterozygote *Fmr1* knockout females. *Fmr1* knockout animals (originally derived from 129/OLA ES cells from B. Oostra), and had been back-crossed to C57BL/6 for numerous generations and provided by Dr Steve Warren. This cross allowed all four genotypes to be present in progeny as littermates: KO, WT, KO/YAC and WT/YAC. Mice were genotyped for the knockout allele by PCR (25). Testing was performed on F1 male littermates. The mice were 3–4 months-old at the beginning of testing. Mice were housed three to five per cage in a room with a 12-h light–dark cycle with access to food and water *ad lib*. An experimenter that was blind to the genotypes of the mice conducted all experiments. Mice were tested in two batches. A total of 26 KO, 33 KO/YAC, 26 WT and 21 WT/YAC males were tested in all assays except for the light–dark exploration. Only the second batch was tested for the light–dark assay consisting of 11 KO, 14 KO/YAC, 15 WT and 11 WT/YAC animals.

Behavioral assays. Tests were performed essentially as described (42) with the following modifications. The rotorod test was performed on mice over a period of 3 days with four trials each day. The Morris water maze task consisted of eight trials, each in blocks of four, over a period of 4 days. After trial 32, the subjects were given a single probe trial.

Statistical analysis

Data were analyzed using two- (Genotype X Batch) or three-way (Genotype X Batch X Repeated Measure) analysis of variance (ANOVA). The present experimental design enabled us to use *a priori* planned comparisons to further analyze any main effect of genotype or interaction between genotype and batch, or genotype and repeated measure. The following planned comparisons were made: KO versus WT, KO versus KO/YAC, WT versus WT/YAC and YAC transgene versus no transgene. The software program Statistica (Statsoft) was used for this analysis.

ACKNOWLEDGEMENTS

We are indebted to Paul Overbeek and Gabby Schuster of the Baylor College of Medicine Mental Retardation Research Center (MRRC) transgenic mouse core lab for their assistance in generating YAC transgenic mice. We thank Hannes Vogel for pathological analysis of the brain sections and Jennifer Seifert and Laura Kirkpatrick for animal help. We would also like to thank Lisa Yuva-Paylor for technical assistance with the behavioral experiments and Danna Morris and Nessan A. Bermingham for technical advice. This work was supported in part by NIH grants HD35576 and HD20521 to S.T.W. who is

also an investigator of the Howard Hughes Medical Institute. The MRRC (grant HD24064) and NIH grants GM52982, HD29256 and HD38038 to D.L.N. supported this work.

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