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A Transgenic Mouse that Carries the Human Fragile-X (FMR1) Gene: Neonatal Lethality, Fragile-X Protein Expression and GABA-A Receptor beta-subunit Expression

by

Jason Scalia

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

2003

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The City University of New York

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Specific Aims

The research designs presented herein were developed to address three specific issues surrounding the action of the human FMR1 transgene in the FMR1-FVB/N transgenic mouse. These goals are: 1) to determine at what point during development the presence of the FMR1 transgene results in mortality of female transgenic mice, and; 2) for the time period revealed by (1), to determine if differences exist between the levels of FMRP/*FmrP* expressed in the brains of transgenic female mice and the brains of mice which do not exhibit increased mortality, and; 3) for the same time period, to determine if differences exist between the levels of $GABA_AR$-β protein expressed in the brains of transgenic female mice and the brains of mice which do not exhibit increased mortality.

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List of Abbreviations

ANOVA	analysis of variance
CAPS	3-cyclohexylamino-1-propranesulfonic acid
CMF-PBS	calcium-magnesium free phosphate-buffered saline
dH_2O	distilled water
DMEM	Dulbecco's modified essential medium
d.p.c.	days post coitus
FCS	fetal calf serum
FISH	fluorescence *in situ* hybridization
FMR1	human fragile-X mental retardation gene
Fmr1	murine fragile-X mental retardation gene
FMRP	human fragile-X mental retardation protein
FmrP	murine fragile-X mental retardation protein
FXR	fragile-X related
FXS	fragile-X syndrome
GABA	γ-amino-butyric acid
$GABA_AR$	γ-amino-butyric acid receptor type A
Genotypes:	
KO	*Fmr1* knockout male
KO/KO	*Fmr1* knockout female
KO/KO/PAC	*Fmr1* knockout female carrying the PAC-FMR1 transgene
KO/PAC	*Fmr1* knockout male carrying the PAC-FMR1 transgene
WT	wild-type male
WT/PAC	wild-type male carrying the PAC-FMR1 transgene
WT/WT	wild-type female
WT/WT/PAC	wild-type female carrying the PAC-FMR1 transgene
HBSS	Hank's balanced salt solution
IPTG	isopropylthio-β-galactosidase
Kb	kilobase
KO	denotes murine *Fmr1* knockout allele
LB	Luria-Bertani media
NMDA	*N*-methyl-D-aspartate
NMDAR	*N*-methyl-D-aspartate receptor
PAC	P1 artificial chromosome containing the human FMR1 clone
PMSF	Phenylmethylsulfonyl Fluoride
P*n*	postnatal day (*n*)
PVDF	polyvinylidene difluoride
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TG	denotes human FMR1 transgene
TRIS	tris[hydroxymethyl]aminomethane
VYS	viceral yolk sac
WT	denotes wild-type *Fmr1* allele
YT	yeast extract-tryptone media

which developed from this observation has allowed the characterization of the phenomenon and provides the initial analysis of a sex specific mortality which is associated with the presence of the transgene.

This work consists of three studies. These studies are hypothesis driven designs which combine to describe TG female mortality with respect to its developmental timing, and with respect to the expression of both the transgene and the $GABA_A$ receptor β-subunit in the brain during this time. The primary incentive for focusing on the brain as a target for protein expression studies was derived from antecedent knowledge of the involvement of FMR1 in brain function.

Fragile-X syndrome (FXS) is a genetically determined mental retardation which results from the silencing of the FMR1 gene. With few exceptions the cause of gene silencing is the aberrant expansion of a CGG repeat in the FMR1 promoter region (Peiretti et al., 1991; O'Donnell and Warren, 2002). The expanded repeat becomes a target for methylation which leads to gene inactivation and the loss of the gene product, FMRP. Since the FMR1 gene is located on the X chromosome, FXS is more pronounced in males than in females. Males suffering from FXS often possess physical abnormalities such as enlarged ears, elongation of the face, macroorchidism and hyperextensibility of the joints. However; the hallmark of FXS in humans is mental retardation.

This aspect of the disorder has driven much of the research surrounding the neurological basis of FXS. Our knowledge of FMRP/*FmrP* function in the central nervous system (CNS) comes largely from two types of studies involving rat and mouse models; those involved in cellular functions, and behavioral studies. Research into the cellular functions of *FmrP* in the CNS has yielded information concerning the tissues in

which it is expressed, its cellular and subcellular localization, putative biochemical functions and its involvement in establishing neuronal cytoarchitecture. The behavioral studies which have been performed have mainly focused on learning and memory. These studies have examined such features as long-term potentiation (LTP), associative and non-associative memory, and spatial learning. These rodent studies examine the functions of the endogenous *Fmr1* gene in the rat and mouse, and utilize the *Fmr1* KO mouse model for FXS.

FmrP is expressed in the CNS during development and throughout maturity (Bakker et al., 2000). Studies in juvenile and adult rodents show that *FmrP* is expressed in neurons where it is localized in dendritic spines (Irwin et al., 2000), cytoplasm and the nucleus (Bakker et al., 2000). FMRP has been shown to possess RNA binding activity and it is suggested that it may function in translational regulation or mRNA localization (Siomi, et al., 1993; Brown et al., 1998). FMRP also exhibits protein binding activity and has been shown to interact with itself and two related paralogs, fragile-X related proteins 1 and 2 (FXR1; FXR2) (Zhang et al., 1995). There is evidence that translation of *FmrP* can be regulated in an activity dependent manner; unilateral whisker stimulation was shown to increase *FmrP* levels in the contralateral somatosensory cortex in the rat (Todd and Mack, 2000). *Fmr1* mRNA was increased in the rat hippocampus after electroconvulsive shock (Valentine et al., 2000). In rat synaptosomes containing entire synapses and the sub-synaptic compartment, *FmrP* levels were increased by treatment with the group I metabotropic glutamate receptor (mGluR) agonist dihydroxyphenylglycine (DHPG) (Weiler et al., 1997).

receptor activity are involved in the early refinement of synaptic connectivity (Aamodt and Constantine-Paton, 1999). Studies in mice with engineered lesions to the NMDA receptor demonstrate the importance of receptor function for the survival of neonates. Kutsuwada et al., (1996) and Poon et al., (2000) report neonatal mortality associated with independent disruptions of NMDA receptor function. In both situations, death occurs within the first 24 hours after birth and is associated with a disruption of brainstem regulatory function. Kutsuwada et al., (1996) observed failure to suckle, abnormal respiratory regulation and excessive long-term depression (LTD) in the nucleus tractus solitarius (NTS) in pups not expressing the NMDA receptor subunit NR1. Poon et al., (2000) observed similar symptoms in pups lacking the ε2 NMDA receptor subunit. Mutant pups exhibited a failure to suckle and abnormal patterns of neurogenesis in the brainstem trigeminal complex. These studies indicate that disparate lesions to the NMDA receptor converge to disrupt critical brainstem regulatory systems.

The mechanisms underlying plasticity of the GABAergic system are thought to be regulated by synaptic activity during prenatal and postnatal development and respond to both the overall level of synaptic activity and to synaptic calcium fluctuations (Aamodt and Constantine-Paton, 1999; Ganguly et al., 2000). While the GABAergic system responds to synaptic activity, GABA mediated synaptic transmission is also an important feature of embryonic synaptogenesis and synapse maturation in the early postnatal brain (Ganguly et al., 2000). Like the branch of the glutamatergic system which is mediated by NMDA receptors, elements of the GABAergic system have been shown to have an effect on the viability of neonatal mice. DeLorey et al., (1998) observed a high incidence of neonatal mortality in mice which do not express the $GABA_A$ receptor β_3-subunit. Due to

findings such as these, and due to our own observation of reduced $GABA_A$ receptor expression in adult KO mice we sought to explore whether alterations in Fragile-X protein levels and $GABA_A$ receptor levels were present in the FMR1-FBV/N transgenic strain.

Diagnostic Assays for Sex Determination and Transgene Carrier Status

INTRODUCTION

The line of investigation described herein presents the PCR based diagnostic assays used for the purpose of sex determination and for differentiating animals which carry the FMR1 transgene from animals which harbor only the wild-type *murine Fmr1* allele. Included in these assays is a semi-quantitative approach used to differentiate hemizygous transgene carriers form homozygous transgene carriers. Furthermore fluorescent in situ hybridization (FISH) on mouse chromosomes (Akeson and Davisson, 2000) was used to verify the FMR1 transgene insertion into a mouse chromosome. We also required the use of homozygous transgenic animals during the course of various experiments. Fluorescent in situ hybridization allows an independent verification of PDR diagnostic techniques which assay the zygosity state of transgnen carriers.

Chromosomal FISH employs, as its name implies, the delivery and hybridization of a nucleotide probe to target chromosomal material which exists as an *in situ* preparation fixed onto a microscope slide. The probe may be labeled with an antigen and subsequently detected by direct or indirect immunofluorescence or, as we have chosen, the probe may be directly labeled with a fluorochrome. While hybridization to chromatin is possible, isolable chromosomes are required for identification by chromosome banding techniques. Cells in metaphase are therefore the target material of choice for cytogenetic analysis.

The PAC 16666 construct was used as the template for the generation of a fluorescently labeled hybridization probe by the nick translation method. The efficacy and specificity of this probe was tested on metaphase material which was derived from homozygous TG male mice. This genotype was selected for this design since probe specificity could be determined by the number of positively staining chromosomes. Specifically, two hybridization signals on morphologically similar chromosomes would demonstrate both the specificity of the probe for the human transgene, and that the transgene has a single point of insertion. The observation of a third signal on a morphologically distinct chromosome was to be accepted as evidence that the probe contained crossreactivity for the *murine Fmr1* sequence. Human cytogenetic material was tested in parallel with mouse material as a positive control for hybridization of the probe to the human sequence.

We have determined that the PAC 16666 construct may reliably be used as a template for the production of a fluorochrome labeled probe. The signal generated by FISH analysis is strong, reproducible and , as expected, does not pick up the *murine Fmr1* sequence.

METHODS and MATERIALS

DNA Preparation and PCR Reaction Conditions

Template DNA was isolated from VYS or tail samples by incubation for 2 hours at 55°C in an extraction buffer containing: 10 mM Tris-Cl, pH 8.5; 50 mM KCL; 1.5 mM $MgCl_2$; 0.01% gelatin; 0.45%, NP-40; 0.45% Tween-20; 0.1mg/ml Proteinase K (Malumbres et al., 1997). After extraction, samples were heated to 95°C for 15 minutes to inactivate the Proteinase K, then centrifuged 2' x 16,000 g to remove undissolved material. VYS and tail samples were digested in 100-200 µl of extraction buffer.

The sex of each embryo was determined by PCR with the primer pair Sry1:Sry2 (Hogan et al., 1994), which amplifies a sequence in the sex determining region of the Y-chromosome. Transgene carrier status was determined by PCR with S1:S2 primers (Baker et al., 1994). These primers amplify a sequence in both human FMR1 and mouse *Fmr1* templates; however, they yield a product of a different size for each species: mouse ~ 500 bp; human ~ 450 bp.

Individual 25 µl reactions were prepared from large scale reaction mixes. 0.5 µl of template were added to 24.5 ul of a reaction mix containing: 0.7 U Platinum *Taq* polymerase (Gibco/BRL); 20 mM Tris-Cl, pH 8.3; 50 mM KCl; and 0.2 mM dNTP (Sigma). $MgCl_2$ was added to a final concentration of 1.5 mM for use with the S1:S2 primer pair or 2.0 mM for use with the Sry1:Sry2 primer pair. Forward and reverse primers were added to a final concentration of 2 µM or 1 µM for S1:S2 and Sry1:Sry2, respectively. Reactions were performed in an MJ PTC-100 thermocycler under the

Forty eight hours after the yeast injection, mice were injected intraperitoneally with 0.1 ml 0.5% colchicine and sacrificed by CO_2 asphyxiation ten minutes later. The femurs and tibias were dissected, stripped of muscle and the diaphyses were isolated. Bone marrow was expressed by flushing HBSS through the medullary canal with a 27 GA needle. The marrow was collected in a total volume of 8 ml HBSS and triturated by several passes through a fire polished Pasteur pipette. The cell suspension was then centrifuged 10' x 350g at 10°C. Approximately 500 ul of the supernatant was retained and the cells resuspended by passage through a pipette. A hypotonic treatment was administered to isolate nuclei by adding 7.5 ml 0.075 M KCl dropwise while gently mixing the suspension. The suspension was then incubated for 25' at 37°C. A 500 ul volume of fixative (3:1 methanol/acetic) was added and the suspension was centrifuged 10' x 350g at 10°C. 500 ul of the supernatant was again retained above the pellet, 2 ml fixative were added and the nuclei resuspended. This centrifugation and fixation step was repeated three times prior to slide preparation.

Slide Preparation and Storage of Cytological Material

Metaphase spreads were prepared for chromosomal FISH analysis by dripping the suspension of fixed nuclei onto wet slides from a height of approximately 20". Slides were allowed to dry at room temperature in a humidified chamber (36-40% relative humidity). Unused suspension was stored under fixative at 4°C.

Probe Preparation

Amplification and Isolation of the p16666 Construct

Escherichia coli containing the complete genomic FMR1 sequence as a 106 Kb (approximate) P1 construct (p16666) were grown from a lyophilized stock as follows. A small amount of the lyophilized stock was aseptically transferred to and resuspended in 75 to 150 ul TY broth (tryptone 8 g/l; yeast extract 5 g/l; NaCl 5 g/l). The cell suspension was then streaked for isolation and incubated for 16-18 hr at a temperature of 37°C on LB plates (10 g/l tryptone; 5 g/l yeast extract; 10 g/l NaCl; pH 7.0; 1.5% agar) supplemented with 25 ug/ml kanamycin. Single colonies were used to inoculate 75 ml batch cultures of TY broth supplemented with kanamycin (25 ug/ml). Cultures were incubated for 16-18 hr at a temperature of 37°C in an orbital shaker (240 rpm). 2.5 ml from the 18 hr culture were used to inoculate 75 ml of fresh TY broth. This culture was incubated at a temperature of 37°C in an orbital shaker (240 rpm) for 1.5 hr. After 1.5 hr, IPTG was added to a final concentration of 500 uM and the culture was incubated for an additional 4 hr. The cell suspension was chilled on ice then centrifuged for 10' x 10,000g at 4°C. The bacterial pellet was processed for plasmid isolation with the Qiagen, Inc. MIDI-PREP kit as per the manufacture's instructions for the retrieval of high molecular weight plasmids. The size of the isolate was confirmed by restriction digest with *Pst-I* and agarose gel electrophoresis.

Generation of a Direct-Labeled Fluorescent Probe

p16666 DNA was labeled with a fluorochrome (SpectrumRed™, Vysis, Inc.) using the Vysis, Inc. Nick Translation Kit (Cat.No. 32-801300), and prepared for hybridization according to the manufacturer's instructions. Briefly, 0.5 ug p16666 DNA were mixed in a 50 ul reaction containing 5ug DNase I; 5ug DNA ploymerase I; 10 uM dTTP; 20 uM each dATP, dCTP and dGTP; 10 uM fluorochrome conjugated dUTP; 50mM Tris-Cl, pH 7.2; 10 mM $MgSO_4$; 1 mM DTT. The reaction was incubated 2hr at a temperature of 15°C and brought to a stop by heating for 10' at 70°C. To confirm that the probe was of the appropriate size (300-600bp), a 9ul sample of the reaction product was analyzed by agarose gel electrophoresis. Care was taken at all stages to minimize exposure of the labeled probe to light.

Approximately 100 ng of probe were used for each cytogenetic preparation. The following protocol for a 100 ng sample was scaled up as required and care was taken at all stages to minimize exposure of the labeled probe to light. Approximately 100 ng of labeled probed were mixed with 1-2 ug Cot-I DNA (Cat No. 32-800028). Probe and blocking DNA were mixed with 0.1 volumes 3M sodium acetate, pH 5.2 and 2.5 volumes 100% ethanol. DNA was precipitated by a two hour incubation at -20°C and centrifuged for 20' x 16,000 g. After removal of the supernatant, the probe was dried under N_2(g) and resuspended in a 70% solution of LSDWCP hybridization buffer (Cat. No. 32.804826).

Hybridization Conditions and Microscopy

Hybridization and microscopy were performed by personnel in the Department of Cytogenetics, NYS-IBR. Human material was handled only by said personnel and was designated as residual and anonymous.

Slides containing metaphase spreads were denatured by incubation for 2' at a temperature of 70°C in a 70% solution of deionized formamide in 2x SSC, and dehydrated in an ethanol series: 70% x 1'; 85% x 1'; 100% x 1', and allowed to dry completely. The probe was denatured by a 5' incubation at 73°C and applied to the slide preparation which was subsequently mounted with a coverslip and sealed with rubber cement. Hybridization was allowed to occur overnight (16-20 hr) at 37°C. Slides were washed for ~ 2' in 0.4 X SSC; 0.3% NP-40, 73oC, then transferred to 2X SSC; 0.1% NP-40, RT for 1'. Slides were then air dried prior to preparations for microscopy. Coverslips were mounted with a DAPI II counterstain (Cat. No. 32-804831), and visualized using epifluorescence microscopy.

PCR Based Diagnostic Assay of Transgene Zygosity State

For purposes of the present study and for the selection of homozygous transgenic sires in Chapter 3, it was necessary to establish a means whereby, the zygosity state of TG mice could be determined. This assay is based upon the fact that the S1:S2 primer pair (Bakker et al., 1994) amplifies regions within both the human FMR1 sequence and in the *murine Fmr1* sequence. These products are of different lengths, and may thus be differentiated by electrophoretic analysis. We sought to determine whether the difference in the molar ratios of template between hemizygous animals and homozygous animals would be revealed in the amount of PCR reaction product and could be visualized. Hemizygous TG males have a molar ratio of 1:1 for the two templates. Homozygous males have a molar ratio of 1:2 for the *Fmr1* and FMR1 alleles, respectively. Band intensities of the PCR reaction product were compared by densitometric analysis of electrophoretic results. When ratios of *murine* derived to human derived product were used as the diagnostic parameter, a clear difference was detected between templates drawn from hemizygous and homozygous animals (Figure 2). These results were later confirmed by extensive pedigree analysis (data not shown).

Electrophoretic Analysis of Diagnostic PCR

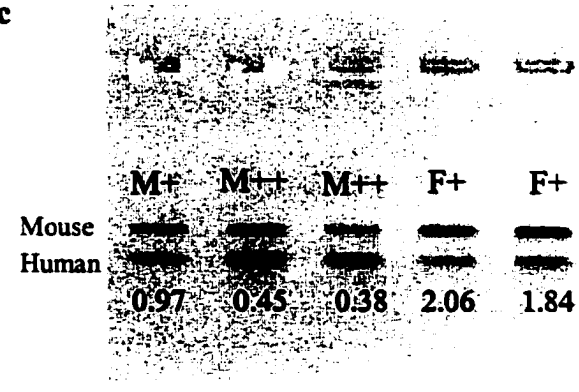


Figure 2. Results from agarose gel electrophoresis for the PCR based zygosity assay are shown. Male and female template sources are indicated by 'M' and 'F', respectively. Transgene zygosity is indicated by a single '+' (hemizygous) or a double '+' (homozygous). Band intensity ratios of mouse derived product (upper band) to human derived product (lower band) are given below each lane. Female homozygotes are not pictured above.

This assay was performed in exactly the same manner as the TG carrier state PCR diagnostic save for the fact that 25 amplification cycles were used instead of 30. This modification was employed in order to reduce the possibility that band intensities would show saturation in densitometric analyses. As a conservative measure to prevent the misdiagnosis of the homozygous carrier state, male subjects with a mouse/human band intensity ratio greater than 0.68 were assumed hemizygous pending pedigree analysis. Female subjects with a mouse/human band intensity ratio greater than 1.5 were treated likewise. These parameters selected post-hoc and *a priori* form our pilot work with this assay.

Restriction Analysis of p16666 Isolate

The p16666 construct was demonstrated by pulse-field gel electrophoresis as being approximately 100 Kb in length (Dobkin et al., unpublished). Though we had used the same bacterial transformants during the amplification and isolation of the construct, we nevertheless examined a Pst-I restriction digest and agarose gel electrophoresis in order to approximate the length of the construct and confirm our results using the pulse-field analysis as standard. We estimate the length of the p16666 isolate to be approximately 106 Kb in length (Figure 3).

Electrophoretic Analysis of the p16666/Pst-I Restriction Product

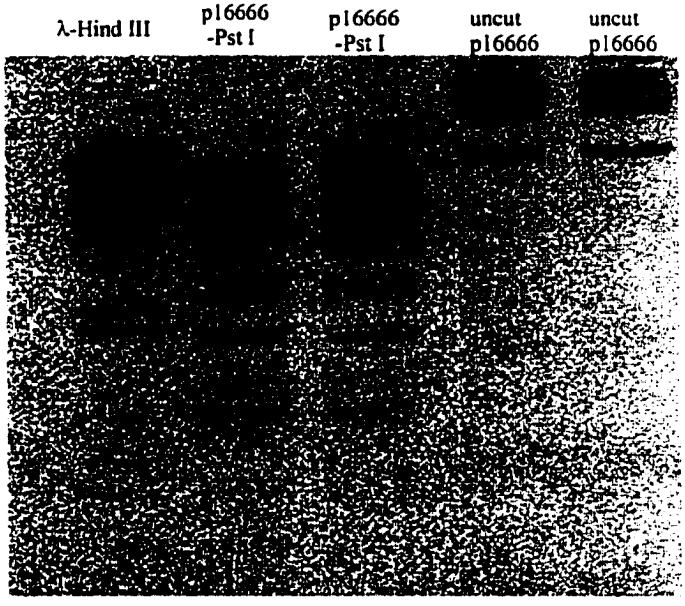


Figure 3. Two separate isolates were processed in parallel by digestion with Pst-I, and were electrophoresed in conjunction with a λ-Hind III marker and undigested p16666. The two Pst-I digests and the two uncut p16666 isolates correspond in lane position.

Hybridization of PAC 16666 Based Probe with Transgene

A probe with specificity to sequences within the human FMR1 transgene was generated from the PAC 16666 construct by the incorporation of a fluorochrome-tagged nucleotide analog using nick translation. The probe was tested by hybridization with cells derived from adult male homozygous transgenic mice. As a positive control for probe hybridization to human FMR1, the probe was tested in parallel by hybridization to a sample of cytogenetic material from a human female which was supplied by the Department of Cytogenetics at the New York State Institute for Basic Research. Figure 4 represents typical results.

Specificity of the probe for the human transgene is confirmed by the presence of two signals in the mouse metaphase spread at similar locations along the length of each chromosome (Figure 4a) and is corroborated by the fact that signal localizes to a distal site on the long arm of both human X chromosomes (Figure 4b). While the preparation was not examined by G-banding, the FMR1 locus, Xq27.3, is nearly the most distal point on the X chromosome long arm. Furthermore, the signal seen in every interphase nucleus which was captured in full is numerically consistent with the signal seen in the metaphase spreads. These observations and the nearly complete absence of background signal strongly support the conclusion that this probe design is highly specific for the human transgene.

Visualization of the FMR1 Transgene in Mouse Chromosomes by Fluorescence in Situ Hybridization

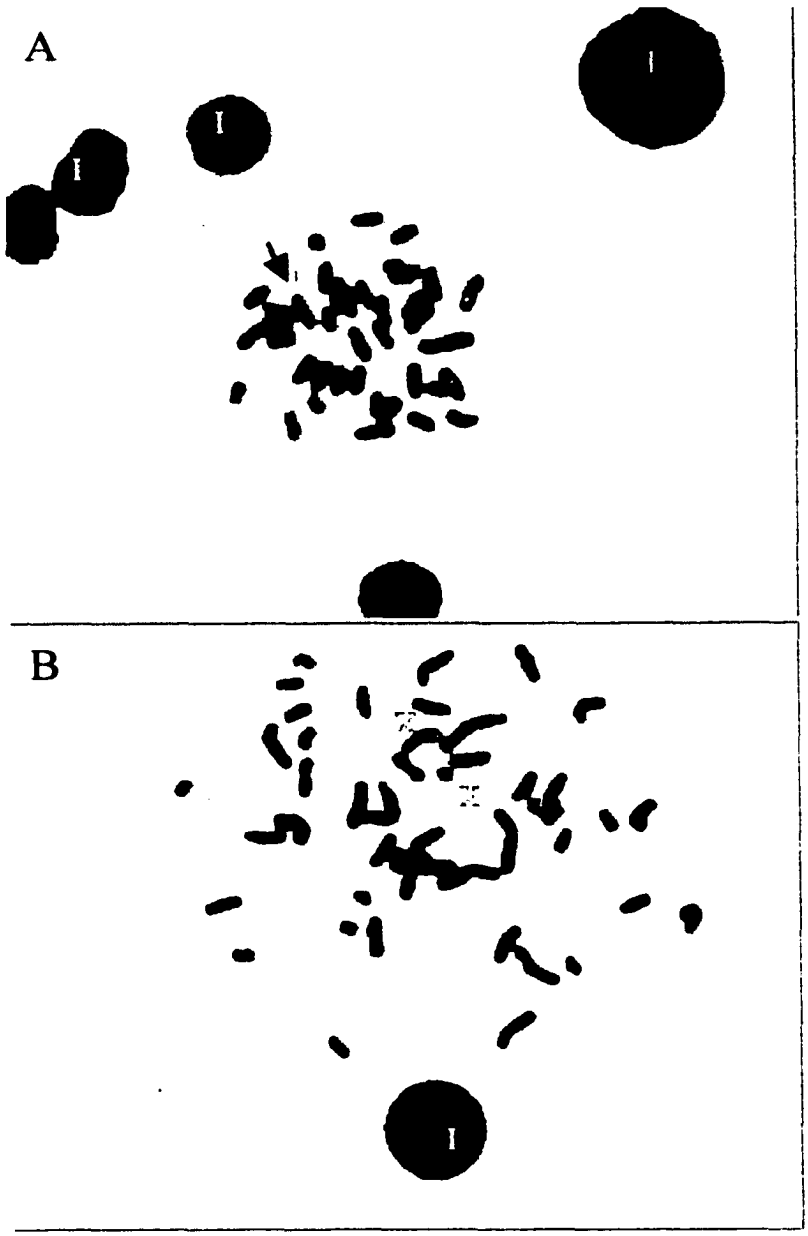


Figure 4. Signal from the hybridization probe (seen as gray spots) is present in interphase nuclei (I) and is localized to metaphase chromosomes in *A* and *B*. A) Cytogenetic material was derived from a homozygous TG male. The probe hybridized to both transgenic alleles. The arrow indicates a metaphase chromosome oriented so that signal is perceived on both chromatids. B) Cytogenetic material from a human female demonstrates hybridization of the probe to a distal site on both X chromosomes. Chromatin and condensed chromosomes appear black in *A* and *B*.

Inheritance of the Transgene

INTRODUCTION

Prior to the design of this thesis a founder FMR1 TG mouse had been provided by Robert Bauchwitz (Columbia University), and an inbred TG strain was being established on site in the IBR Animal Colony. The FVB/N strain was used as the genetic background upon which a population of congenic TG mice was to be produced. To establish this new strain the founder TG mouse, a hemizygous TG male C57BL/6-CBA hybrid, was mated with a wild-type female of the FVB/N strain. Female TG progeny were subsequently mated with FVB/N males in order to outcross the C57BL/6-CBA genome (Figure 5). This mating regimen was continued serially until the eighth backcross generation. At that time it was observed that females carrying the FMR1 transgene appeared to be produced at a reduced frequency relative to wild-type females (Table 1; Figure 6). Our standard practice during the husbandry of transgenic mice was to wait until the time of weaning (21 days postnatal) before scoring progeny for sex and transgene carrier status, and we noted no signs of dead juvenile mice. We were thus guided to the conclusion that the observed deficit of TG females resulted either from mortality *in utero* and the subsequent absorption of aborted embryos, or death during the early postnatal period.

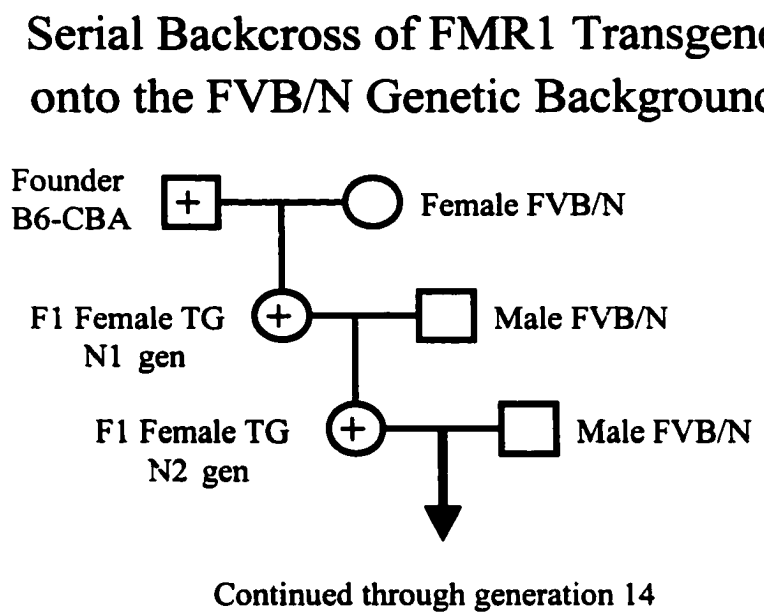


Figure 5. Schematic depiction of the serial backcross mating regimen used to generate the FVB/N-FMR1 transgenic strain diagramed to the third backcross generation. Males are denoted by squares and females are denoted by circles. The '+' symbol denotes the presence of the FMR1 transgene (TG).

Similar observations were reported by Peier et al., (2000). That study presents an FMR1 transgenic mouse strain which harbors a yeast artificial chromosome (YAC) clone containing the entire human FMR1 gene. They note a decrease in the proportion of hemizygous transgenic progeny, and the complete absence of homozygous transgenics among the offspring of transgene carriers. Their observations do not however, include a measure of sex specificity with regard to the deficit of YAC hemizygotes. The YAC-transgenic mice are reported to contain two to three copies of the transgene inserted at an autosomal location and, as is the case with our PAC-TG mice, show a 10-15 fold increase in total FMRP/*FmrP* levels. Peier et al., (2000) did not rigorously pursue the issue of reduced transmission of the YAC transgene and so are reserved with respect to the conclusion that the phenomenon is directly related to FMR1 expression. Yet, they do offer germline instability or FMRP overexpression as possible sources of the deficit in transgenic progeny. We began an investigation of the phenomenon in our FMR1-FVB/N strain.

Two experiments were designed to address the issue of unequal gametogenesis and to determine the developmental time period during which TG females were dying. The general methodology employed to address these questions involved large scale crosses between transgenic and wild-type animals and the subsequent analysis of F1 progeny to determine sex and transgene carrier status. Data obtained from such analyses are nominal scale and thus testable by the Chi-square statistic against expected Mendelian ratios for sex and transmission of the transgene. Given the possibility that TG female mortality was either occurring *in utero* or shortly after birth, experiments were designed

for the collection of progeny during both time periods. By controlling the developmental period over which progeny were collected, this design allowed us to test the following hypotheses: H_1) the observed deficit of TG females appears *in utero*; H_2) the observed deficit of TG females results from death within the first 24 hours after birth. The possibility that the deficit of TG females results from failures during gametogenesis is not directly tested by this design; however, it is understood that if H_1 is refuted gametogenesis must occur in a normal manner. We also evaluated whether the sex of the parent donating the transgene would have an effect on TG female mortality. As described below, we merely retested H_2 using females as the parent of origin rather than males.

Our findings demonstrate normal transmission of the transgene *in utero*. Thus, we discount germline instability or death during embryogenesis as sources of the reduction in the numbers TG females. Furthermore, we observed a statistically significant reduction in the number of TG females within the first 24 hours after birth. These data were derived from experiments using males as the parent of origin for the transgene. The incidence of TG females was also reduced when females were used as the parent of origin, but not to a statistically significant degeree.

METHODS and MATERIALS

Experimental Organisms

Wild-type FVB/N male and female mice were purchased from Taconic Farms Inc. (Germantown, NY) at ages ranging from six to eight weeks and housed for two weeks prior to use. Hemizygous transgenic animals of the FVB/N-FMR1 strain were selected from FVB/N backcross generations 8-14. Homozygous transgenic mice were selected from the F2 progeny of FVB/N backcross generations 8-12.

Husbandry Conditions

Animals were maintained at an average temperature of 22°C in a filter isolated Thorin rack on a 12 hr. photoperiod. Bedding material, food, water and all hardware were sterilized prior exposure within the colony, and all personnel entering the colony were attired in barrier garments. Food and water were available *ad libitum*.

Experimental Design

A statistical comparison of observed and expected genotypic frequencies of the progeny of transgene carrying mice was chosen as the method whereby the initial observation of TG female lethality would be examined in detail. Two mating schemes were designed in order to determine the developmental period during which TG female mortality first occurred. Initially, hemizygous TG males were mated with wild-type FVB/N females, and embryos were collected and scored for both sex and transgene carrier status. Secondly, homozygous TG males were mated with wild-type FVB/N females and progeny were collected 48 hours after birth and assayed for sex. Within this

design, germline transmission of the transgene and fetal mortality were examined by measures of fetal genotypic frequencies. TG female mortality during the postnatal period was assessed by examining the sex ratio of the progeny of homozygous TG males mated with wild-type FVB/N females. Parent of origin effects were assessed by the same design used to examine the postnatal period save only that homozygous TG females were used as the parent of origin for the transgene rather than homozygous TG males.

Embryo Analysis

Embryos were generated by mating hemizygous TG males with virgin FVB/N females. Each male was mated with one female at any given time. Mated females were examined each morning and afternoon for the presence of a coitus plug as per Hogan et al., (1994). Females presenting with a coitus plug in the afternoon were designated as 0 d.p.c. and those presenting in the morning were designated as 0.5 d.p.c. Females which had copulated were removed from the presence of the male and housed individually until embryos were collected.

Pregnant females were sacrificed by CO_2 asphyxiation at 9.5, 11, 11.5, 12, 12.5, 13, 15, 16, and 17.5 d.p.c., and the embryos surgically removed. The viability of each embryo was confirmed by morphological examination, observation of heartbeat (11-16 d.p.c.) and by response to tail pinch (16 & 17.5 d.p.c.). The VYS was removed from each embryo as a source of template DNA for later PCR analysis.

Postnatal Analysis

Pups used for sex ratio determinations were generated by mating homozygous TG males with wild-type FVB/N females. Females were examined daily for the presence of a coitus plug and those presenting with a plug were housed alone for the duration of the pregnancy and monitored twice daily as they approached term.

Pups used for sex ratio determinations were examined for viability and the presence of milk in their stomachs shortly after birth and were collected from brood cages two days after birth. The pups were sacrificed by CO_2 asphyxiation, and sexed by direct anatomical observation of the gonads. Tail samples (5-10 mm) were collected at this time as a DNA template source for PCR analysis. A tissue sample for PCR analysis was taken from all pups which died prior to P2.

Statistical Analyses

All nominal scale data were tested against predicted genotypic frequencies using the Chi-square statistic calculated with Statistica 6.0 (StatSoft) and are presented in tabular format. The expected frequency for all same sex transgenic progeny v. wild-type progeny was calculated as a 1:1 ratio based upon the observation that the PAC-FMR1 transgene is localized to an autosome. The expected sex ratio for the FVB/N strain of 52% female to 48% male was taken from a reproduction study performed on the FVB/N strain by Taconic Farms, Inc. (Germantown, NY) which they graciously supplied at our request.

RESULTS

Transgene Segregation in utero

Transgene induced mishaps during gametogenesis or the death and subsequent absorption of embryonic mice could result in the observed deficit of TG females and would not have been otherwise observed during the standard course of animal husbandry. Furthermore; during the initial matings between TG females and wild-type FVB/N males (Figure 5), complete data pertaining to all potential genotypic frequencies were not collected. At that time, the incidence of wild-type male progeny was not recorded. It was thus not known whether TG males were under represented relative to wild-type males. By monitoring the genotypic frequency of embryos produced by matings between hemizygous TG males and wild-type FVB/N females (Figure 7), we anticipated the opportunity to directly observe the loss of either TG genotype during an early stage. Since embryos were collected at several time points, we expected any aborted embryos to be apparent. Very few aborted embryos were observed and the majority appeared to be decidual reactions and, being of maternal origin, could not be scored for genotype.

A total of 172 embryos consisting of 39 WT/PAC, 45 WT , 41 WT/WT/PAC, and 47 WT/WT were collected and genotyped. Chi-square analysis (Table 2) indicates that these values do not differ from expected values by a statistically significant degree: d.f. = 3; $\chi^2 = 0.887$; $p \leq 0.827$. These data indicate that survivorship of TG mice is not affected *in utero*, and suggest that any possible influence of the FMR1 transgene on spermatogenesis does not extend to the differential production of viable transgenic zygotes.

Transgenic Females are Underrepresented in Backcross Generations N1-N8

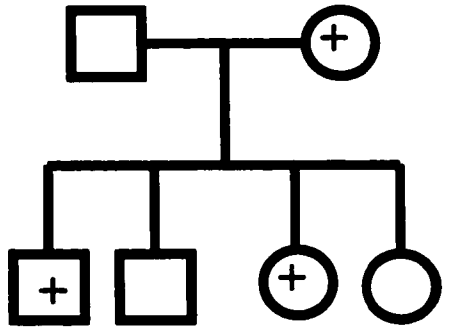


Figure 6. Pedigree schematic representing ratio of TG females to wild-type females born through backcross generation N8. Males are denoted by squares and females are denoted by circles. The '+' symbol denotes the presence of the FMR1 transgene (TG).

Chi-Square Analysis: Transgenic Females are Underrepresented Relative to Wild-Type Females

Genotype	Observed (O)	Expected (E)	$(O-E)^2$	$(O-E)^2 / E$
Wild-type Females	48	37	121	3.27
TG Females	26	37	121	3.27

Total (O) = 74

$\chi^2 = 6.54$

d.f. = 1

$p \leq 0.0105$

Table 1. The difference between the observed ratio of transgenic females to wild-type females differed significantly form the expected ratio of 1:1. d.f. = 1; $\chi^2 = 6.54$; $p \leq 0.0105$.

Design Schematic for *in utero* Analysis of Transgene Segregation

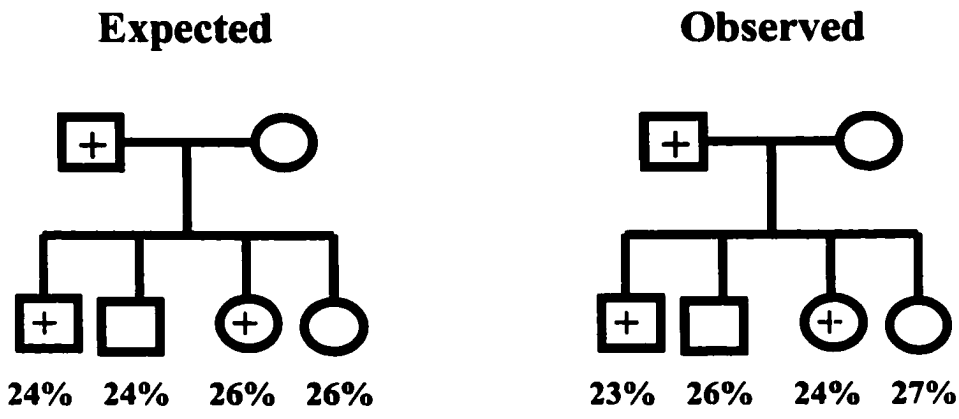


Figure 7. Pedigree schematic representing mating scheme, expected Mendelian ratios, and observed genotypic ratios for segregation of the FMR1 transgene *in utero*. Males are denoted by squares and females are denoted by circles. The '+' symbol denotes the presence of the FMR1 transgene (TG).

Chi-square Analysis: Observed Genotypic Frequencies for all Embryos

Genotype	Observed (O)	Expected (E)	$(O-E)^2$	$(O-E)^2 / E$
TG + Males	39	41.28	5.20	0.126
TG - Males	45	41.28	13.84	0.335
TG + Females	41	44.72	13.84	0.309
TG- Females	47	44.72	5.20	0.116
Total (O) =	172			$\chi^2 = 0.887$
		d.f = 3		$p \leq 0.827$

Table 2. A statistically significant difference between the observed and expected genotypic frequencies was not observed. Expected values assume random segregation of transgene and a sex ratio for FVB/N strain of: F 52%; M 48%. d.f. = 3; $\chi^2 = 0.887$; $p \leq 0.827$. The data indicate no reduction in the number of male or female carriers of the transgene.

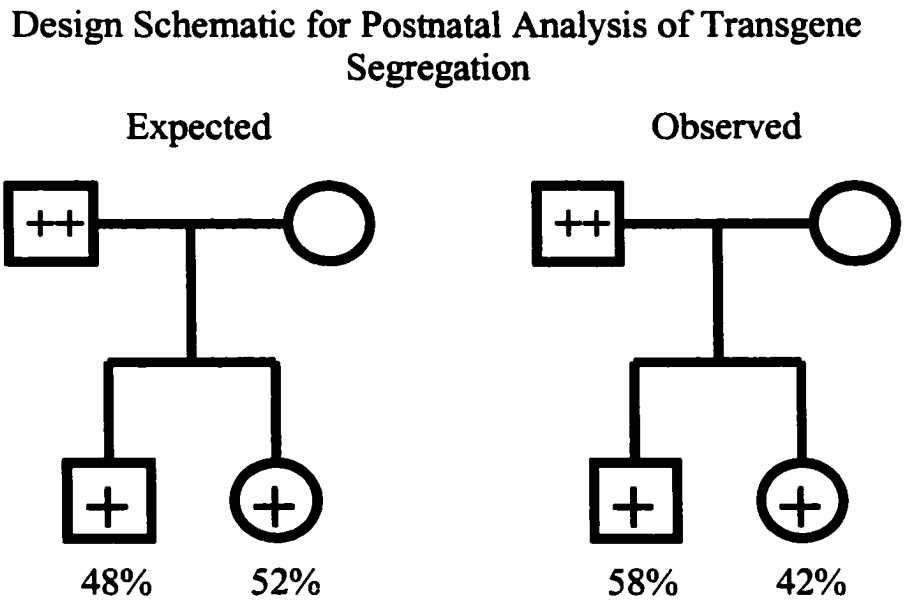


Figure 8. Pedigree schematic representing mating scheme for postnatal analysis, the expected Mendelian ratios, and observed genotypic ratios for segregation of the FMR1 transgene. Males are denoted by squares and females are denoted by circles. The '+' symbol denotes the presence of the FMR1 transgene (TG).

Chi-Square Analysis: Observed Frequencies of Male and Female Carriers of the FMR1 Transgene (Male TG Donor)

Genotype	Observed (O)	Expected (E)	$(O-E)^2$	$(O-E)^2 / E$
TG+ Male	109	90.24	351.94	3.90
TG+ Female	79	97.76	351.94	3.60
Total (O) = 188			d.f. = 1	$\chi^2 = 7.5$ $p \leq 0.006$

Table 3. Chi-Square analysis of male and female transgenic pups indicates a statistically significant difference in the sex ratio as a result of female lethality. d.f. = 1; $\chi^2 = 7.5$; $p \leq 0.006$. Expected values assume a sex ratio for FVB/N strain of: F 52%; M 48%. The mating regimen employed homozygous TG males as the parent of origin for the transgene.

Several pups were found dead in the birthing cages within 12-24 hours of birth. While many of these had been cannibalized to the point where sex determination was impossible or were so decayed that DNA isolation failed to yield suitable template for PCR analysis, a number were recovered nearly whole. Ten such pups were recovered. Seven of these were female, and none were found to possess any stomach contents indicating that they had died prior to the onset of suckling. Chi-Square analysis (table not shown) of the sex ratio fit for the summed count of live and dead pups does not, however, indicate a full recovery of the normal sex ratio when the dead specimens are included: d.f. = 1; $\chi^2 = 5.8$; $p \leq 0.016$.

A decrease in the frequency of TG females was observed when homozygous TG females were mated with wild-type FVB/N males (Figure 9) but was not shown to be statistically significant (Table 4). Of the 84 progeny scored, 46 (54.8%) were male and 38 (45.2%) were female. While a reduction of TG females was observed, it was not shown to be statistically significant.

Design Schematic for Postnatal Analysis of Transgene Segregation:
Parent of Origin Effects

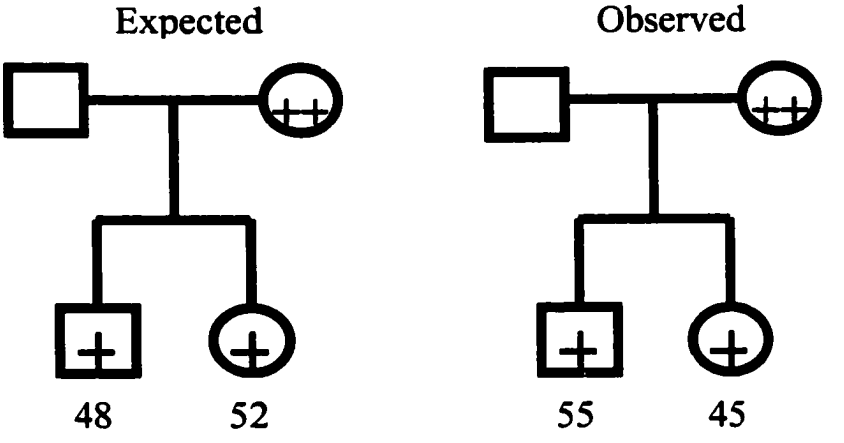


Figure 9. Pedigree schematic representing mating scheme for postnatal analysis of parent of origin effects, the expected Mendelian ratios, and observed genotypic ratios for segregation of the FMR1 transgene. Males are denoted by squares and females are denoted by circles. The '+' symbol denotes the presence of the FMR1 transgene (TG).

Chi-Square Analysis: Observed Frequencies of Male and Female Carriers of the FMR1 Transgene (Female TG Donor)

Genotype	Observed (O)	Expected (E)	$(O-E)^2$	$(O-E)^2 / E$
TG+ Male	46	40.32	32.26	0.80
TG+ Female	38	43.68	32.26	0.74

Total (O) = 84

$\chi^2 = 1.54$

d.f. = 1

$p \leq 0.214$

Table 4. Chi-Square analysis of male and female transgenic pups does notindicate a statistically significant difference in the sex ratio as a result of female lethality. d.f. = 1; $\chi^2 = 1.54$; $p \leq 0.214$. Expected values assume a sex ratio for FVB/N strain of: F 52%; M 48%. The mating regimen employed homozygous TG females as the parent of origin for the transgene.

CONCLUSIONS

These findings suggest that the presence FMR1 transgene is not detrimental to the viability of the embryo. This conclusion; however, must be regarded with a degree of reserve. The *in utero* sampling performed in this study is a measure of embryo viability through embryonic day 17.5. This is not a complete survey of the 19 day gestational period, and embryonic viability is not directly tested during the final 36 hours of fetal development. Yet it is clear, that at some point in the immediate perinatal period, a significant portion of TG females is lost. During the observed period of embryonic development, approximately 51% of the viable transgene carrying progeny were determined to be female. By the end of the first 24 hours after birth only 42% of the surviving TG progeny were female.

Embryonic development during the last 36 hours of gestation involves, among other things, the modification of organ systems vital to life outside the womb. The cardiovascular system is already well developed by 17.5 d.p.c. and begins the final reorganizations for pulmonary circulation within hours before birth. The remnants of the fetal architecture of the heart, i.e. the foramen ovale and ductus arteriosus, disappear shortly after birth. The respiratory system undergoes extensive ramification during this period. At this time alveolar proliferation and vascularization proceed rapidly toward the development of the functional postnatal lung. While the brainstem regulatory systems for the control of cardiac and respiratory function are in place, barring any postnatal refinements of cortical projections, the major part of their necessity to function in a dynamic environment is likely buffered by maternal life support.

At the time of birth these systems have in place the prior development necessary to carry out coordinated function, and developmental abnormalities might only then affect survivorship. Thus, the postnatal environment may be seen to possess more rigorous requirements for the maintenance of viability than does the late prenatal environment. Given this observation, an investigation with the design of the *in utero* study presented above directed toward the late gestational period could elucidate this issue with a high resolution survey of TG female survivorship during late embryogenesis.

Expression of the FMR1 Transgene

INTRODUCTION

One aspect of our goal toward the characterization of the FMR1-FVB/N transgenic strain is to assess the degree to which the transgene is expressed. We sought to compare FMRP/*FmrP* levels in hemizygous TG mice with wild-type FVB/N mice. We also had available an *Fmr1* knockout (KO) strain (Bakker et al., 1994) which was bred onto the FVB/N genetic background. With this strain, we had begun breeding hemizygous TG mice which were homozygous for the KO allele for the purpose of measuring FMR1 specific expression. Such specimens were deemed necessary for the determination of FMR1 specific expression since the only commercially available antibody against FMRP is crossreactive with the *murine* protein. Given the neurological dysfunction present in humans with FXS we chose the brain as the target tissue for protein analysis. In addition to providing one element in the characterization of the FMR1-FVB/N strain, we planned to use protein measures in neonatal brain to test the specific hypothesis that the mortality of TG females is due to the overexpression of FMRP/*FmrP* in the TG female brain relative to unaffected genotypes.

We examined FMRP/*FmrP* levels in the neonatal brain by western blot and found that TG females possess total FMRP/*FmrP* levels which are approximately 10-15 times that wild-type mice. However, protein levels in male and female TG mice have no

statistically significant difference, nor is there any such difference between carriers of the FMR1 transgene which differ in having a wild-type or *Fmr1* KO background.

METHODS and MATERIALS

Brain Dissection

All brains were collected from healthy, neonatal pups which were less than 18 hours old, of normal appearance, and were found to have milk in their stomachs. Upon removal from the birthing cage, pups were rapidly ($\leq$ 3 min.) transferred to a pre-warmed cardboard box and maintained at a temperature of 37°C. Pups were anesthetized by cooling on ice and sacrificed by decapitation. Whole brains were removed and snap frozen in liquid nitrogen until processed for protein extraction. Brain dissections were carried out at 0°C in CMF-PBS. Tail samples were collected from each pup at this time to serve as a PCR template source for genotype determination and were processed as described above (Chapter 3).

Protein Extraction

Whole brains were removed from liquid nitrogen, weighed and immediately placed on dry ice. Each tissue sample was homogenized in five volumes of an extraction buffer containing: 20 mM Tris-Cl, pH 7.5; 1 mM EDTA; 80 mM Beta-phosphoglycerate; 50 mM Sodium Fluoride; 0.5% Sodium deoxycholate; 1 mM Sodium Orthovanadate; 1 mM PMSF; 10 ug/ml Aprotinin; 10 ug/ml Leupeptin; 1% NP-40 and 0.1% SDS. Homogenization was performed on wet ice using a motor driven, Teflon pestle and a smooth glass Dounce with a clearance of between 0.08 and 0.15 mm. All samples were allowed to thaw for five minutes on ice in extraction buffer prior to homogenization.

Lysates were then centrifuged for 30' x 16,000g at 4°C to pellet nuclei, and the cleared lysates were frozen at -70°C for storage.

SDS-Polyacrylamide Gel Electrophoresis and Electroblot Transfer

The protein concentrations of cleared lysates were determined by colorimetric analysis using the Pierce, BCA Protein Quantification Kit as per the manufacturer's instructions. Lysates were measured in duplicate, and all quantifications were performed in parallel with BSA standards diluted in the extraction buffer.

Electrophoresis was performed essentially as described in Gallagher et al., (1997a;b). Protein samples were diluted with extraction buffer to a concentration of 1.0 ug/ul for FMRP/*FmrP* analysis and 2.0 ug/ul for $GABA_AR$ analysis. 15 ul samples were vortexed briefly, and boiled for 3.5 minutes in one volume of a β-mercaptoethanol loading buffer (0.125 M Tris-Cl, pH 6.8; 4% SDS; 20% Glycerol (v/v); 1.33M β-mercaptoethanol; 0.02% Bromophenol Blue), then chilled on ice for 5 minutes. Samples were electrophoriesed for 45 minutes through a 1.5 cm 3.9% acrylamide (37.5:1 acrylamide:bis-acrylamide) stacking gel at 80 V in a Tris-Glycine buffer system. Proteins were then separated for approximately 2 hours at 120 V in a 6 cm 10% acrylamide (37.5:1) resolving gel. All gels were cast to a thickness of 1.5 mm. Proteins were transferred to Millipore, Immobillon-P PVDF membranes using a Hoefer Electroblot Transfer Apparatus. The transfer was carried out in an alkaline, CAPS/methanol buffer (2.213 g/l CAPS, pH 11.0; 10% methanol) for one hour at 400 mA.

ml solution/membrane and were performed on a rotary shaker. Membranes were removed from the alkaline phosphatase wash solution and incubated for 5 minutes, at room temperature, in the dark, in a 1:500 dilution of the CDP-Star reagent in the manufacture's reaction buffer. Membranes were then placed into transparent, acetate folders and chemiluminescence was recorded with a digital camera.

Densitometric Analysis

Digital images were analyzed using Aida densitometry analysis software (Raytest). Immunoreactive bands were demarked within rectangular analysis regions and background noise was subtracted from each individual band by the perimeter background subtraction method. Occasionally, the perimeter background fields overlapped adjacent bands. In these cases the regions selected for background determination were rectangular areas spanning the breadth of the gel and located immediately above or below the immunoreactive areas. Signal intensities for FMRP, *FmrP* and $GABA_AR$-β were all normalized to the sample's β-actin signal by the following equation:

Target signal [background adj.] / Lane β-actin signal [background adj.]

Sample Sizes and Statistical Analysis

Sample availability allowed the following sample number (n) and replicate (r) regimen for $GABA_AR$-β analysis:

KO/KO n = 3, r = 2,4,4; KO/KO/PAC n = 5, r = 2; WT/WT n = 3, r = 4,3,3;
WT/WT/PAC n = 4, r = 3,3,2,2; WT n = 3, r = 4,4,2; KO.PAC n = 5, r = 2 KO n = 3, r = 4,4,2.

For FMRP/*FmrP* analysis, two replicates were performed for each of three brains of each genotype.

Data Analysis

Overall genotype effects on protein expression were determined by main-effects analysis of variance. Specific differences between genotypes were revealed by post hoc comparisons of means using the Tukey HSD test. All analyses were performed using Statistica 6.0 (StatSoft).

RESULTS

FMRP/FmrP Expression in Neonatal Mice

Protein extracted from whole brains harvested from neonatal pups was processed by western blot and immunologically probed for FMRP/*FmrP* using the monoclonal antibody MAB2160 (Chemicon). Quantitative analysis was performed on male and female specimens from wild-type and *Fmr1* KO backgrounds, and included specimens which were either hemizygous for the FMR1 transgene or did not carry the TG allele. β-actin was used as an internal control for sample loading in all analyses. Due to the high levels of expression seen in mice harboring the TG allele, the maximum quantity of total protein in each sample was limited to 15 ug to avoid signal saturation. While this proved adequate for comparisons between mice which carried the TG allele and wild-type mice, this low level of sample did not allow a direct analysis of the TG(-)/KO animals. MAB2160 exhibits crossreactivity with the FXR proteins (Peier et al., 2000), thus it is possible that this effect proved limiting with our low protein assay paradigm. *Fmr1* KO animals are not included in the FMRP/*FmrP* analyses presented.

Total FMRP/*FmrP* levels in all TG carriers tested were shown to be present at levels which were 10-15 times greater than that of wild-type animals (Figure 10; Table 6). Given the previously mentioned caveat regarding MAB2160 crossreactivity, this may be a slight underestimation of the TG associated increases in protein level. This increase in protein level is shown to be statistically significant by analysis using main-effects anova (Table 5) and a post hoc Tukey HSD test (Table 6).

ANOVA: Genotype Effect on FMRP/FmrP Expression

GENERAL EFFECT	SS	Degr. of Freedom	MS	F	p
Intercept	23.056	1	23.056	138.947	0.00001
Genotype	9.684	5	1.937	11.673	0.00063
Experiment	5.648	5	1.130	6.807	0.00039
Error	4.148	25	0.166		

Table 5. Analysis of variance illustrating statistically significant effects of neonate genotype on FMRP/*FmrP* levels in brain.

Post-Hoc Analysis of Means: Genotype Effect on FMRP/FmrP Expression

Genotype	KO/KO/PAC (1.056)	WT/WT (0.093)	WT/WT/PAC (1.412)	WT/PAC (1.218)	WT (0.103)	KO/PAC (0.919)
KO/KO/PAC	NA	0.004*	0.779	0.996	0.005*	0.999
WT/WT	0.004*	NA	0.0002*	0.0007*	1.00	0.021*
WT/WT/PAC	0.779	0.0002*	NA	0.989	0.0002*	0.411
WT/PAC	0.996	0.0007*	0.989	NA	0.0007*	0.893
WT	0.005*	1.00	0.0002*	0.0007*	NA	0.023*
KO/PAC	0.999	0.021*	0.411	0.893	0.023*	NA

Tukey HSD Test
Error: Between MS = 0.327; d.f. = 30

Table 6. Crosswise comparison by genotype. Parenthesis in upper row indicate group means. All comparisons yielding alpha values below 0.05 are indicated with an asterisk. Self comparisons are designated non-applicable (NA).

Immunological Characterization of $GABA_AR$-β Expression

Introduction

In addition to measuring FMRP/*FmrP* levels, we measured protein levels of the $GABA_A$ receptor β-subunit ($GABA_AR$-β) in neonatal brain. ElIdrissi et al., (in preparation) have demonstrated a reduction in the levels of $GABA_AR$-β expressed in the neocortex, hippocampus and brainstem of adult *Fmr1* KO mice. Given the disruption to $GABA_AR$-β levels seen in the absence of *Fmr1* gene product, we sought to examine whether the expression of the human FMR1 transgene had an influence on the expression of this receptor, and potentially link that effect with TG female mortality.

The GABAergic system functions as one of the primary excitatory neurotransmitter systems in brain during the developmental period during which TG females suffer mortality, and is involved in the early establishment of synaptic connectivity. Disruptions to brain development at this time and prior to this time might be expected to have an adverse effect on brain function and survival outside the womb. Our data did not indicate any statistically significant differences in $GABA_AR$-β levels between any of the genotypes tested, yet this must be assessed in conjunction with a potentially confounding observation of a large degree of within group variation in target protein levels.

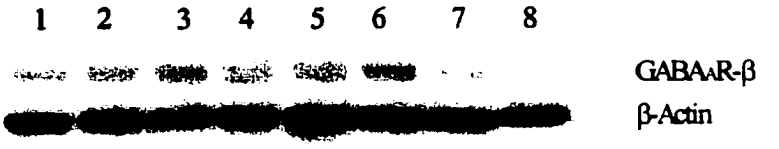
Results

$GABA_AR$-β Expression in Neonatal Brain

Neonatal levels of $GABA_AR$-β were analyzed in a manner similar to that used for FMRP analysis (see Methods and Materials, Chapter 4) and all eight genotypes available were included. Protein levels were shown to slightly lower in females hemizygous for the FMR1 transgene than was the case for wild-type females or TG males (Figure 11a). However, wide variation existed between individuals within each genotype group. Statistical analysis by analysis of variance do not indicate that $GABA_AR$-β levels differ by a statistically significant degree in any of the mice regardless of transgene carrier status, sex or the presence of the *Fmr1* KO allele (Table 7).

$GABA_AR$-β Levels in Neonatal Brain

A



B

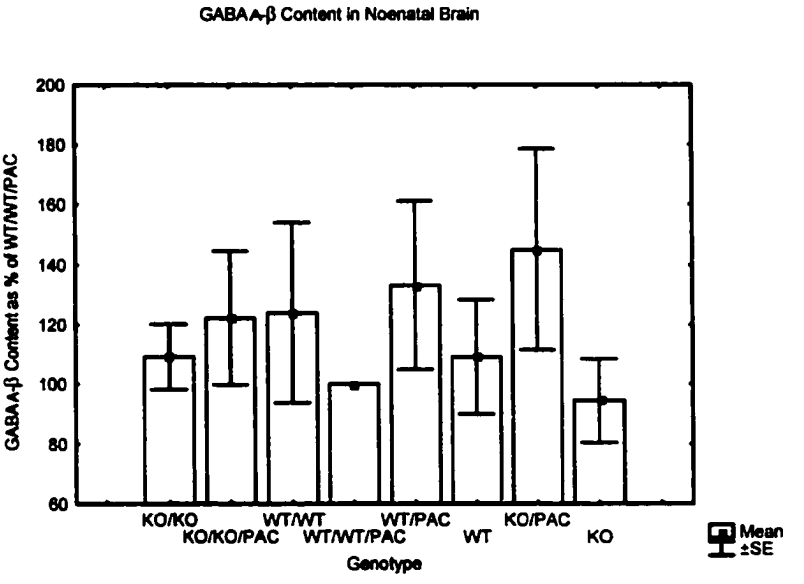


Figure 11. A) Western blot probed for $GABA_AR$-β and β-actin. The samples in lanes 1-6 follow the genotype order presented in *B*. B) Plot represents the relative levels of FMRP/*FmrP* in neonatal brain. Values are derived from pooled immunoblot analyses and are normalized to the TG female. The presence of the transgene is designated by (PAC). Wild-type or knockout *Fmr1* alleles are indicated by (WT) and (KO), respectively. Females are represented by the three leftmost plots and males by the three right plots.

ANOVA: Genotype Effect on $GABA_AR$-β Expression

GENERAL EFFECT	SS	Degr. of Freedom	MS	F	p
Intercept	63.855	1	63.855	652.635	0.00001
Genotype	0.904	7	0.129	1.320	0.258
Error	5.479	56	0.098		

Table 7. ANOVA demonstrate no between group difference in the levels of $GABA_AR$-β in neonatal brain.

The high degree of variation observed between individuals of the same genotype is possibly due to the assay paradigm used for immunoblot evaluation. An effort was made to assay protein samples which contained the minimum possible amount of total extracted protein. This was deemed necessary to allow the simultaneous measurements of $GABA_AR$-β target signal and the β-actin control signal. Since both the β-actin bands and the $GABA_AR$-β were visualized simultaneously, the length of time each blot was exposed for photography was determined by the signal saturation of the stronger of the two bands; the β-actin band. This strategy proved adequate for the quantification of FMRP levels. However, β-actin signal approached saturation very rapidly when an amount of total protein equaling 30 ug was processed by western blot. This value was chosen as the maximum quantity which would be processed in the $GABA_A$-R-β assays. We believe that this methodology returned a $GABA_AR$-β target signal which was not

sufficiently powerful, for the limited camera exposure period, relative to the levels of the background noise inherent in our chemiluminescent visualization procedure. It should be possible to mitigate this problem by implementing the following changes to the experimental methodology.

Our method involved probing western blots with a primary antibody solution which contained a mixture of two antibodies. We included the anti-β-actin antibody in the solution with the anti-$GABA_AR$-β antibody. This forced the simultaneous visualization of both targets, and may have served to reduce the absolute intensity of the $GABA_AR$-β signal. According to instructions provided by Amersham Life Sciences, the use of single antibody probe solutions on reinforced nitrocellulose membranes followed by target visualization, probe removal, and subsequent re-probing steps for β-actin will enhance the signal of individual target proteins. This modification would allow an extension of the exposure time during the $GABA_AR$-β visualization sequence as the β-actin signal would not be present as a limiting factor. Furthermore, an increased amount of total protein could be loaded for the SDS-PAGE sequence of the assay, thus enhancing the signal to noise ratio. The β-actin assay sequence would not necessarily be affected by the increased amount of protein loaded for SDS-PAGE since the amount of chemiluminescent substrate applied during the second visualization phase of the assay could be reduced to accommodate the increased β-actin signal strength which would accompany the increase in protein loading. The net effect of these modifications would be to increase the relative signal intensity for $GABA_AR$-β with the likely result that a reduction of within group variation would follow. The consequent reduction of within

group variation ought to allow a semi-quantitative comparison of $GABA_AR$-β levels which approaches the reliability of the analysis of FMRP expression.

DISCUSSION

The previous chapters detail our characterization of the FMR1-FVB/N transgenic mouse. The work which is presented arose from data gathered during our initial efforts to produce a congenic strain which carried the FMR1 transgene in the FVB/N genome. During the early course of this breeding program, we observed what seemed to be a deficit in the proportion of transgene carrying females among the progeny of our inbred line. A closer examination of this phenomenon revealed the reduction in TG female progeny to be statistically significant. We resolved to characterize this phenomenon, and have determined the developmental period during which a significant portion of TG females suffer a lethal effect from the human FMR1 transgene. By tracking the survivorship of TG females from mid-gestation through postnatal day 2, we have established that mortality occurs during the perinatal period with the majority of TG females dying within 24 hours of birth. The information provided by these studies is, of course, limiting with respect to the causality of TG female lethality. We therefore examined the hypothesis that, during the early postnatal period, FMRP/*FmrP* levels in TG female brain differed from those in wild-type females.

Immunoblot analysis of FMRP/*FmrP* levels indicated a 10-15 fold increase of protein expressed in TG female brain relative to wild-type female brain. These analyses also included the measurement of protein levels in male TG and wild-type mice as well as male and female mice which harbored the FMR1 transgene but carried a knockout mutation in the *murine Fmr1* allele. All mice carrying the FMR1 transgene were shown

to express FMRP at high levels relative to wild-type mice, yet FMRP levels did not differ by a statistically significant degree between any of the transgene carriers. Of the transgene carrying genotypes, TG males had been rigorously assayed for survivorship and they were shown not to be adversely affected by the transgene.

These findings provoke questions concerning the nature of the difference between TG females and wild-type females which might underlie TG female mortality, and what condition exists for TG males which, apparently, makes them refractory to this perinatal mortality. These are broad questions, yet they address two specific categorical issues. In the first case, TG females differ from their wild-type counterparts only in that they harbor foreign genetic material in the form of a transgene. This case suggests a transgene specific cause for TG female mortality, but it is complicated by the second case. TG males do not appear to suffer from perinatal mortality. Thus, the presence of the FMR1 transgene becomes a necessary but not sufficient explanation for the perinatal mortality evidenced by TG females; the pathology itself is as yet unexplained.

We observe that while TG females express the FMR1 transgene to a much higher degree than wild-types express the endogenous *Fmr1* gene; TG males do so as well. Since TG males do not appear to be affected by a cost to early survivorship, one of two phenomena would seem to underlie TG female mortality. It is either the case that the differences in physiology between males and females results in different effects secondary to transgene expression and the female specific secondary effects incur a cost to survivorship or, that the effects downstream of transgene expression are similar yet spare males due to some feature of male physiology.

It is known, for instance, that sex specific morphometric and functional dimorphisms exist in several of the hypothalamic nuclei (Searles et al., 2000). While the best characterized of these are studied in adults with respect to behaviors such as reproductive activity and differential seizure thresholds, nearly all are associated with dimorphisms in the GABAergic system (Nett et al., 1999; Searles et al., 2000). Though $GABA_AR$-β is downregulated in the KO mouse, we can only speculate on the involvement of FMRP in these dimorphic areas. It is interesting to note, however, that in rodents the sexual dimorphisms of the hypothalamic nuclei are established by steroid hormone action during the immediate perinatal period, and GABAergic sexual dimorphisms in the striatum are established during mid to late gestation (Ovtscharoff et al., 1992). Thus there are sexual dimorphisms in the brain which are generated during the developmental period associated with TG female mortality, and these dimorphisms are expressed in a neurotransmitter system which is linked to *FmrP* function.

This relates to a fundamental question raised by our research; does FMRP have a different developmental function in females than in males? It is not intended that this question include gross dimorphic effects e.g., the male phenotype macroorchidism of FXS but rather, more cryptic effects regarding the development of the central nervous system. Of certain relevance to this question is the finding that TG female mortality likely occurs shortly after birth. This suggests that a portion of TG females fail to develop the physiology required to meet the specific demands of survival without maternal life support. Concerning this specific issue, a confirmation of TG female survival during the last 36 hours of gestation using the design presented in Chapter 3 is necessary. Were this to be confirmed, it would be reasonable to hypothesize that some form of dysgenesis in

neural substrates such as those for suckling behavior, cardiorespiratory function or distress vocalization might be present in TG females. Substantial deficits in any of these behaviors will affect early postnatal survival and mild deficits could, however, be survivable. This is not an exhaustive list of potential targets for the pathological effect of transgene expression but may serve as a design guide for further investigation into the etiology of TG female mortality. The specific issues to be examined must carry the reasonable expectation that they might result in postnatal mortality, but accommodate our finding that TG female lethality does not affect all representatives of the genotype.

If FMRP function is essentially the same in males and females, it would be a necessary observation that TG females exhibit a different pattern of transgene expression than males or that they exhibit a different quantitative effect on a system which is shown in males to be affected by FMRP/*FmrP*. A step toward addressing these questions is to examine an augmentation to the research we have already performed. Sex related differences in the secondary effects of transgene expression may exist at a level which was not examined by our design. It is possible that changes in the regulation of $GABA_AR$-β expression do exist but are not revealed by the present methodology. Further examination of this question is warranted. The observation that the absence of *FmrP* in the mouse affects $GABA_AR$-β expression provides a strong basis for the hypothesis that the overexpression of the human homolog would also affects $GABA_AR$-β levels. Further, it is unreasonable to ignore the importance of the GABAergic system to the developing nervous system. The technique modifications presented in Chapter 5 may reveal transgene effects on $GABA_AR$-β expression and could be augmented by a region specific analysis of protein levels in brain. The analysis of regional FMRP

expression may also reveal a difference between TG males and TG females which was masked by the use of whole brain as a tissue source for protein analysis. Furthermore, the examination of protein expression during the postnatal period may be an inadequate test with respect to these analyses. If it is the case that the *in utero* development of the brain is a critical feature of TG female mortality, TG males and females may only differ in transgene expression levels during embryogenesis.

Females express both *Fmr1* alleles during embryogenesis prior to X-inactivation. In the mouse brain, it is approximated that X-inactivation is not completed until embryonic day 12.5 (Tan et al., 1993). It is possible that total FMRP/*FmrP* levels differ between males and females during embryogenesis and converge by postnatal day 1. Considering the large difference in protein levels between transgenic mice and wild-type mice at P1, the potential difference due to pre-X-inactivation gene dosage might be presumed to be negligible. However, a technical issue must be addressed before such a conclusion is drawn as it may be the case that an overestimation of FMRP content is inherent in our assays. The antibody used during the immunoblot assays demonstrates crossreactivity with both FMRP and *FmrP*. This antibody, MAB2160, was developed using human FMRP as the inducing antigen, and may have a higher affinity for FMRP than for *FmrP*. At the present time, this question remains largely unanswered in the existing literature. This caveat may be addressed by performing SDS-PAGE and immunoblot signal quantification on purified protein samples. *FmrP* is available for isolation form wild-type animals and FMRP, as expressed in the mouse, is available from KO/TG animals. Should the signal strength differ between the two proteins in such an

assay, the ratio of the two signals may be applied as a scalar to FMRP/*FmrP* quantification assays and a true measure of transgene expression could then be obtained.

The assays just described address refinements to our general design for the examination of transgene expression. These refinements are specifically directed toward the goal of increasing the resolution of a search for differences in transgene expression patterns between TG females and TG males and toward a more rigorous examination of $GABA_AR$-β levels in the brain. The finding that FMRP/*FmrP* or $GABA_AR$-β levels do vary between male and female transgenics would direct the investigation into TG female mortality toward the specific brain structures or developmental periods wherein the differences were found. Such investigations may still fail to reveal the underlying cause of TG female lethality but, by including the KO mouse in the experimental designs as a complementary experimental subject, further knowledge is sure to be forthcoming regarding the function of FMRP in the central nervous system.

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