Mouse models
for the fragile X syndrome

Muismodellen
voor het fragiele X syndroom

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Promotor:	Prof.dr. B.A. Oostra
Overige leden:	Prof.dr. C.I. de Zeeuw Prof.dr. J.A. Grootegoed Dr. L.B.A. de Vries
Copromotor:	Dr. R. Willemsen

Omslag: Rob Wighman (illustratie) en Anneke van Kempen (vormgeving)

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Voor mijn ouders
Aan Hans, Cas en Maeve

Contents

Chapter 1

General Introduction

1.1 Clinical aspects of the fragile X syndrome

Fragile X syndrome is an X-linked human disease. The clinical manifestations were first described by Martin and Bell in 1943, and only in 1969 the association with a fragile site on the X chromosome was found (Lubs 1969). Culturing cells of patients in medium depleted of folic acid made the fragile site on the X chromosome visible in a proportion of the cells. In 1991 the gene and its gene defect involved in the fragile X syndrome were identified (Verkerk et al. 1991) and molecular diagnosis became available.
The main characteristic feature of the fragile X syndrome is mental retardation. In males the mental retardation can vary from mild to severe (De Vries et al. 1993). Unlike most X-linked disorders, also females can be affected. Fifty to 70 % of females carrying a disease-causing mutation also show mental impairment, although generally less severe compared to males with a disease-causing mutation (Rousseau et al. 1991a; Rousseau et al. 1991b; Smits et al. 1994; Taylor et al. 1994; De Vries et al. 1996b).
Patients may show behavioural abnormalities such as hyperactivity, decreased attention span and a number of autistiform features (Cohen et al. 1991; Fisch 1993). In approximately 20% of fragile X males epileptic seizures are observed (Hagerman 1996). Male patients show physical characteristics such as a long face and large everted ears. Macroorchidism (enlargement of the testes) is seen in most adult patients and develops mostly during and after puberty (reviewed in PhD thesis of De Vries, 1997) (De Vries et al. 1998).

1.2 The *FMR1* gene

1.2.1 The CGG repeat in the *FMR1* gene

The gene involved in the fragile X syndrome is designated ***F***ragile X ***M***ental ***R***etardation gene *1* (*FMR1*) (Verkerk et al. 1991) and is located on the X chromosome at q27.3. The gene spans 38 kb containing 17 exons (Eichler et al. 1994), including a polymorphic CGG repeat in the 5' untranslated region (Fu et al. 1991). Exons 12, 14, 15 and 17 show alternative splicing, generating a number of different mRNAs and protein isoforms (Ashley et al. 1993b; Verkerk et al. 1993). All elements necessary for proper *FMR1* expression *in vivo* are present in a 2.8 kb fragment including most of the 5' region of *FMR1* and the CGG repeat (Figure 1) (Hergersberg et al. 1995).
The polymorphic CGG repeat found in the normal population can vary between 5 and 50 units and has an average length of 30 CGG units (Fu et al. 1991). The repeat is transmitted to the next generation in a stable fashion and the gene is transcribed and translated into protein.

Affected individuals with the fragile X syndrome have a repeat containing more than 200 to several thousands of CGG units. This long repeat is called a full mutation. Generally the full mutation is methylated, as is the promoter region of *FMR1*. Due to this methylation, transcription is inhibited and no protein is produced. Absence the fragile X protein FMRP, or presence of the protein at very low levels, evolves in the characteristic features of the fragile X syndrome (Pieretti et al. 1991; Hansen et al. 1992; Sutcliffe et al. 1992; Verheij et al. 1993).

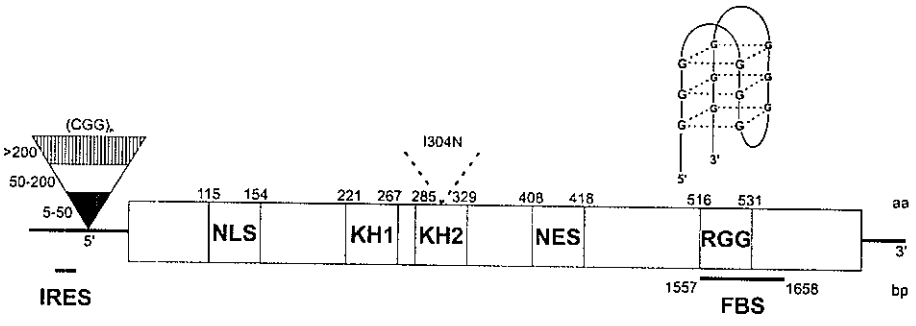

Figure 1. Schematic representation of the *FMR1* mRNA and protein, showing the known domains: IRES, internal ribosome entry site; NLS, nuclear location signal; KH, K-protein homology domain; NES, nuclear export signal; RGG box, Arg-Gly-Gly triplet; FBS, FMRP binding site. In addition, the I304N missense mutation found in a single, severely affected patient is indicated. In the 5'-UTR the different repeat classes are shown. The G-quartet structure present in the mRNA is depicted above the RGG box for which it encodes and to which it binds.

The alleles that contain a repeat of 50 to 200 CGG units are called premutation alleles. Upon transmission to the next generation, the premutation repeat may be instable. Only through maternal transmission, a premutation allele can expand to a full mutation allele. Increasing length of the premutation increases the chance of repeat expansion to a full mutation (Figure 2) (Devys et al. 1993; Feng et al. 1995a; Feng et al. 1995b; Bat et al. 1997; Tassone et al. 2000c).

High-end normal alleles form a fourth class of repeats. These repeats comprise 40 to 50 CGG units and are referred to as 'grey zone' alleles or protomutations (Oostra and Chiurazzi 2001). Upon transmission, these protomutations may increase or decrease a few CGG units in length, forming a group of alleles being at risk to become a premutation.

Most fragile X patients with a full mutation show, in different cells, different lengths of the CGG repeat, which is visible as a smear on a Southern blot. The presence of a premutation next to the full mutation repeat is found in 20% to 40% of the male patients, and is referred to as somatic 'length mosaicism' (Nolin et al. 1994; Rousseau et al. 1994). Since premutation alleles are normally transcribed and translated (Devys et al. 1993; Feng et al. 1995a) these mosaic patients show FMRP expression in some of their cells.

Nevertheless, their intellectual impairment seems to be similar to that found in males with a full mutation only (De Vries et al. 1993; Rousseau et al. 1994).
Almost all identified fragile X patients have an expanded CGG repeat. One severely affected male fragile X patient was found to have a point mutation in exon 10, leading to the substitution of the amino acid isoleucine to asparagine at position 304 (Ile304Asn or I304N) (Figure 1) (De Boulle et al. 1993). As is described later, this is a very conserved amino acid in an important domain of the FMR1 protein.

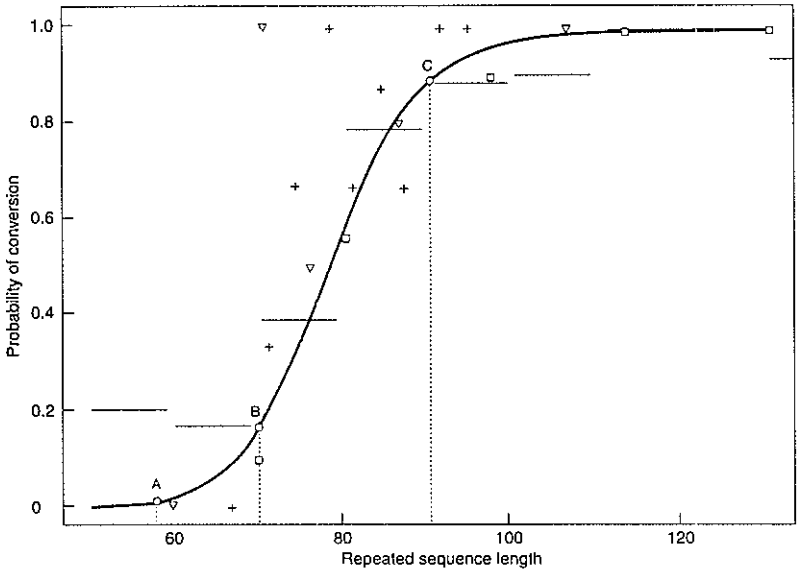

Figure 2. The probabilities of conversion from premutation repeat to full mutation repeat upon transmission from female carriers to children as a function of the repeat length in female carriers. Experimental data from (Fu et al. 1991)(+), (Heitz et al. 1992)(□), (Fisch et al. 1995)(-), data of Sherman listed in (Morris et al. 1995)(∇). The continuous line represents the fit to experimental frequencies obtained in (Morris et al. 1995). Points marked A, B and C correspond to the number of 58, 70 and 90 repeats, respectively, in premutation alleles. This figure is adapted from (Bat et al. 1997).

1.2.2 Inheritance

Remarkably, only transmission through the female germ line can give rise to a full mutation in the next generation. Transmission through the male germ line, irrespective whether the male has either a full mutation or a premutation in all his somatic cells, results always in a premutation in the daughters (Reyniers et al. 1993).
Oocytes from full mutation female foetuses have been shown to contain a full mutation without a detectable premutation (Malter et al. 1997). In male foetuses with a full

mutation in their somatic cells, the developing testes also show absence of FMRP in most cells. This is at week 13 of foetal development, whereas at week 17 some primordial germ cells are found to be positive for FMRP expression (Malter et al. 1997). After birth, FMRP expression in the testis becomes more prominent, as was found in a two-year old boy where approximately 50% of the immature germ cells are stained for the presence of FMRP (R. Willemsen, personal communication). Adult full mutation males show only a premutation in their sperm cells, while FMRP expression is seen in early spermatogonia only (de Graaff et al. 1995; Reyniers et al. 1993). The most likely model, explaining these findings, is the model in which the full mutation is transmitted in the oocyte and the mutation is regressed to a premutation size during spermatogenesis (Malter et al. 1997).
The somatic mosaicism often seen in patients, can be explained by instability that occurs postzygotically. Instability in the full mutation range can occur, but also regression of a full mutation to a premutation can occur in some cells in somatic tissues. For the male germ line, we have to assume that during testis development a regression of the full mutation takes place resulting in a premutation in sperm cells after puberty (De Graaff et al. 1996; Malter et al. 1997). Thus, in the male germ line a mechanism of selection, in favour of the premutation, takes place (De Graaff et al. 1996). This selection mechanism should be rather subtle and is not based on the presence or absence of FMRP, since complete absence of the protein, as is the case in the knockout mouse (Bakker et al. 1994) and a in family in which the promoter region is deleted (Meijer et al. 1994), reveals normal sperm maturation and normal fertility.

1.2.3 Methylation of *FMR1*

The presence of a full mutation repeat generally coincides with methylation of most cytosines in this full mutation CGG repeat and in the upstream CpG island of the promoter region. This methylation silences the transcription of *FMR1* resulting in the absence of its protein FMRP. The moment of methylation and inactivation of the *FMR1* gene appears to be after fertilisation during early embryonic development (Malter et al. 1997).
Next to 'length mosaicism' as described earlier, some individuals with a full mutation show 'methylation mosaicism'. A proportion of their leukocytes contains an unmethylated full mutation and FMRP can be detected in these cells.
Full mutation males with a high proportion (> 60%) of leucocytes with an unmethylated CGG repeat and promoter region have been reported (McConkie-Rosell et al. 1993; Hagerman et al. 1994; Smeets et al. 1995; De Vries et al. 1996a; Wohrle et al. 1996; Taylor et al. 1999; Tassone et al. 2000a). These males generally show a less severe phenotype, some are even normal, and they are called "high-functioning" males. They produce FMRP, but the number of cells that express FMRP is decreased compared to

normal males (Hagerman et al. 1994; Smeets et al. 1995; De Vries et al. 1996a; Tassone et al. 1999). Surprisingly the *FMR1* mRNA levels in these males were reported to be significantly elevated (Tassone et al. 2000a). An explanation for these elevated mRNA levels can be found in the hypothesis of a compensatory mechanism, which is described in Paragraph 1.3.3.

The question was put forward by Chiurazzi et al. whether demethylation of a methylated full mutation would result in reactivation of the *FMR1* gene (Chiurazzi et al. 1998; Chiurazzi et al. 1999). Demethylation studies would provide insight in the role of methylation in the fragile X syndrome and could be the basis for a therapeutic approach. Indeed, several *in vitro* studies confirm that demethylation of a methylated *FMR1* promoter reactivates the gene and leads to protein expression (Chiurazzi et al. 1998; Chiurazzi et al. 1999; Coffee et al. 1999). Methylation is playing a key role in inactivating *FMR1* and thus in development of fragile X syndrome.

1.2.4 Instability of the CGG repeat

Stability of the CGG repeat seems to be dependent on several factors. Concerning the structure, the length of the repeat is a risk factor for repeat instability (Eichler et al. 1994; Kunst and Warren 1994). Length variation is found at the 3' end of the repeat. Also the presence of interspersed AGG repeats seems to play a crucial role in repeat stability. In normal alleles, AGG repeat units are most often found downstream of 9 or 10 CGG units (Eichler et al. 1996). Loss of AGG interspersions leads to alleles with longer perfect CGG repeat tracts, which are prone to expand to a premutation repeat, especially when the number of pure CGG units is over 30. Also the number of AGG interspersions may be a risk factor (Nolin et al. 1999; Crawford et al. 2000). Premutation alleles have either no or only one AGG repeat unit and are therefore unstable. They may expand to a different sized premutation or to a full mutation (Oberlé et al. 1991). Perfect CGG repeats with 65 to 100 CGG repeat units have a high risk to grow exponentially upon maternal transmission to the next generation (Figure 2) (Devys et al. 1993; Feng et al. 1995a; Feng et al. 1995b; Bat et al. 1997; Tassone et al. 2000c).

Non-structural, *cis*-acting factors may be related to a specific haplotype background. Dombrowski et al. found, in a population-based study, that grey zone alleles or protomutations are often associated with a specific fragile X syndrome-related haplotype (Dombrowski et al. 2002). But, in a much smaller number of unstable events, other authors did not find evidence for a specific haplotype as a risk factor for instability (Sullivan et al. 2002).

Trans-acting factors might be involved in repeat instability (Nolin et al. 1996; Burman et al. 2000;). White et al. proposed the influence of RAD27, the yeast homolog of human FEN1, as a *trans*-acting factor on instability. RAD27 is necessary for correct processing

of Okazaki fragments during DNA replication and its absence strongly increases array instability (White et al. 1999).
Also, the parental origin of the transmitted repeat seems to be a risk factor for repeat instability (Sullivan et al. 2002). In the normal and protomutation repeat length range, paternally transmitted CGG repeats are less stable than maternally transmitted repeats. This is in contrast with CGG repeats in the premutation range, which are more unstable upon maternal transmission, while a paternally transmitted premutation repeat never expands to a full mutation. These findings suggest the involvement of different mutational mechanisms or different selection processes in males compared to females (Sullivan et al. 2002).
Studies in yeast and *E. coli* suggest that direction of replication, genetic background (including repair systems), transcription and growth conditions also have an influence on repeat instability too (Shimizu et al. 1996; Wells 1996; White et al. 1999).

1.2.5 CGG Repeat expansion

The molecular mechanism of CGG repeat expansion is still not clear. It is likely that a multistep process is involved (Morton and Macpherson 1992; Kolehmainen 1994; Richards and Sutherland 1994), since it has never been observed that a normal repeat expands to a full mutation repeat upon one transmission. One of the mechanisms that is assumed to be involved in the initial expansion of trinucleotide repeats is slipped-strand mispairing between DNA strands during DNA replication (Schlotterer and Tautz 1992) or possibly repair (Sinden 2001). Pausing of DNA polymerases allows the formation of secondary structures on the nascent lagging strand (Darlow and Leach 1998; Kang et al. 1995; Usdin and Woodford 1995), which, if not correctly excised, should result in small (up to 10 CGG repeat units) increase (Wells 1996).
For the expansion of premutation repeats to full mutation repeats it is thought that extremely unstable structures are formed in the nascent lagging strand due to slippage on both ends of Okazaki fragments when these fragments contain solely CGG repeats (Richards and Sutherland 1994). Incorrect repair will than lead to large expansions, but might also result in contractions or deletions.

1.3 Expression of the *FMR1* protein

The protein encoded for by *FMR1* is the **F**ragile X **M**ental **R**etardation **P**rotein (FMRP). Using Western blot analysis different splice variants of FMRP were recognised by specific antibodies. They have a molecular mass of between 70 to 80 kD (Verheij et al. 1993).

In human, *FMR1* expression levels and tissue distribution were studied using Northern blot analysis detecting mRNA (Hinds et al. 1993). Brain and testis showed the highest levels of mRNA expression. In lung, kidney and placenta *FMR1* expression was quite high and liver, pancreas and skeletal muscle showed low or no expression of *FMR1* (Hinds et al. 1993). Protein studies showed that FMRP is expressed predominantly in the cytoplasm of cells (Devys et al. 1993; Verheij et al. 1993), but *in vitro* experiments showed that some isoforms are found in the nucleus (Eberhart et al. 1996; Fridell et al. 1996; Sittler et al. 1996; Willemsen et al. 1996). High levels of FMRP expression were found in neurons of the brain, spermatogonia in the testis, and in connective tissues, particularly in epidermal layers which are actively dividing (Devys et al. 1993).
Expression of the *Fmr1* gene in mouse could be studied more extensively using tissues from mice in immunohistochemical studies and mRNA *in situ* hybridisation experiments (Devys et al. 1993; Hinds et al. 1993; Bakker et al. 2000a; De Diego Otero et al. 2000). *Fmr1* mRNA expression is turned on early in embryonic development and is high in all embryonic tissues. In successive stages of embryonic development, expression diminishes and in adult mice tissue-specific expression of *Fmr1* mRNA is found (Hinds et al. 1993). In protein studies using embryonic and adult mouse tissues the same expression pattern of Fmrp is found (Bakker et al. 2000a; De Diego Otero et al. 2000).
In adult mice, *in situ* mRNA studies showed moderate levels of *Fmr1* expression in kidney, liver, colon, uterus, thyroid, and lung. No *Fmr1* mRNA is found in muscle, heart or aorta. High levels of *Fmr1* mRNA expression are detected in adult brain, testes, ovaries, thymus, oesophagus and spleen (Hinds et al. 1993).
In mouse brain, Fmrp is most intensely expressed in the granular layers of the hippocampus, cortex, in motorneurons, and in Purkinje cells of the cerebellum (Devys et al. 1993, Hinds et al. 1993; Bakker et al. 2000a). High concentrations of Fmrp are found in the somata and proximal dendrites of neurons, but not in the axons (Devys et al. 1993; Verheij et al. 1993; Feng et al. 1997b; Tamanini et al. 1997). Using synaptoneurosomes from the cortex of the rat brain, *Fmr1* mRNA is also detected in the post-synaptic dendritic areas. Upon dendritic stimulation mRNA is translated into protein and the presence of Fmrp in this postsynaptic area suggests a function in the normal maturation of synaptic connections (Comery et al. 1997; Feng et al. 1997b; Weiler et al. 1997).
Fmrp expression is found in the cytoplasm of primordial germ cells of the immature testis and in early spermatogonia localised on the basal membrane of the tubuli seminiferi in the mature testis (Devys et al. 1993; Tamanini et al. 1997; Bakker et al. 2000a).

1.3.1 Localisation signals

In exon 14 of the *FMR1* gene, a sequence coding for a nuclear export signal (NES) was identified (Figure 1) (Fridell et al. 1996) and Tamanini et al. showed that the nuclear

export of FMRP is mediated via the exportin 1 pathway (Tamanini et al. 1999b). *In vitro* (over)expression studies with FMRP lacking the NES showed a strong localisation in the nucleus with exception of the nucleolus (Sittler et al. 1996; Willemsen et al. 1996).
FMRP also contains a nuclear localisation signal (NLS) between residues 115 and 154 (Figure1) (Eberhart et al. 1996; Bardoni et al. 1997) that directs the protein in an energy-dependent manner to the nucleus. So far, the nuclear import receptor for FMRP has not been identified.
The presence of both an NES and NLS in FMRP suggests that the protein can shuttle between the nucleus and the cytoplasm. Together with its function in mRNA processing it has been proposed that FMRP might play a role in specific mRNA export from the nucleus to the cytoplasm (Figure 3) (Feng et al. 1997b).
In *FMR1* mRNA, just upstream of the CGG repeat, the sequence of an internal ribosome entry site (IRES) has been identified (Figure 1). This element is thought to help promotion of translation in dendrites (Chiang et al. 2001).

1.3.2 RNA binding properties

Although the precise function of FMRP has not yet been completely elucidated, many characteristics have been identified. FMRP contains two hnRNP K homology domains (KH domains) in the middle part and an arginine/glycine-rich RNA-binding motif (RGG box) at the carboxy terminus (Figure 1). These domains have been described for proteins involved in RNA binding, and their presence in FMRP suggest that FMRP has RNA binding properties (Ashley et al. 1993a; Mattaj 1993; Siomi et al. 1993; Burd and Dreyfuss 1994).
KH domains have been found in single or multiple copies in different proteins. Their only common property is that they all function in close association with RNA. KH domain-containing proteins play a major role in regulating cellular RNA metabolism since they are capable to bind *in vitro* single strand RNA, messenger RNA, and ribosomal RNA.
RGG boxes also show RNA-binding activity and have been found in a considerable number of nuclear and nucleolar RNA-binding proteins (Kiledjian and Dreyfuss 1992). The RGG box of FMRP bears striking similarity to those found in the nucleolar protein fibrillarin (Siomi et al. 1993).
The RNA-binding domains in FMRP appear to be functional, since FMRP directly binds homopolymeric RNA (poly Y and poly G) *in vitro* (Siomi et al. 1993) and has selectivity for a fraction of mRNAs expressed in brain, including its own mRNA (Ashley et al. 1993a; Brown et al. 1998).
Interestingly, on the other hand the mutant FMRP with the Ile304Asn mutation, which is located in the second KH domain (Figure 1), has impaired RNA-binding capacity (Siomi et al. 1994; Verheij et al. 1995; Feng et al. 1997a). Evidence derived from structural

analysis indicates that this mutation disrupts the folding of one of the KH domains (Musco et al. 1996).

Another characteristic of FMRP is that the protein co-sediments with translating ribosomes (polyribosomes) on sucrose gradients (Khandjian et al. 1996), and immuno-electronmicroscopy analysis confirms the close proximity of FMRP to free ribosomes, membrane-bound ribosomes and polyribosomes (Willemsen et al. 1996; Feng et al. 1997b). The association of FMRP with ribosomes occurs via RNA-forming messenger ribonuclear protein (mRNP) particles (Eberhart et al. 1996; Tamanini et al. 1996; Corbin et al. 1997; Feng et al. 1997a).

Recent studies have shown that FMRP can suppress translation of bound mRNA in an *in vitro* translation assay, suggesting that FMRP may modulate mRNA translation and/or influence mRNA instability *in vivo* (Laggerbauer et al. 2001; Li et al. 2001). This inhibition of translation was found to be dosage-dependent (Laggerbauer et al. 2001). In this same assay the *FMR1* protein containing the mutation Ile304Asn failed to repress translation, most likely due to its inability to form homo-oligomers. Therefore also oligomerisation of FMRP is required for inhibition of translation (Laggerbauer et al. 2001).

Immunoprecipitation studies revealed several other proteins to be part of FMRP-containing mRNP particles (Ceman et al. 1999), including the proteins homologous to FMRP, namely FXR1P and FXR2P (Zhang et al. 1995; Ceman et al. 1999). Also nucleolin, a known component of mRNP particles, was identified (Ceman et al. 1999). Other proteins in the mRNP particles were found to be NUFIP1 (***NU***clear ***F***MRP ***I***nteracting ***P***rotein 1), which is a novel RNA binding protein (Bardoni et al. 1999), and CYFIP 1 and 2 (***CY***toplasmatic ***F***MRP ***I***nteracting ***P***rotein 1 and 2), both present in the synaptic terminals of neurons (Schenck et al. 2001). The identification of mouse Y box-binding protein 1/p50 as a part of the FMRP-associated mRNP particle suggests the involvement of FMRP in translation modulation (Ceman et al. 2000).

Recent reports on close examination of the RGG box region have revealed new insights in the function of FMRP. Although FMRP has a high propensity to interact with RNA in a non-specific manner, it is demonstrated that FMRP binds with its RGG box to an intramolecular G-quartet of its own mRNA (Figure 1). This binding is specific and with high affinity (Schaeffer et al. 2001). A 101-nucleotide fragment in the 3' end of the *FMR1* coding sequence was identified harbouring this G-quartet structure (***F***MRP ***B***inding ***S***ite, FBS) (Figure 1). This region codes for the RGG box in FMRP (Schaeffer et al. 2001). Binding of FMRP to its own mRNA could indicate that FMRP is involved in negative regulation of *FMR1* mRNA transcription or in controlling its breakdown (Schaeffer et al. 2001).

FMRP does not only bind its own mRNA, but also roughly 4% of other mRNA species. Using *in vitro* RNA selection and microarrays many of these mRNAs were identified

(Brown et al. 2001; Darnell et al. 2001). Nearly 70% of the identified mRNAs contain a G-quartet structure (Brown et al. 2001). A subset of these mRNAs shows an alteration in their polysome distribution in FMRP null cells. Several of these mRNAs encode proteins involved in synaptic function (for example *Munc13*) and neuritic extension and neuronal development (for example *MAP1B*) (Brown et al. 2001; Darnell et al. 2001). The altered polysome distribution in the absence of FMRP may transcriptionally dysregulate these mRNAs, which in turn has its effect on neuronal development and synaptic function. This model is supported by findings in the fruit fly *Drosophila melanogaster*. Inactivation of *dfxr*, the *Drosophila* homolog of the *FXR* genes (Wan et al. 2000), leads to dysregulation of the *futsch* gene. *Futsch*, the homolog of human *MAP1B*, is involved in development of the nervous system of the fly, and dysregulation of *futsch* results in defects in the nervous system (Zhang et al. 2001).

FMRP present in the mRNP particles might be involved in selecting certain mRNAs via binding of the G quartet structure and transporting them, in the neurons, into the dendrites to the postsynaptic site (Figure 3). Indeed, recent studies using PC12 cells showed that FMRP-containing mRNP particles are trafficking from the cell body of the neuronal cell to its dendritic-like extensions. This transport appears to be mediated via microtubules (R. Willemsen, personal communication). At the postsynaptic site, the mRNAs can be expressed in response to synaptic activity.

These findings suggest that failure of FMRP to transport and regulate expression of certain mRNAs plays a key role in pathogenesis of fragile X mental retardation.

1.3.3 Regulation of FMRP

Recent studies in premutation carriers showed that the length of the CGG repeat has an inverse correlation with the FMRP level, while it has a positive correlation with the *FMR1* mRNA level (Tassone et al. 2000b; Kenneson et al. 2001). Tassone et al. described premutation carriers with more than 100 CGG repeats having a reduced number of FMRP-positive cells, but with at least 5-fold elevated mRNA levels (Tassone et al. 2000b). These results suggest that the low or absent FMRP level is due to a post-transcriptional defect probably in the translation process (Tassone et al. 2000b). In an earlier study Feng et al. described transcripts with more than 200 CGG repeats being associated with stalled 40S ribosomal subunits (Feng et al. 1995b). In this way, the linear 40S migration along the 5'untranslated region (containing the CGG repeat) is impeded, resulting in translational inhibition.

These findings have led to the hypothesis of a compensatory mechanism. If the translation of *FMR1* mRNA is less efficient or defective, low FMRP levels initiate via feedback induction *FMR1* transcription. The resulting high *FMR1* mRNA level can only partially compensate the defect, since a normal FMRP level is not achieved in cells with large

premutation repeats (Tassone et al. 2000b; Kenneson et al. 2001). It is yet unknown whether the FMRP level itself is the crucial factor responsible for this compensatory mechanism, or whether it is coupled to the length of the CGG repeat (Tassone et al. 2000b; Kenneson et al. 2001).

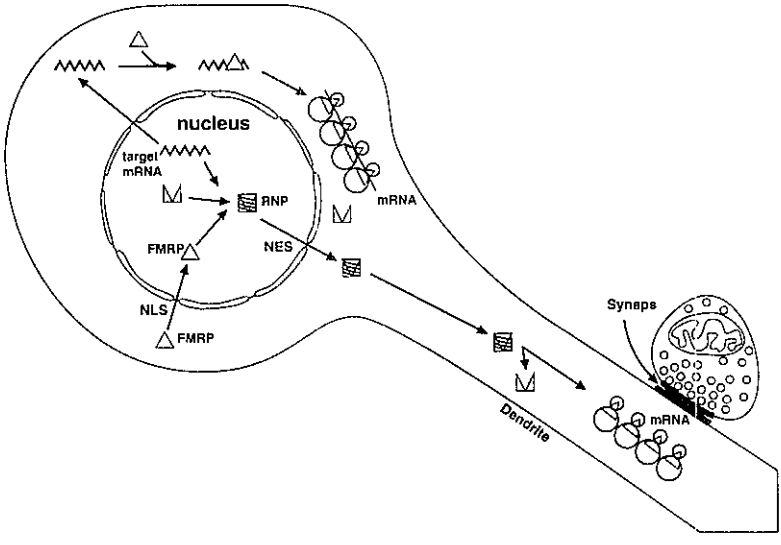

Figure 3. A hypothetical model for the function of FMRP. In normal individuals FMRP is transported to the nucleus and assembles into an mRNP particle, containing specific mRNAs and other unknown proteins. The mRNP particles are transported to the cytoplasm and the mRNA is presented to the ribosomes in the perikaryon and in the dendrites. An alternative route is the transport of mRNAs to the perikaryon in mRNP particles that do not contain FMRP. These mRNAs can bind FMRP, after which they are presented to ribosomes. This is also the route taken in fragile X patients lacking FMRP. In fragile X patients however, the absence of FMRP means that the mRNAs are not presented to the ribosomes properly, leading to free mRNAs that cannot be translated efficiently. NES, nuclear export signal; NLS, nuclear location signal.

1.3.4 Homologs of FMRP

As mentioned before, FMRP is associated with FXR1P en FXR2P, which are both very homologous to FMRP. The similarity at amino acid level throughout the amino-terminal and central regions is 86% and 70%, respectively. The corresponding genes, *FXR1* and *FXR2* (***F***ragile ***X*** ***R***elated gene *1* and *2*), respectively, have been cloned. *FXR1* is located on chromosome 3q28 (Coy et al. 1995) and *FXR2* on 17p13.1 (Zhang et al. 1995). The protein product of *FXR1* (FXR1P) consists of two major isoforms of 70 and 78 kD. The *FXR2* protein (FXR2P) has a molecular mass of 95 kD. Both FXR1P and FXR2P are co

expressed with FMRP in adult human brain in the cytoplasm of neurons (Tamanini et al. 1997). Two novel isoforms of FXR1P of 81 and 84 kD were identified, and they have been found exclusively in skeletal muscle, heart, and differentiated cultured myoblasts (Khandjian et al. 1998), and in testis (Huot et al. 2001). FMRP, FXR1P and FXR2P form a small family of proteins, the FXR proteins (***FMR1 cross***-reacting ***R***elative), related by structure, possible originating from a common ancestor gene.

In human adult testes the FXR proteins are differentially expressed. FMRP expression is found only in the cytoplasm of spermatogonia. FXR1P is also expressed in the cytoplasm of more mature spermatogenic cells including spermatocytes and spermatids, whereas FXR2P is, at a lower level, present in all cells of the tubules seminiferi (Tamanini et al. 1997). A similar expression pattern has been described for the FXR proteins in adult mouse testis (Bakker et al. 2000b). A more detailed study of Fxr1p in testis of the mouse showed that all isoforms, including the 81 and 84 kD isoform, are expressed in the cytoplasm of almost all spermatogenic cells, ranging from pachytene to spermatozoa (Huot et al. 2001). At the subcellular level, Fxr1p was found to be associated with microtubules in the flagella of the spermatozoa (Huot et al. 2001). This microtubule-association and the presence of Fxr1p in the myocontractile bands in striated muscle (Khandjian et al. 1998; Dube et al. 2000), suggest a function of Fxr1p in movement and motility that is energy dependent (Huot et al. 2001).

Like FMRP, both FXR1P and FXR2P contain KH domains, RGG boxes, an NES and an NLS. The three proteins can form homo- and heterotypic interactions in the yeast two-hybrid system as well as *in vitro* (Zhang et al. 1995) via a common region in the amino termini (FXR oligomerisation domain) (Siomi et al. 1996).

Unlike FMRP, some isoforms of FXR1P and FXR2P show a nucleolar targeting signal in their carboxy terminus (Tamanini et al. 2000). FXR1P can shuttle between cytoplasm and nucleoplasm similarly to FMRP, while FXR2P can shuttle between cytoplasm and nucleolus, suggesting that they may transport in part different RNAs or have different physiological functions (Tamanini et al. 1999a). Also only FMRP, and not FXR1P or FXR2P, recognizes mRNAs containing a G-quartet structure, indicative for a distinct function for FMRP (H. Moine, personal communication).

The identification of NUFIP, a novel RNA-binding nuclear protein (Bardoni et al. 1999) that interacts with FMRP, but not with FXR1P or FXR2P is confirming the hypothesis that the three proteins interact with different proteins or sites within the nucleus. They may have specific rather than overlapping functions there, although they appear to coalesce, at least partially, in the cytoplasm, forming a common mRNP particle.

Until now, no mutations have been described in FXR1 or FXR2.

1.4 Scope of this thesis

The aim of the studies described in the present thesis has been to obtain insight in the physiological function of the fragile X mental retardation protein (FMRP), the mechanism of repeat amplification and the pathogenesis of the fragile X syndrome. For this purpose mouse models have been generated and characterized.
An overview of the clinical aspects of the syndrome, and the *FMR1* gene and FMRP in relation to the fragile X syndrome is provided in Chapter 1.
Chapter 2 describes, in a broad spectrum, the characterization of different mouse models, created to study the fragile X syndrome, using our own work (Chapter 4) and the work of other researchers.
Chapter 3 is a general discussion facing the problems encountered in behavioural tests to characterize mouse models (Paragraph 3.1), and Paragraph 3.2 speculates on the (in)ability of therapeutic strategies for the fragile X syndrome.
Chapter 4 presents the published experimental work. A knockout mouse has been created (Paragraph 4.1), in which FMRP is absent, to address the question whether this mouse could be a model system to study the fragile X syndrome. This mouse model has been used to introduce an *FMR1* transgene in order to test restoration of the normal phenotype (Paragraph 4.2).
To understand more about the effect of the absence of FMRP and the development of mental retardation, it is necessary to study the expression of FMRP during (embryonic) development and in adult life. Localisation studies using antibodies directed against FMRP, FXR1P and FXR2P have learned us more about the localisation of the three proteins and their possible function (Paragraph 4.3 and 4.4).
Since in human fragile X patients the primary cause of the syndrome is expansion of the CGG repeat in the *FMR1* gene, a mouse model has been developed with an expanded, unstable CGG repeat in its *Fmr1* gene. This mouse model enables us to study the timing and mechanism of CGG repeat expansion (Paragraph 4.5).

Chapter 2

Mouse models for the fragile X syndrome

2.1 Model organisms

The identification of a gene involved in human disease is just a first step on the long road to the understanding of the biological basis of the disease. To unravel the biological mechanisms involved in normal and abnormal functioning of a disease gene, a model system might be of great help.

Studying biochemical processes in human cells and tissues *in vivo*, especially in the brain, is for many obvious reasons very difficult. The use of human cells that can be immortalised as a permanent growing cell line enables us to study many aspects of biological processes *in vitro*.

Due to the growing number of genome projects, it has become clear that there is an amazing degree of conservation among genes that participate in similar pathways across a wide spectrum of species. Together with the technical ability to introduce disease-causing mutations in an organism, this high degree of conservation makes it possible to use simple organisms to study human disease (reviewed by van Heyningen 1997). For example, the use of yeast has shed light on DNA replication and DNA repair in cancer, and mechanisms involved in cellular signalling. Recently, yeast was shown to be a simple model for cellular aging that may provide clues to mammalian aging events (Frohlich and Madeo 2001). Yeast is also used as a model organism to study determinants involved in DNA repeat instability in general (Shimizu et al. 1996; Wells 1996; White et al. 1999; Jin and Warren 2000).

Genes involved in development and differentiation are widely studied in the nematode worm *Caenorhabditis elegans* and the fruit fly *Drosophila melanogaster*. One homolog of the *FXR* genes has been identified in *Drosophila* and is used in neurodevelopmental studies (Wan et al. 2000; Zhang et al. 2001; Dockendorff et al. 2002; Morales et al. 2002), which has led to new insights in the function of the *FXR* proteins.

The recent development of new technologies, like RNA interference, makes it possible to use non-mammalian vertebrates, like the frog *Xenopus leavis* and the zebra fish *Danio rerio*, to study genes involved in early development of the vertebrate. The advantage of using these lower vertebrates, is that they are easy to maintain and manipulate. Their central nervous system is not as complicated as that in mammalian species, and well described, and the embryos are transparent, which makes it possible to view tissue formation and organogenesis inside the embryo from the outside.

Although lower vertebrate organisms and also worms and flies can serve as good models, there are many reasons to use a mammalian system when specific details and aspects need to be studied. The laboratory mouse is, for practical and technical reasons, the mammal of choice in functional studies. The occurrence of many spontaneous mutations in mice revealed insight in numerous processes. The technologies to experimentally modify the genome of the mouse, like embryonic stem (ES) cell

technology or DNA-microinjection into fertilised oocytes, made it possible to create precise models.

2.2 The fragile X knockout mouse

The *FMR1* gene is highly conserved among species (Verkerk et al. 1991), although the size of the human CGG repeat is longer than in other mammals (Deelen et al. 1994). Like in humans, the mouse *Fmr1* gene is also located on the X chromosome and, dependent on the mouse strain, the CGG repeat contains 9 to 11 CGG units. The murine homolog Fmrp shows 97 % identity in amino acid sequence compared to the human FMRP (Ashley et al. 1993b). The expression level and the localisation of the murine Fmrp in different tissues is very similar to the level and localisation seen in humans (Abitbol et al. 1993; Bächner et al. 1993; Devys et al. 1993; Hinds et al. 1993b; Bakker et al. 2000a; De Diego Otero et al. 2000). This makes the mouse a good organism to use as a model for the fragile X syndrome.
To mimic the fragile X syndrome in the mouse, several options are open. In humans, the mechanism of the disease is the growing CGG repeat, followed by methylation and inactivation of the *FMR1* gene. In the mouse, it is not known whether the endogenous *Fmr1* CGG repeat can grow, and in this way result in inactivation of *Fmr1*. Also it is technically very difficult to manipulate long, pure CGG repeats for transgenic purposes. Since the fragile X phenotype in the human is caused by the absence of FMRP, the first attempt to make a mouse model was to disrupt the murine *Fmr1* gene. With this disruption the gene was inactivated leading to the absence of Fmrp in the mouse. The procedure and results of this attempt to mimic the human fragile X phenotype in the mouse are described in Paragraph 4.1 (Bakker et al. 1994).
Similar to male human patients, *Fmr1* knockout mice develop progressive macroorchidism with time (Bakker et al. 1994; Kooy et al. 1996; Slegtenhorst-Eegdeman et al. 1998). Cognitive function and behavioural profile of the knockout mice are in line with behavioural findings in human fragile X patients (Bakker et al. 1994; Kooy et al. 1996; Paradee et al. 1999; Peier et al. 2000; Musumeci et al. 2000; Van Dam et al. 2000; Mineur et al. 2002; Nielsen et al. 2002). As the knockout mice lack Fmrp and show abnormalities comparable to human fragile X patients, it is a potentially valid model to provide insight into the physiological function of FMRP and the pathogenesis of the fragile X syndrome.

2.2.1 Macroorchidism

Macroorchidism found in the *Fmr1* knockout mouse has been studied in more detail by Slegtenhorst-Eegdeman et al. (Slegtenhorst-Eegdeman et al. 1998). In general, the size

of the testis is mainly determined by the number of Sertoli cells that support the proliferation and differentiation of the germ cells (Sharpe 1993). In the knockout mice, it appeared that the proliferative activity of the Sertoli cells was significantly higher, resulting in an increase in spermatogenic cell number and testicular weight. Follicle-stimulating hormone (FSH) plays an important, but not essential, role in Sertoli cell proliferation (Kumar et al. 1997). Sertoli cells are most sensitive to the mitogenic activity of FSH at the end of the foetal period and shortly after birth (Orth 1982). The circulating FSH level was measured in knockout mice, but not found to be elevated compared to that in wild type littermates. This observation is similar in human fragile X patients showing no evidence for an increased FSH level (Nielsen et al. 1982; Moore et al. 1991). Determination of the level of FSH receptor mRNA in the testis showed a slight increase, which, however, was not significant (Slegtenhorst-Eegdeman et al. 1998). These findings suggest that elements of the FSH signal transduction pathway are not involved in development of macroorchidism in the fragile X syndrome.
Absence of FMRP in developing germ cells as a primary cause of development of macroorchidism is not very likely, since in affected human male foetuses FMRP expression could be detected in the primordial germ cells (Malter et al. 1997). The primary cause of the increased testis size in *Fmr1* knockout mice may be found in Sertoli cells. Sertoli cells of the wild type neonatal mouse show a high expression of Fmrp at postnatal day three. This expression disappears at postnatal day 14, suggesting a function for Fmrp in Sertoli cells during early postnatal life (Bakker et al. 2000a). In knockout mice, and also in human fragile X patients, this high early postnatal expression is not present. This might indicate that the absence of Fmrp during this postnatal period leads to dysregulation of Sertoli cell proliferation, and consequently to development of macroorchidism in adult life.

2.2.2 Neuroanatomy

The neuroanatomy of the *Fmr1* knockout mouse was studied in more detail using *in vivo* high-resolution magnetic resonance imaging (MRI) (Kooy et al. 1999). MRI studies in human fragile X males show a decrease in size in the posterior vermis (Reiss et al. 1991a), but *Fmr1* knockout mice do not show differences in this area compared to wild type littermates (Kooy et al. 1999). Along with the vermis hypoplasia, a 25 to 35% increase in size of the 4th ventricle has been reported in human patients (Reiss et al. 1988; Reiss et al. 1991a; Reiss et al. 1991b), but knockout mice did not show these differences (Kooy et al. 1999). The caudate nucleus, together with the lenticular and thalamic nucleus, is forming the subcortical grey and allows the cerebral cortex to affect behaviour. The caudate nucleus is significantly larger in volume in fragile X male patients (Reiss et al. 1995). In *Fmr1* knockout mice, it was not possible to identify each

nucleus individually, but no evidence was found for an increase in subcortical grey volume (Kooy et al. 1999).

Using 3D-reconstruction, total brain volume of knockout mice was compared with the total brain volume of wild type mice. No significant differences were found (Kooy et al. 1999), in contrast to the human situation where fragile X brains appeared larger than control brains.

In conclusion, no evidence was found for differences in neuroanatomy between *Fmr1* knockout mice and wild type mice in those brain areas where human fragile X patients show differences with non-fragile X patients. However, the structural differences in neuroanatomy between human fragile X patients and controls may have been overestimated in the limited number of previous studies (Jakala et al. 1997). The basis for studying vermis size was the correlation between vermis size and autistiform behaviour. It was found that vermis size is reduced not only in patients with autism, but also in a percentage of patients suffering from neurogenetic syndromes without autistiform features (Schaefer et al. 1996).

It may well be that the mouse model does simply not resemble the human disease with respect to brain structure, although the same neuropathologic abnormalities of dendritic spines have been reported in both human and mouse (see Paragraph 2.2.6) (Hinton et al. 1991; Comery et al. 1997; Irwin et al. 2001).

Recently, altered sizes and distributions of hippocampal intra- and infrapyramidal mossy fibre (IIPMF) terminal fields in *Fmr1* knockout mice were described (Ivanco and Greenough 2002; Mineur et al. 2002). It has been shown that these hippocampal fibres are involved in spatial learning tasks (Crusio and Schwegler 1987; Schwegler and Crusio 1995; Schwegler et al. 1990), but the precise consequences of these alterations in knockout mice are still unclear.

2.2.3 Mean corpuscular haemoglobin

Biochemical abnormalities in human fragile X patients are limited to a lowered cyclic AMP production (Berry-Kravis and Huttenlocher 1992), and an increased mean corpuscular haemoglobin (MCH) level (Langenbeck et al. 1984). The difference in MCH in human patients was small, but statistically significant. The authors argued that the increase in MCH level was a result of a minor disturbance in the folate metabolism of fragile X patients. The MCH level in erythrocytes from *Fmr1* knockout and wild type mice were measured, but no difference was found (Reyniers et al. 1996). It is therefore unlikely that Fmrp has an effect on MCH.

2.2.4 Behavioural tests

Along with the cognitive dysfunction accounting for deficits in short-term memory and visual-spatial disabilities (Maes et al. 1994), a complex of behavioural abnormalities is seen in human fragile X patients. These abnormalities include social avoidance, hyperactivity, abnormal response to sensory stimuli, and repetitive, stereotypic autistiform behaviour (Brown et al. 1986; Fisch 1992; Reiss and Freund 1992). The *Fmr1* knockout mouse is used as a model to study the cognitive dysfunction and behavioural abnormalities.

Extensive behavioural tests have been performed to study cognitive functions of the knockout mice (Bakker et al. 1994; Kooy et al. 1996; D'Hooge et al. 1997; Paradee et al. 1999; Dobkin et al. 2000; Peier et al. 2000; Van Dam et al. 2000; Mineur et al. 2002). A mouse behavioural test that measures spatial learning and memory is the Morris water maze task (Morris et al. 1982; Morris 1984). This task is highly dependent on hippocampal function. Since FMRP expression is high in hippocampus, but absent in *Fmr1* knockout mice, these mice were tested in the Morris water maze to analyse their hippocampal function. The knockout mice showed an impaired performance during learning and reversal trails, but the differences are rather subtle and highly dependent on the genetic background of the mouse strain used (Kooy et al. 1996; D'Hooge et al. 1997; Paradee et al. 1999; Dobkin et al. 2000; Peier et al. 2000; Van Dam et al. 2000; Mineur et al. 2002). In addition, tests measuring exploration, hyperactivity, anxiety, conditioned fear and aggression were performed (Bakker et al. 1994; Kooy et al. 1996; D'Hooge et al. 1997; Paradee et al. 1999; Dobkin et al. 2000; Peier et al. 2000; Van Dam et al. 2000; Mineur et al. 2002). It is clear from these studies that *Fmr1* knockout mice show increased activity and exploratory behaviour, but no differences were seen in conditioned fear or aggression compared to control mice. Finally, tests measuring anxiety give conflicting results probably due to differences in experimental setting or interpretation (Peier et al. 2000; Mineur et al. 2002).

The results of the behavioural tests illustrate the complexity of the effects of *Fmr1* deficiency on brain function. It has become clear that only extensive behavioural validation of the *Fmr1* knockout mouse will benefit the use of the model in pathophysiological and therapeutic studies.

2.2.5 Long-term potentiation and long-term depression

Malperformance of the Morris water maze task is indicative for dysfunction of the hippocampus (Morris et al. 1982). Studies on other, unrelated knockout mouse models have shown that also long-term potentiation (LTP) is involved in malperformance of the

Morris water maze task (Grant et al. 1992; Silva et al. 1992a; Silva et al. 1992b; Bach et al. 1995; Sakimura et al. 1995; Wu et al. 1995). The physiological significance of LTP is still unknown, but it is generally assumed that there is a link between learning and memory, synaptic plasticity, and LTP. LTP is an input-specific and persistent increase in the strength of synaptic connections that is the major model mechanism for information storage in the brain (Bliss and Collingridge 1993). Electrophysiological studies in CA1 hippocampal slices from *Fmr1* knockout mice and wild type mice were performed to investigate the involvement of *Fmr1* in early-phase LTP, but no differences were found (Godfraind et al. 1996). Also short-term potentiation was similar in this study. Additionally, *Fmr1* expression in brain after seizures was monitored, to investigate whether *Fmr1* is involved in the later stages of LTP (LTP3) as an immediate early gene. No alterations in the amount of *Fmr1* mRNA could be detected in total brain or hippocampus up to 150 minutes after the first seizures, making it unlikely that *Fmr1* is an immediate early gene involved in LTP3 (Godfraind et al. 1996).

There is evidence suggesting that some mechanisms of synaptic plasticity are common to both hippocampus and cortex (Aniksztejn and Ben-Ari 1991; Pelletier and Hablitz 1996). Li et al. studied LTP in the cortex of mice and, although no changes in hippocampal LTP were found, studies on cortical LTP showed a significant reduction in knockout mice (Li et al. 2002).

Another study examining late-phase long-term potentiation, the protein synthesis-dependent form of long-term potentiation, did also show no differences between knockout and wild type mice (Paradee et al. 1999). These results suggest that Fmrp may not have an influence on long-term potentiation in the hippocampus or that any influence is too subtle to be detected by the techniques used.

A second form of hippocampal synaptic plasticity, which is protein synthesis-dependent, is long-term depression (LTD) triggered by activation of group 1 metabotropic glutamate receptors (mGluR) (Huber et al. 2000; Huber et al. 2001; Snyder et al. 2001). This type of LTD is protein dependent and is not observed when mRNA translation is inhibited (Huber et al. 2000; Snyder et al. 2001). Since it is known that FMRP is synthesised in response to mGluR activation by glutamate (Weiler and Greenough 1999), the involvement of FMRP in hippocampal LTD has been investigated (Huber et al. 2002). In *Fmr1* knockout mice, the hippocampal LTD is selectively enhanced compared to wild type mice. This finding is in agreement with the function of FMRP as an inhibitor of translation (Laggerbauer et al. 2001; Li et al. 2001; Zhang et al. 2001). In the absence of Fmrp, translation of mRNAs is not inhibited resulting in enhanced hippocampal LTD (Figure 4). The hypothesis is that this enhanced hippocampal LTD could interfere with the formation and maintenance of strong synapses required for normal brain function (Huber et al. 2002). It is also suggested that the enhanced activity- and mGluR-dependent synapse turnover is underlying the slowed development of

dendritic spines in the cerebellar cortex (Nimchinsky et al. 2001) and thereby affecting the normal function of the cerebral cortex (Huber et al. 2002).

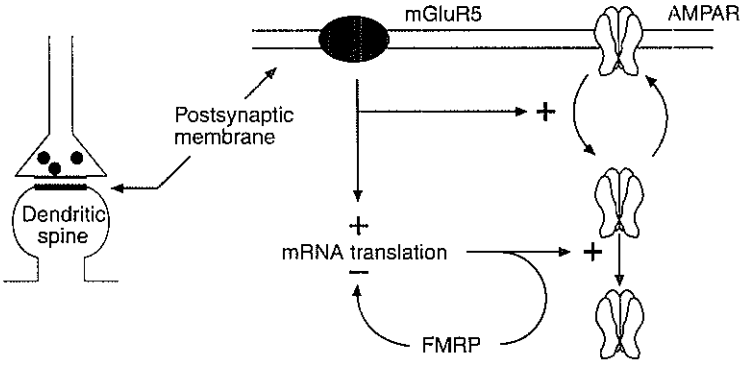

Figure 4. Model. One expression mechanism for LTD of synaptic transmission is the internalization of AMPA receptors. Activation of mGluR5 stimulates the internalization of AMPA receptors. The stable expression of this modification requires protein synthesis, which is proposed to be negatively regulated by FMRP synthesized in response to mGluR activation. Therefore, in the absence of FMRP, LTD magnitude is increased. This figure is adapted from (Huber et al. 2002).

2.2.6 Synaptic plasticity

Both *FMR1* mRNA and FMRP are present in dendrites, and the expression level is increased by activation of metabotropic glutamate receptors (Weiler et al. 1997; Weiler and Greenough 1999) linking FMRP to synaptic function. Glutamate receptors, such as NMDA and AMPA receptors, are localised in the brain and play a critical role in learning and memory (Bliss and Collingridge 1993; Danysz et al. 1995; Asztely and Gustafsson 1996; McHugh et al. 1996; Lu et al. 2001; Tsien 2000). For the AMPA receptors, it has recently been found that they have an important function in synaptic plasticity (Musleh et al. 1997; Nayak et al. 1998; Zamanillo et al. 1999). Together with the reduced LTP in cortical slices, it was of interest to study the expression of GluR1, an AMPA receptor subunit, in the cortex of *Fmr1* knockout mice. It was shown that cortical GluR1 expression is reduced in the absence of Fmrp, probably leading to depressed cortical synaptic plasticity (Li et al. 2002).

In both human fragile X patients (Rudelli et al. 1985; Hinton et al. 1991; Irwin et al. 2001;) and adult *Fmr1* knockout mice (Comery et al. 1997), abnormalities in dendritic spines have been described. The spines are unusually long, resembling immature spine morphology, and are increased in density. Absence of FMRP may be involved in a defect in spine maturation and pruning (Comery et al. 1997). Spine motility is believed

to represent postsynaptic participation in synapse formation (Dailey and Smith 1996). An abnormality in spine motility early in postnatal life could affect synaptogenesis, and therefore it is of interest to determine spine motility in the absence of Fmrp. Nimchinsky et al. showed that dendritic spines *in vivo* in the intact brain of *Fmr1* knockout mice were abnormal, early in postnatal life (Nimchinsky et al. 2001). These abnormalities in the somatosensory cortex were most pronounced during the period of greatest synaptogenesis in that region (White et al. 1997), which therefore supports the hypothesis that Fmrp plays a role in synaptogenesis in the normal brain. This early disturbance of synaptogenesis could be reflected at that developmental time point by abnormalities in spine morphology and density and later by cognitive defects resulting from improperly established connections (Nimchinsky et al. 2001). In contrast, *in vitro* studies (Steward et al. 1998) do not detect the spine abnormalities found in the *in vivo* study performed by Nimchinsky et al., probably due to differences in methods used (Nimchinsky et al. 2001).

Near synapses, protein synthesis occurs, and particularly during development polyribosomal aggregates are formed. Polyribosomal aggregates in spines increase during experience-dependent synaptogenesis (Greenough et al. 1987). Some protein synthesis appears to be regulated directly by synaptic activity. This synthesis can be monitored in synaptoneurosomes. Synaptoneurosomes are preparations highly enriched in pinched-off, resealed presynaptic processes attached to resealed postsynaptic processes that retain normal function of neurotransmitter release. When synaptoneurosomes are stimulated with glutamate, mRNA is rapidly taken up into polyribosomal aggregates, and proteins are synthesised (Greenough et al. 2001). One of these proteins is Fmrp. Greenough et al. reported that the protein synthesis in response to glutamate is dramatically reduced in synaptoneurosomes derived from the cerebral cortex of *Fmr1* knockout mice (Greenough et al. 2001). This is in contrast with reports of others describing a function of Fmrp in inhibition of mRNA translation. Absence of Fmrp, as is the case in *Fmr1* knockout mice, would than result in upregulation of mRNA translation (Laggerbauer et al. 2001; Li et al. 2001; Zhang et al. 2001; Huber et al. 2002).

In conclusion, all data obtained with studies on dendritic spines point in the direction of an impairment of mechanisms that promote synapse maturation and pruning in the *Fmr1* knockout mouse. Fmrp plays a role in the neural maturation process, and its absence evolves in cognitive and behavioural impairments as observed in the fragile X syndrome.

2.2.7 Seizures

More than 50% of prepuberal fragile X patients show typical EEG abnormalities, whereas seizures are observed in more than 20% of the patients (Musumeci et al. 1999). Since *Fmr1* knockout mice have never been reported to have spontaneous epileptic seizures, acoustic stimulation was used to provoke audiogenic seizures in the knockout and wild type mice. The susceptibility to audiogenic seizures (AGS) is greater in knockout mice compared to wild type mice. These results indicate that absence of Fmrp results in an increase in cortical excitability in knockout mice (Musumeci et al. 2000).
In general, fragile X patients show hyperreactivity to auditory, visual, tactile and olfactory stimuli (Hagerman 1996; Merenstein et al. 1996; Hagerman et al. 1999; Miller et al. 1999). Chen and Toth were particularly interested in the sensory responsiveness of the *Fmr1* knockout mouse. They tested the knockout mice for susceptibility of audiogenic seizures, as did Mucumeci et al., and found an age dependent increased AGS in knockout mice from week 10 onwards (Chen and Toth 2001). Chemical convultants were used to test whether this seizure susceptibility is restricted to the auditory system or is a more general phenomenon. No difference between knockout and wild type mice in seizure susceptibility was found, indicating that knockout mice do have a local, presumably auditory system hyperexcitability (Chen and Toth 2001). This auditory system hyperexcitability was confirmed in a study of the auditory startle response in *Fmr1* knockout and control mice of different genetic backgrounds. The knockout mice exhibited increased auditory startle response amplitudes to low intensity stimuli and decreased responses to high intensity stimuli (Nielsen et al. 2002). Although the decreased responses to high intensity stimuli are difficult to explain it might be that there is an abnormality in secondary brain regions that modulates the primary startle response (Nielsen et al. 2002). The responsiveness of *Fmr1* knockout mice to auditory stimuli is consistent with the sensory hypersensitivity of fragile X patients.
Since epileptic seizures induce neuronal expression of the immediate-early gene product c-Fos (Morgan and Curran 1991) some mice that had experienced an audiogenic seizure were analysed for localisation of c-Fos expression. The c-Fos expression pattern denotes the involvement of specific brain structures in seizure propagation. In this way the neuronal network involved in audiogenic seizures in the knockout mice could be traced from auditory stimulus to the final motor component (Chen and Toth 2001). Based on this study the authors hypothesised that absence of Fmrp in the auditory system of knockout mice results in perturbations in developmental/activity-dependent neuroplasticity, leading to an increased susceptibility to loud, audible sound (Chen and Toth 2001).
As mentioned before, FMRP is expressed in synapses in response to activation of glutamate receptors by glutamate (Weiler et al. 1997; Weiler and Greenough 1999) and

via a negative inhibition pathway FMRP suppresses translation of mRNA (Figure 4) (Laggerbauer et al. 2001; Li et al. 2001; Huber et al. 2002). Studies on mGluR-triggered protein synthesis in the hippocampus suggest that without inhibition of translation, for example performed by FMRP, the threshold for synaptic potentiation is reduced (Raymond et al. 2000) and epileptiform activity can be triggered (Merlin et al. 1998; Wong et al. 1999). This finding might explain the hyperreactivity seen in the absence of FMRP in the fragile X syndrome (Huber et al. 2002).

2.3 Reintroduction of FMRP

Using the *Fmr1* knockout mouse as a model, the *FMR1* cDNA and thus also FMRP could be reintroduced in an attempt to rescue the phenotype, as is described in Paragraph 4.2 (Bakker et al. 2000b). In this study the expression of the protein is constitutive, and in all cell types, but there is no rescue of the phenotype. The timing of expression, the level of expression per cell, and the type of cells in which FMRP is expressed seem to be essential for a rescue (Bakker et al. 2000b).

Peier et al. used a yeast artificial chromosome (YAC), containing the entire human *FMR1* gene with extensive amounts of flanking sequence, for insertion into the genome of the *Fmr1* knockout mouse (Peier et al. 2000). The transgenic protein was expressed 13 to 17 times higher than the endogenous Fmrp. While the *Fmr1* knockout mouse in behavioural tests displays less anxiety-related responses with increased exploratory behaviour compared to wild type mice, the YAC-containing *Fmr1* knockout mice showed opposing behavioural response and additional abnormal behaviours compared to wild type mice. These observations indicate that overexpression of FMRP is harmful to the animal, and this finding has significant implications for gene therapy for the fragile X syndrome (Peier et al. 2000).

Taking into consideration the problems with reintroduction of the *FMR1* gene, a more appropriate approach could be the use of pharmacological agents to reactivate the *Fmr1* gene in patients with a methylated full mutation. Since methylation of the CGG repeat and of the *FMR1* promoter region is the inactivating cause of *FMR1* in the fragile X syndrome, demethylation could be a way to activate *FMR1* and obtain FMRP expression. Studies with demethylating agents in cultured cells from patients have been performed and show promising results, although the agents used are very toxic (Chiurazzi et al. 1998; Chiurazzi et al. 1999). To test this type of re-expression of Fmrp *in vivo*, an animal model, containing a long repeat and showing methylation of the *FMR1* promoter region, would be of much importance.

2.4 A CGG repeat mouse

A mouse model, mimicking several aspects of the fragile X syndrome in considerable detail, would be of great value, not only to a demethylation therapy, but also to shed light on the mechanisms involved in *FMR1* CGG repeat instability and methylation.
Previously, a transgenic mouse model was made containing a fusion gene of a long human repeat of 81 CGG units and the *LacZ* reporter gene. Upon transmission to the next generation, this repeat remained stable (Bontekoe et al. 1997). Even when human sequences normally surrounding the CGG repeat were included in the transgene, no instability was seen (Lavedan et al. 1997; Lavedan et al. 1998). Thus, nearby *cis*-acting elements seem not to be involved in the control of, or sufficient to induce, instability of the repeat. Therefore, placing the long repeat in its endogenous environment may provide the factors needed for repeat instability.
A mouse with a long CGG repeat at the position of the endogenous short mouse CGG repeat has been created as described in Paragraph 4.5 (Bontekoe et al. 2001). The initial length of the long repeat of human origin was 98 CGG repeat units. This repeat is in the human premutation range, showing instability upon maternal transmission to the next generation in the human. In the mouse, this CGG repeat shows mild instability, and stepwise has reached up till now a length of 108 CGG repeat units (Bontekoe et al. 2001). Creating successive generations of mice may finally lead to a full mutation repeat, initiating methylation and inactivation of the *Fmr1* gene. This mouse model will make the study of CGG repeat instability in its mouse endogenous environment possible.
Other mouse models containing other trinucleotide repeats involved in human diseases have been generated. Instability was found in a number of them. These mouse models are reviewed in the PhD thesis of C. Bontekoe (Bontekoe 2001).

2.5 A knockout mouse for *Fxr1* and *Fxr2*

For the homologs of *FMR1*, *FXR1* and *FXR2*, no disease causing mutations have been described yet. The absence of Fmrp in the *Fmr1* knockout mouse does not influence the expression of Fxr1p or Fxr2p markedly (Bakker et al. 2000a). Knockout mice for *Fxr1* and *Fxr2* may reveal a phenotype and could shed light on the functions of Fxr1p and Fxr2p.
The generation of a knockout mouse for *Fxr1* have not been successful until now. The first indications are that mice homozygous for the *Fxr1* knockout allele die shortly after birth (H. Siomi, personal communication). Since *Fxr1* is highly expressed in muscle tissue, and absence of Fxr1p would lead to malfunction of vital organs, a logic explanation would be that heart or lung functions are impaired. A conditional knockout

mouse for *Fxr1*, in which the gene can be turned off at a desired moment, or in a desired organ, is under development (B. Oostra, personal communication).

Generation of a knockout mouse for *Fxr2* has been successful, and has led to mice being vital and fertile (Bontekoe et al. 2002). The *Fxr2* knockout mice have been subjected to the same behavioural test battery, as have been the *Fmr1* knockout mice. *Fxr2* knockout mice show behavioural abnormalities compared to wild type mice. Some of these behaviours resemble those of the *Fmr1* knockout mouse and several others of these are opposing. Both *Fmr1* and *Fxr2* knockout mice are hyperactive in the open-field test and impaired in the rotarod test. On the other hand, *Fxr2*, but not *Fmr1*, knockout mice show poor performance during training trails of the Morris water maze task, and are impaired in the context-dependent conditioned fear test (Peier et al. 2000; Bontekoe et al. 2002). It has to be taken into account that, although the behavioural tests are performed in the same laboratory, they were not performed simultaneously and the genetic background of the two knockout models was different. Still, the results with the *Fxr2* knockout mice are indicative for a function of Fxr2p in those parts of the brain involved in behavioural responses (Bontekoe et al. 2002).

Generation of *Fmr1/Fxr2* double-knockout mice can provide information about possible interaction of Fmrp and Fxr2p, and whether or not these proteins show partial or complete functional complementarity. Studies with the double-knockout mice are in progress (B. Oostra, personal communication).

Chapter 3

General Discussion

3.1 The mouse as a model

Since the identification of the *FMR1* gene in 1991, the gene and its products have been studied thoroughly. In this period more has become clear about the mechanism of CGG repeat amplification, and the characteristics and possible functions of the fragile X mental retardation protein, FMRP. The use of model-organisms contributed considerable to our knowledge.

The studies with model organisms were and are useful to elucidate rather basic questions. The mechanism of repeat expansion, for example, has been studied in yeast (White et al. 1999). Even the fly *Drosophila melanogaster* has been found to contain a gene homologous to the *FXR* genes. Studying this gene revealed insight in the possible involvement of the *FXR* genes in neural development (Wan et al. 2000; Zhang et al. 2001; Dockendorff et al. 2002; Morales et al. 2002). A limitation of the use of *Drosophila* is the presence of only one gene instead of the three FXR genes, *FMR1*, *FXR1* and *FXR2*.

The zebra fish *Danio rerio* is being used to study early embryonic development, but to study more complex processes higher organisms are needed. The mouse is a mammal that resembles the human in many aspects, and has turned out to be a proper organism to study disease. The *Fmr1* knockout mouse was and is used in many experiments, and has revealed many new insights in the processes involved in the fragile X syndrome, as is described in this thesis.

Limitations to the use of the *Fmr1* knockout mouse as a model concern especially the tests to determine behavioural abnormalities. These tests are not only very time consuming but, although large numbers of mice are used, reveal rather subtle differences between knockout mice and control mice.

Especially the latter behavioural studies show that the genetic background of the mouse strain is a determining factor in the *Fmr1* knockout phenotype (Paradee et al. 1999; Dobkin et al. 2000; Van Dam et al. 2000). The genetic background of the mice in the first described experiments was a combination of C57BL/6 and 129/Ola (Bakker et al. 1994). The significant, but subtle increased latencies in the reversal trails of the Morris water maze task observed in knockout mice in these first studies could not be confirmed in studies with mice of the C57BL/6 genetic background (Paradee et al. 1999). In the cross-shaped water maze task, *Fmr1* knockout mice of an FVB/n-129/Ola background showed a pronounced deficiency in their ability to learn the position of a hidden escape platform, in comparison to their normal littermates. Knockout mice of a C57BL/6 background did not differ from their normal littermates in this aspect (Dobkin et al. 2000).

Not only unknown genetic factors may have an influence on the outcome of behavioural tests. One has to be aware of the known genetic factors that may lead to

misinterpretation. For example, water maze tasks require the animal to perceive and use distal environmental cues to locate a hidden escape platform. The FVB/n genetic background shows retinal degeneration involving impaired vision and difficulties in observing the environmental cues. The testing of mice containing FVB/n background for retinal degeneration is essential to avoid misinterpretation of water maze task results. For mice of the C57BL/6 genetic background it is known that they show early-onset hearing loss to pure tones (Carlson and Willott 1998) and a late progressive hearing loss with age (Johnson et al. 1997). This has to be kept in mind when testing mice in experiments using noise, like for determining the auditory startle response (Nielsen et al. 2002) or the susceptibility to audiogenic seizures (Musumeci et al. 2000).

Genetic background can influence performance of animals in behavioural tests, but also external factors like interlaboratory differences can play a role. Several conditional fear tests performed by different groups using the same conditioning protocol show conflicting results (Paradee et al. 1999; Van Dam et al. 2000), probably due to differences in laboratory-specific environment (Crabbe et al. 1999).

New behavioural tests need to be developed, that may give clear-cut results to discriminate between knockout and control phenotypes. Very recently a successful new test measuring eye blinking has been developed (Koekkoek et al. in press, Journal of Neurophysiology). This test depends on the abilities of animals to condition their eyelid responses. Investigating these abilities has revealed important insights in the mechanism underlying learning and memory. The major area for memory formation and storage underlying classical eyeblink conditioning is probably the cerebellum (McCormick and Thompson 1984; Kim and Thompson 1997; Hesslow and Yeo 1998). Classical eyeblink conditioning can be used to study the molecular mechanisms underlying cerebellar motor learning in mutant mice. The new test, the magnetic distance measurement technique (MDMT), measures the kinetic and frequency domain properties of conditioned and unconditioned eyeblink responses in mice. This technique records eyelid movements in alert freely moving animals with adequate, absolute and continuous determination of their eyeblink movements in time and space while using an electrical shock as the unconditioned stimulus (Koekkoek et al. in press, Journal of Neurophysiology).

The *Fmr1* knockout mice were tested for this eyeblink conditioning. It has been shown that the eyeblink conditioning of *Fmr1* knockout mice is comparable to the eyeblink conditioning of mice lacking a functional cerebellar cortex. This implicates that the *Fmr1* knockout mice have a defect in their cerebellar learning (B. Koekkoek, personal communication). Impairment of cerebellar function is also implicated by the study of Huber et al (Huber et al. 2002) (Paragraph 2.2.5).

In conclusion, the MDMT is a good discriminative test to distinguish wild type from *Fmr1* knockout mice.

3.2 Therapeutic approaches

The *Fmr1* knockout mouse has been used to reintroduce *FMR1* in the germ line, in order to restore the fragile X phenotype in the mouse (Bakker et al. 2000b). It has become clear from this and other studies, that it is necessary to have expression of FMRP at sufficient levels, in cells that normally express *FMR1*, and probably at the precise developmental moment (Bakker et al. 2000b; Peier et al. 2000). Gene therapy via the germ line is not an option in the human situation, for ethical reasons. Somatic gene therapy would preferably direct the *FMR1* gene to neurons of the brain, to avoid or overcome mental retardation. But for this type of therapy dividing cells are needed, and neurons are in general non-diving cells. Furthermore *FMR1* needs to be directed to the cells that normally express *FMR1*, be expressed at a sufficient level and at the precise developmental moment. Therefore, somatic gene therapy is no option to treat the fragile X syndrome, in the near future.

Replacement therapy using FMRP could be another option. It has become clear from the studies described in this thesis, that absence of FMRP has its influence on neuronal development early in postnatal life. Therapeutic attempts should therefore be undertaken early after birth. But the widespread expression of Fmrp in all tissues during normal embryonic development (De Diego Otero et al. 2000), ending up in tissue-specific expression in adult tissues (Bakker et al. 2000a), might indicate that Fmrp is needed during embryogenesis. This would urge application of the protein to an affected embryo *in utero*, and would be complicating the therapy even more.
Apart from the time point to start with protein replacement therapy, it is important to deliver the protein to the target organ and to the specific cells within this organ. In this case, the brain would be the target organ, and the neurons the specific cells, to prevent abnormalities in neuronal development. The problems to overcome would be to pass the blood-brain barrier that prevents most proteins to enter the brain, and to deliver the protein into the cells that normally express *FMR1*. Attempts with a fusion-protein containing FMRP fused to a TAT peptide, directing the fusion protein through the barrier into cells (Green and Loewenstein 1988; Schwarze et al. 1999), are being undertaken now (S. Reis, personal communication). Also the amount of FMRP entering the cells must be controlled. It has become clear that low levels of FMRP will not be sufficient to overcome the phenotype (De Vries et al. 1993; Rousseau et al. 1994; Bakker et al. 2000b), and a high level FMRP is harmful to the patient (Peier et al. 2000). But the recent findings in the hippocampus make clear that the function of FMRP is rather sophisticated and tightly regulated in a controlled pathway (Figure 4) (Huber et al. 2002). Therefore, just supplying the protein may not have the right effect. Than, it

would be preferable to have an active *FMR1* gene, to overcome the pathology seen in the fragile X syndrome.

Inactivation of the *FMR1* gene is, in most fragile X patients, due to methylation of the *FMR1* promoter region. Studies in cultured cells from human fragile X patients using demethylating agents showed that it is possible to reactivate the *FMR1* promoter (Chiurazzi et al. 1998). Before using these demethylating agents in an animal model, some problems need to be encountered first. It has to be investigated at what developmental time point the demethylating agent is best administered, and how to administer the demethylating agents. Another problem to solve concerns the toxicity of the presently known demethylating agents, with respect to the aspecificity of the demethylation and thus an unwarranted stimulatory effect on induction of oncogenic gene expression. This form of treatment does not seem to be an option in the near future either.

Recently, a model was proposed in which an increase in FMRP in the hippocampal synapse normally serves to limit expression of long-term depression (LTD) by inhibiting group 1 metabotropic glutamate receptor (mGluR)-dependent translation of other synaptic mRNAs (Figure 4) (Huber et al. 2002). It was suggested that absence of FMRP leads to exaggerated LTD and/or mGluR function, resulting in the behavioural features of the fragile X syndrome. In therapeutic terms, one could think of the use of antagonists of mGluRs to restore the behavioural abnormalities (Huber et al. 2002). This therapeutic approach needs to be studied more thoroughly.

A long way of solving problems is still ahead, before the therapeutic value of some of the approaches described above hopefully will be proven. Also ethical issues need to be considered. For example, it will require extensive debate when replacement therapy will be applied to adult patients who have missed some of the intellectual and emotional development of the brain.
In the near future, studies in different mouse models, as described in the present thesis, which aim to gain information about possible therapeutic strategies, will be of benefit to tray to unravel the precise function of FMRP.

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Chapter 4

Experimental work

4.1 Publication 1

Fmr1 knockout mice: A model to study fragile X mental retardation

The Dutch-Belgian Fragile X Consortium

Group 1: Cathy E. Bakker[1], Coleta Verheij[1], Rob Willemsen[1], Robert van der Helm[1], Frank Oerlemans[2], Marcel Vermey[3], Anne Bygrave[4], André T. Hoogeveen[1], and Ben A. Oostra[1]

[1]Dept. of Clinical Genetics, [3]Dept. of Pathology, Erasmus University Rotterdam, The Netherlands
[2]Dept. of Cell Biology and Histology, University of Nijmegen, The Netherlands
[4]Laboratory of Gene Structure and Expression, National Institute for Medical Research, Mill Hill, England

Group 2: Edwin Reyniers[1], Kristel De Boulle[1], Rudi D'Hooge[2], Partick Cras[2], Désiré van Velzen[1], Guy Nagels[2], Jean-Jacques Martin[2], Peter P. De Deyn[2], John K. Darby[1], Patrick J. Willems[1]

[1]Dept. of Medical Genetics, [2]Dept. of Neurochemistry and Behavior, University of Antwerp, Belgium

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Fmr1 Knockout Mice: A Model to Study Fragile X Mental Retardation

The Dutch-Belgian Fragile X Consortium*

Summary

Male patients with fragile X syndrome lack FMR1 protein due to silencing of the *FMR1* gene by amplification of a CGG repeat and subsequent methylation of the promoter region. The absence of FMR1 protein leads to mental retardation, aberrant behavior, and macroorchidism. Hardly anything is known about the physiological function of *FMR1* and the pathological mechanisms leading to these symptoms. Therefore, we designed a knockout model for the fragile X syndrome in mice. The knockout mice lack normal Fmr1 protein and show macroorchidism, learning deficits, and hyperactivity. Consequently, this knockout mouse may serve as a valuable tool in the elucidation of the physiological role of *FMR1* and the mechanisms involved in macroorchidism, abnormal behavior, and mental retardation.

Introduction

The fragile X syndrome is the most frequent form of inherited mental retardation in humans, with an incidence of 1 in 1250 males and 1 in 2500 females (reviewed by Oostra et al., 1993). The clinical syndrome includes moderate to severe mental retardation, autistic behavior, macroorchidism, and facial features such as a long face with mandibular prognathism and large, everted ears (Hagerman, 1991). The gene involved in the fragile X syndrome (*FMR1*) is located in Xq27.3, a region that cytogenetically displays a fragile site. The molecular basis for this disease is a large expansion of a triplet repeat $(CGG)_n$ in the 5′ untranslated region of the *FMR1* gene (Verkerk et al., 1991; Oberlé et al., 1991; Yu et al., 1991; Fu et al., 1991). In the normal population, this CGG repeat is polymorphic, with a repeat length ranging from 6–53 units (Fu et al., 1991). Carrier males (normal transmitting males) and carrier females show a repeat length of 43–200 CGGs (premutation) and are asymptomatic. The full mutation is characterized by a large repeat containing over 200 CGGs. As a result of repeat amplification, the *FMR1* promoter and the CGG repeat itself become methylated, leading to silencing of transcription and translation of the *FMR1* gene (Pieretti et al., 1991; Verheij et al., 1993). Males with a full mutation are affected, and 50%–70% of the females with a full mutation allele show mild to moderate mental impairment (Rousseau et al., 1991).

The FMR1 protein shows hardly any homology to other known proteins, and little is known about its function. Recently, RNA binding studies have shown that FMR1 protein is able to bind its own messenger as well as 4% of the human fetal brain mRNAs (Ashley et al., 1993b). Two types of domains that are known to bind RNA, two KH domains and a RGG box, have been identified in FMR1 protein (Siomi et al., 1993; Ashley et al., 1993b).

The *FMR1* gene is highly conserved among species (Verkerk et al., 1991), and the murine homolog *Fmr1* shows 97% homology in amino acid sequence (Ashley et al., 1993a). The expression pattern of *FMR1* at the mRNA

*The Dutch-Belgian Fragile X Consortium is comprised of the following groups:

Group 1:

Cathy E. Bakker,[1] Coleta Verheij,[1] Rob Willemsen,[1] Robert van der Helm,[1] Frank Oerlemans,[2] Marcel Vermey,[3] Anne Bygrave,[4] André T. Hoogeveen,[1] and Ben A. Oostra[1]†

[1]Department of Clinical Genetics
[3]Department of Pathology
Erasmus University
3000 DR Rotterdam
The Netherlands
[2]Department of Cell Biology and Histology
University of Nijmegen
6500 HB Nijmegen
The Netherlands
[4]Laboratory of Gene Structure and Expression
National Institute for Medical Research
Mill Hill
London NW7 1AA
England

Group 2:

Edwin Reyniers,[1] Kristel De Boulle,[1] Rudi D'Hooge,[2] Patrick Cras,[3] Désiré van Velzen,[1] Guy Nagels,[2] Jean-Jacques Martin,[3] Peter P. De Deyn,[2] John K. Darby,[1] Patrick J. Willems[1]†

[1]Department of Medical Genetics
[2]Department of Neurochemistry and Behavior
[3]Department of Neuropathology
University of Antwerp
2610 Wilrijk
Belgium

†*Correspondence should be addressed to Ben A. Oostra and Patrick J. Willems.*

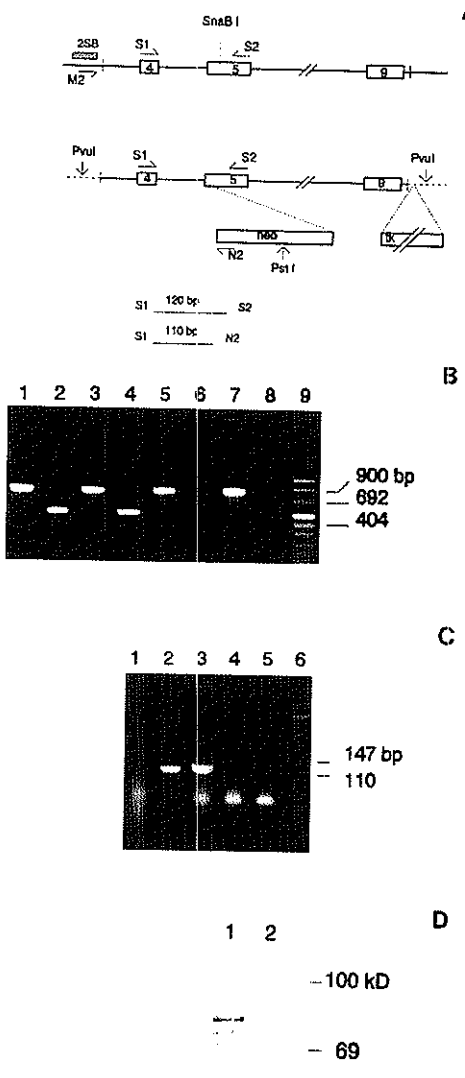

Figure 1. Construction and Characterization of the *Fmr1* Knockout Mice

(A) A schematic drawing of the genomic organization of the *Fmr1* locus (upper part) and a schematic drawing of targeting vector pMG5 (lower part). The genomic sequence is interrupted at the SnaBI site of exon 5 by a *neo* cassette. The *tk* cassette is depicted 3′ of the genomic sequence. Primers M2, N2, S1, and S2, probe 2SB, and the PstI sites (close to each other) are indicated.

(B) PCR analysis of ES clone CB5.2 (lane 1) and tail DNA from a wild-type mouse (lane 2), a heterozygous female (lanes 3 and 4), a mutant male (lanes 5 and 6), and a mutant female (lanes 7 and 8). Primer M2 and N2 were used to detect the ~800 bp fragment of the knockout allele (lanes 1, 3, 5, and 7), and primer S1 and S2 were used to detect the 465 bp fragment of the wild-type allele (lanes 2, 4, 6, and 8). Lane 9 contains a size marker.

(C) RT-PCR analysis of a wild-type (lanes 1 and 3) and a knockout (lanes 2 and 4) mouse testis using primers S1 and N2 (lanes 1 and 2) producing a 110 bp fragment in the mutant testis, and primer S1 and S2 (lanes 3 and 4) producing a 120 bp fragment in the wild-type testis. Lane 5 contains a blank sample. Lane 6 shows a size marker.

(D) Western blot analysis of *Fmr1* expression in brain of a wild-type (lane 1) and a knockout (lane 2) mouse showing protein bands between 67 kDa and 74 kDa only present in the control mouse.

and protein level is very similar in different tissues of humans and mice (Hinds et al., 1993; Bächner et al., 1993a, 1993b; Devys et al., 1993; Abitbol et al., 1993), which makes the mouse a good animal model in which to study the fragile X syndrome.

To study the function of *FMR1* in the development of human mental retardation, we developed a mouse in which the *Fmr1* gene is inactivated. These knockout mice lack normal *Fmr1* RNA and protein and show enlarged testes, impaired cognitive function, and aberrant behavior. This animal model might shed light on the function of *FMR1* and the pathophysiological mechanisms that lead to mental retardation and behavior abnormalities in humans.

Results

Generating a Mouse Containing an Inactivated *Fmr1* Gene

Mutant *Fmr1* mice were generated by homologous recombination of a targeting vector into the mouse germline using the embryonic stem (ES) cell technology (Mansour et al., 1988). We constructed a homologous recombination targeting vector, pMG5, containing 5.7 kb of mouse genomic *Fmr1* DNA (Figure 1A). Exon 5 was interrupted by the positive selection marker gene neomycin (*neo*). The negative selection marker gene thymidine kinase (*tk*) was inserted 3′ of the genomic sequence in the polylinker of the vector. Vector pMG5 was introduced in E14 ES cells, and selection for homologous recombination events was performed using medium containing G418 and gancyclovir. A clone (CB5.2) found to be positive for a homologous recombination event by polymerase chain reaction (PCR) (Figures 1A and 1B) and Southern blotting (data not shown) was injected into C57BL/6J blastocysts and transferred to pseudopregnant females. Twelve highly chimeric males were crossed with C57BL/6J wild-type females for determination of germline transmission. Two transmitted the ES cell genome to part of their offspring and three showed 100% transmission of the ES cell genome (all females contained the knockout allele). On the DNA level, the presence of the knockout allele in these female mice could be confirmed by PCR analysis (Figure 1B) and Southern blot hybridization (data not shown), similar as used for screening the ES cell clones. F1 heterozygous females were crossed with C57BL/6J wild-type males to obtain knockout males and with the chimeric males to obtain females homozygous for the knockout allele. PCR analysis showed the absence of the wild-type

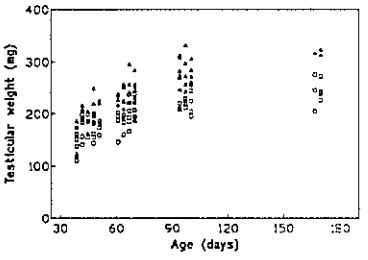

Figure 2. Combined Testicular Weight of Both Testes of Knockout Mice and Control Mice in Time

Knockout mice are shown by closed circles and control mice by open circles. The data are derived from 59 mutant mice and 59 normal littermates.

Table 1. Testicular Weight[a] of Mutant Mice Versus Control Littermates at Different Ages

Age (days)	Mutant Mice	Control Mice	P Value
40–50	190 ± 8 (n = 19)	168 ± 5 (n = 22)	0.02 < P < 0.05 (t = 2.3)
60–70	239 ± 5 (n = 21)	196 ± 5 (n = 19)	P < 0.001 (t = 5.6)
90–100	280 ± 6 (n = 16)	219 ± 6 (n = 11)	P < 0.001 (t = 7.11)
160–170	319 ± 5 (n = 3)	244 ± 9 (n = 7)	P < 0.001 (t = 7.12)

[a] Testicular weight is taken as the combined weight of both testes.

allele in these mutants (Figures 1A and 1B). The male knockouts were shown to be negative for proper *Fmr1* RNA in their testes by reverse transcription (RT)–PCR (Figures 1A and 1C) and to be negative for normal Fmr1 protein in their testes, kidney, liver (data not shown), and brain (Figure 1D) by Western blotting using antibodies raised against a FMR1 fusion protein.

Reproductive Fitness

As it has been suggested that *FMR1* is essential in gametogenesis (Bächner et al., 1993a, 1993b), we studied the reproductive fitness of mutant mice. Crossings were performed between the knockout males and wild-type females and between the homozygous knockout females and wild-type males in order to test the fertility of the mutants. We did not detect a negative selection against germ cells with a mutant *Fmr1* gene at any stage in the breeding experiments. Heterozygous females and both male and female knockout mice were fertile, and the females had normal litter sizes with the expected distribution of offspring with the mutant allele. The transmission of the mutant *Fmr1* gene corresponded well to the expected Mendelian segregation ratios of an X chromosomal gene. (data not shown). The observation that both male and female knockout mice without any protein expression are fertile and have the same size of progeny as controls indicates that *Fmr1* is not necessary for spermatogenesis and oogenesis in mice. Furthermore, mutant mice appear perfectly viable, the oldest knockouts having lived over 250 days.

Phenotype and Major Neurological Functions

Mice lacking *Fmr1* do not exhibit an overt phenotype, but ascertainment of physical features in mice is impaired by a lack of appropriate measurement systems. Macroorchidism is one of the key features of the fragile X syndrome, present in more that 90% of adult males (Turner et al., 1980), and it is thought to develop gradually through time. Therefore, we compared testicular weight of knockout and control mice at four different ages. Testes of knockout mice were significantly heavier than control testes in all four age groups, and the difference became progressively more significant through time (Figure 2; Table 1). Total weight in three age-matched groups and the weight of different organs including kidney, heart, spleen, and liver were not significantly different ($P > 0.05$) from that of control littermates (data not shown).

The major neurological functions of the mutant mice including gait, grooming, circadian activity, swimming, feeding, and mating behavior were normal. It is difficult, however, to recognize minor neurological abnormalities in mice. Also, in human fragile X patients, neurological dysfunction is confined to minor abnormalities such as slight hypotonia, hyperactive deep tendon reflexes, extensor plantar responses, clumsy or sluggish gait, and persistence of pinch synkinesia (Finelli et al., 1985; Vieregge and Froster-Iskenius, 1989; Wisniewski et al., 1991).

Pathological Examination

Light microscopic examination of kidney, heart, spleen, liver, and lung of mutant and normal mice revealed no abnormalities (data not shown). This is consistent with the absence of abnormalities of these organs in human fragile X patients (Hagerman, 1991). Special attention was paid to the brain and testes, as both organs are anatomically and functionally abnormal in human fragile X patients.

Histological studies of human fragile X testes have revealed limited abnormalities: testicular enlargement seems to be due to interstitial edema or increased amount of interstitial tissue (Turner et al., 1975). However, microscopic examination of the testes of mutant mice revealed no structural differences as compared with controls, including a normal pattern of tubule size, a normal amount of interstitial mass, and normal spermatogenesis (Figure 3). No difference was found between the ovaries of control animals and homozygous knockout female mice (data not shown).

Brain weight of seven mutant mice (462 ± 10 mg) was not significantly ($p > 0.05$) different from that of five age-matched (all mice were between 60 and 70 days old) control littermates (467 ± 15 mg). The brains of eight knockout and four wild-type littermates were macroscopically and microscopically normal. The following structures were examined and were found to be normal by light microscopy: frontal, temporal, and occipital cortices, striatum,

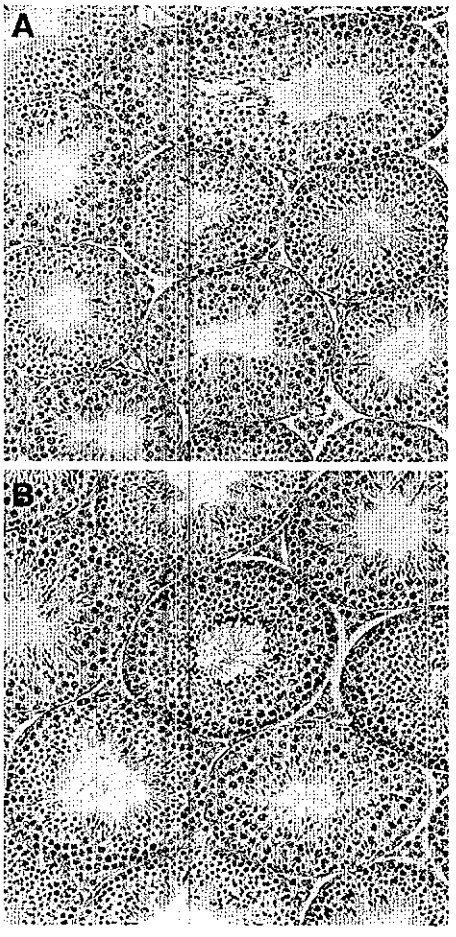

Figure 3. Light Microscopic Micrographs of Paraffin Sections of Testis Normal (A) and Mutant (B) mice (117×). There is no difference in morphology between normal and mutant mice.

corpus callosum, hippocampus, third and lateral ventricles, plexus choroideus, hypothalamus, cerebellum with nucleus dentatus, fourth ventricle, and brain stem. The cortex also had normal numbers of pyramidal neurons and lamination. There was normal myelination of the white matter. Immunohistochemistry showed a normal number of astrocytes with glial fibrillary protein immunoreactivity in the glia limitans and the white matter. Figures 4A and 4B illustrate a cresyl violet staining of normal and mutant brain. We concentrated our pathological survey on hippocampus and cerebellum as the neurons in these structures have the highest expression of *FMR1* (Abitbol et al., 1993; Hinds et al, 1993). To study eventual pathological abnormalities in or near the hippocampal pyramidal cells, we stained coronal sections of the hippocampus with neuron-specific enolase (NSE) antibody (as shown in Figures 4C and 4D) and neurofilament-M antibody (Figures 4E and 4F). The gross architecture and the CA1–CA3 pyramidal and other neurons of the hippocampus were normal. All cerebellar layers were unremarkable, including the molecular and inner granular layer. As the Purkinje cell layer of the cerebellum also shows high *FMR1* expression, we stained sagittal sections of the cerebellum with antibodies against L7 and neurofilament-M (Figures 4G and 4H), but could not detect any abnormality in the mutant mice. Also, the number of cerebellar Purkinje cells in mutant mice (32 ± 1 mm^{-2}; $n = 6$) was not significantly different from that in control littermates (30 ± 1 mm^{-2}; $n = 3$).

Behavioral Tests

The behavior of the knockout mice seemed normal. The mice appeared to have normal social interaction with littermates and human investigators. General assessment of the behavioral state of the mice according to Irwin (1968) was normal, but detailed social interaction studies are being performed now. Behavioral tests of male knockout and male control mice presented here include the passive avoidance task, the exploratory behavior test, and the motor activity test.

Passive Avoidance Task

In this task, mice are placed in a brightly lit compartment of a step-through box. Upon entrance into the dark compartment of the box, they receive an electric shock. The passive avoidance task measures the latency of the mice to enter the dark compartment 24 hr after initial exposure to the shock. Knockout mice performed like normal controls in this test as the mean entrance latency of 11 mutant males (241 ± 34 s) was not significantly different from that of 12 normal littermates (247 ± 29 s).

Exploratory Behavior Test

An exploratory behavior test was performed with 15 knockout mice and 16 normal littermates in a two chambered light–dark transition design. In this test, movement in and between two compartments (one lit and one dark) of a box was monitored for 10 min by infrared beams (Figure 5A).

The difference in time spent in the lit compartment was not statistically significant between mutant mice and control littermates (Figure 5B). However, knockout mice did display much more line crossings in the lit compartment than normal littermates. Both the first and the second beam showed a significant difference (two-tailed Student's t test, $p < 0.01$) between the two groups (Figure 5C). Thus, this test revealed that mutant mice display significantly more exploratory behavior than their normal littermates.

Motor Activity Test

In this test, the activity of mutant versus control mice was monitored by counting the number of crossings through three infrared beams in an empty cage over 40 min after placing the mice in the cage. Five mutant mice (310 ± 16 crossings) showed significantly ($p = 0.002$) more crossings than five control littermates (127 ± 31 crossings).

Cognitive Function Analysis

Specific cognitive defects in human fragile X males include deficits in visual short-term memory, and visual–spatial

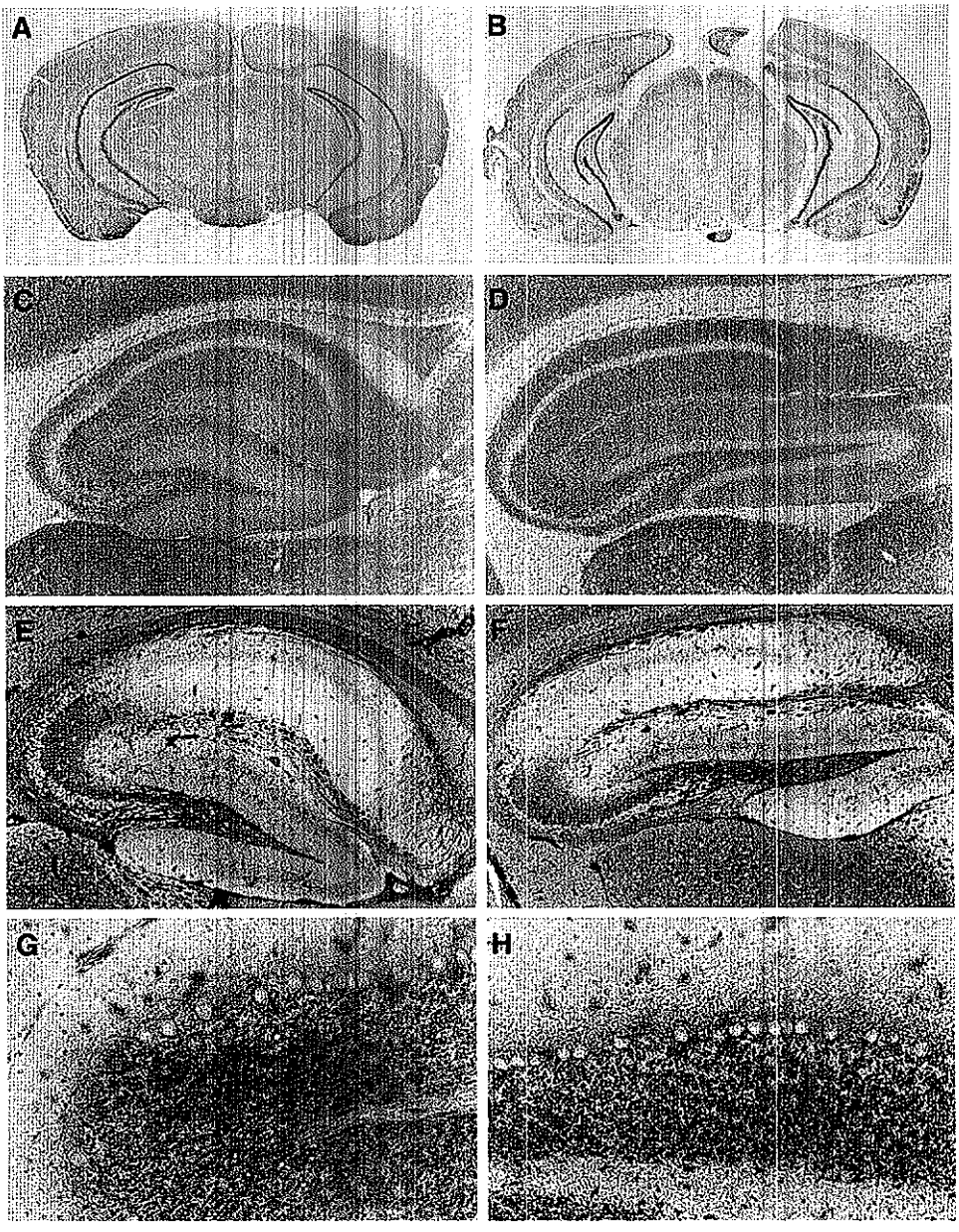

Figure 4. Light Microscopic Micrographs of Cryostat Sections from Brain

Normal (A, C, E, and G) and mutant (B, D, F, and H) mice. (A) and (B) (6.8 ×) show an overview of coronal sections stained with cresyl violet; (C) and (D) (34 ×) are sagittal sections of hippocampus immunostained for neuron-specific enolase; (E) and (F) illustrate sagittal sections of hippocampus labeled for neurofilament-M; (G) and (H) (102 ×) show sagittal sections of cerebellum incubated with antibodies against neurofilament-M. Note that for all parameters studied no difference could be found between normal and mutant mice.

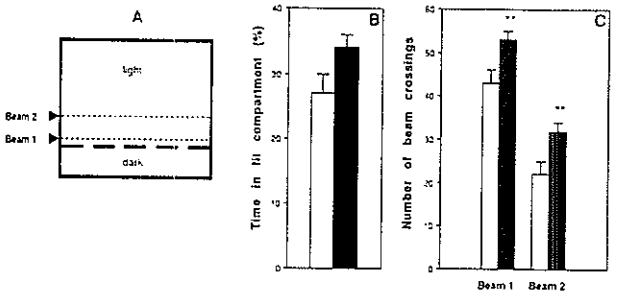

Figure 5. Differences in Exploratory Behavior between Knockout Mice and Normal Littermates

Mean values and SEM are shown. (A) Schematic drawing of the box used in the two chambered light–dark transition test. Dashed lines indicate the position of the two infrared beams through the lit compartment. (B) Comparison between mutant mice (closed box) and normal litter mates (open box) for percentage of time spent in the lit compartment and for number of beam crossings in the lit compartment (C). Asterisks indicate statistical differences between the mutant and age-matched normal littermates (one asterisk, $0.01 < p < 0.05$; two asterisks, $0.001 < p < 0.01$; three asterisks, $p < 0.001$) calculated by a Students' t test.

abilities (Cianchetti et al., 1991; Maes et al., 1994). Since its first description by Richard Morris (1981), the Morris water maze task has been used extensively to study spatial learning in small rodents. In the hidden platform condition of the task, animals are placed in a large circular pool filled with opaque water from which they must learn to escape by locating and climbing onto a platform hidden beneath the water surface. In the visible platform test, the platform is indicated by a clearly visible flag and the mice do rely on proximal cues to find the platform.

We first subjected our mutant mice and their normal littermates to the hidden platform condition of the Morris water maze task. During the 12 learning trials, both knockout mice (one-factor ANOVA, effect of trial number: $F_{(11,156)} = 33.32$, $p = 0.0001$) and their normal littermates ($F_{(11,123)} = 19.93$, $p = 0.0001$) showed highly significant decrease of escape latency and reached similar levels of performance in this task with a latency on the twelfth trial of, respectively, 11.3% and 11.6% of the initial first trial value (Figure 6A). Although knockout mice did reach similar high levels of performance, the knockouts showed longer latencies in the initial training trials as compared with controls (two-factor ANOVA on the first four trials, effect of genotype: $F_{(1,92)} = 6.88$, $p = 0.01$). However, as the interaction trial versus genotype was not significant, there is no difference in the rate of learning between both groups in the training trial. However, when the position of the platform was changed during the reversal trials, mutant mice performed much worse than their normal littermates (two-factor ANOVA on four reversal trials, effect of genotype: $F_{(1,92)} = 37.67$, $p = 0.0001$; interaction effect trial X genotype: $F_{(3,92)} = 7.49$, $p = 0.0002$) (Figure 6B). This indicates that during the reversal trial in the hidden platform condition the rate of acquisition was significantly lower in knockouts than in normal littermates.

Spatial memory was tested by probe trials performed after both the training trials and the reversal trials of the hidden platform condition of the Morris water maze task. If spatial memory is intact, mice spent more than the 25% chance level of their time in the target quadrant that contained the platform in the preceding trial of the hidden platform test. Both the knockouts and the control littermates performed well in the probe trials, and no significant differences ($p > 0.05$) were found. Therefore, both groups show an essentially normal spatial memory in the probe test. It is, however, possible that the knockouts are impaired under more stringent or selective measures of the probe test.

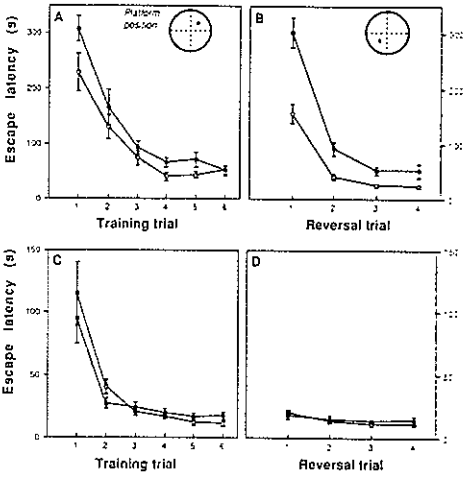

Figure 6. Morris Water Maze Performance of Mutant Mice and Their Normal Littermates

Escape latency and SEM to find a hidden (A and B) of visible (C and D) platform in the Morris water maze were recorded. The escape latency presented here is the sum of four latencies recorded from each corner of the Morris water maze. Hidden platform test with mean escape latency and SEM for 14 male knockouts (closed circles), and 11 normal male littermates (open circles) during six training trials (A) and four reversal trials (B). Visible platform test with mean escape latency and SEM for eight male knockouts (closed circles) and 10 control littermates (open circles) during six training trials (C) and four reversal trials (D). The position of the platform is indicated in the right corner of the graphs.

To exclude that the apparent impaired spatial learning in the hidden platform Morris water maze results from neurological or sensory deficits or lack of motivation, we conducted a visible platform Morris water maze test. In this

condition of the task, the animals do not require the use of a mapping strategy to learn the platform position. Knockouts were not different from their normal littermates in the visible platform condition of the Morris water maze (Figures 6C and 6D). Latency to locate the visible platform in the training trials as well as the rate of decrease of this latency were the same in knockouts and their normal littermates (two-factor ANOVA on first four trials, effect of genotype : $F_{(1,64)} = 0.57$, not significant; interaction effect trial X genotype : $F_{(3,64)} = 0.46$, not significant). Neither were there differences between the latency curves in the reversal trials (two-factor ANOVA on four reversal trials, effect of genotype: $F_{(1,64)} = 1.12$, not significant; interaction effect trial X genotype : $F_{(3,64)} = 0.97$, not significant).

Discussion

In this study, we have produced knockout mice that have no proper *Fmr1* mRNA or protein, which is also the case in human fragile X syndrome. Also, at the clinical level, the knockout mice display abnormalities comparable to those seen in human patients. The combination of macroorchidism and mental retardation in the absence of gross pathological abnormalities of testes and brain is very typical for the fragile X syndrome and is also characteristic of the knockout mice.

Testicular weight of knockout mice at four different ages was significantly greater than that of the control littermates, and macroorchidism gradually develops through time in mutant mice. Also, human fragile X patients develop progressive macroorchidism after puberty (Thake et al., 1985). Consistant pathological abnormalities in testes of human fragile X patients or male knockout mice have not been found, and the macroorchidism seems to be due to an increase in size. The mechanism leading to macroorchidism, however, remains unclear. Nothing is known about the function of *FMR1* in the testis. It has been suggested that expression of the *FMR1* gene is a prerequisite for a proper germ cell proliferation in the testis and in the ovary (Bächner et al 1993a, 1993b), but there is much controversy about the expression of *FMR1* in testes. Studies of RNA (Bächner et al., 1993a, 1993b) and protein expression (Devys et al., 1993) showed high expression of *FMR1* in male germ cells, whereas other studies of RNA expression suggested low expression in male germ cells (Abitbol et al., 1993; Hinds et al., 1993). The present finding of normal fertility of both male and female mutant mice with no *Fmr1* expression indicates that *Fmr1* is not essential in reproduction, at least not in mice. *FMR1* is also not essential in human reproduction in view of the observation that a male fragile X patient without any *FMR1* RNA or protein due to a deletion in *FMR1* encompassing the promoter region has progeny (Meijer et al., 1994). If *Fmr1* expression is not obligate for germline proliferation, the absent Fmr1 protein in the germline of knockout mice cannot be responsible for the development of macroorchidism. This is in line with an earlier observation that fragile X males showing macroorchidism have only the premutation and not the full mutation in their sperm (Reyniers et al., 1993), while their germline epithelium expresses FMR1 (B. A. O., unpublished data). Since no fetal lethality or impaired viability is observed in mutant mice, Fmr1 protein does not play a crucial role in development either.

No gross pathological abnormalities have been described in the brain of human fragile X patients and knockout mice. We studied especially the hippocampus as this brain region is involved in learning and memory and shows high expression of *FMR1*. However, hippocampal abnormalities could not be detected. Reduced size of the posterior cerebellar vermis and pontine region, together with increased size of the fourth ventricle has been noted on magnetic resonance imaging of the brains of human fragile X patients (Reiss et al., 1991a, 1991b). This has also been found in autistic behavior (Courchesne et al., 1988), which is a common feature of fragile X syndrome (Brown et al., 1986; Hagerman et al., 1986). The size of the cerebellum and vermis of the knockout mice is currently being investigated. In autism not associated with fragile X syndrome, a reduction in the amount of cerebellar Purkinje cells has also been reported (Bauman and Kemper, 1985; Ritvo et al., 1986), which may be responsible for the size reduction of the vermis (Murakami et al., 1989). As Purkinje cells have high expression of *FMR1* (Abitbol et al., 1993), we investigated the number of Purkinje cells in the murine cerebellum of the knockouts, but this was not significantly different from that of control littermates. In conclusion, the gross anatomy of the brain of mutant mice is normal.

Human fragile X patients are known to exhibit a more or less specific complex of behavior abnormalities consisting of social avoidance, unusual response to sensory stimuli, hyperactivity, and stereotypic, repetitive behavior in a pattern sometimes consistent with autism (Brown et al., 1986; Hagerman et al., 1986; Cohen et al., 1988; Cianchetti et al., 1991; Reiss and Freund, 1992; Fisch, 1992). Also, the mutant mice show abnormalities such as increased exploratory behavior and motor activity. The increased exploratory activity shown by the mutant mice might indicate slower and less efficient learning of the environment, the result being an increased need to recheck the different chambers. On the other hand, it might also be the result of their hyperactivity.

Cognitive deficits in human fragile X males usually take the form of moderate to severe mental retardation with deficits in visual short-term memory and visual–spatial abilities (Cianchetti et al., 1991; Maes et al., 1994). As the *FMR1* expression is very high in the hippocampus (Abitbol et al., 1993; Hinds et al., 1993) and as the visual spatial disabilities of human fragile X patients might be related to hippocampal dysfunction, we studied the performance of mutant mice in the Morris water maze.

The hidden platform condition of the Morris water maze test shows that mutant mice reached equal levels of performance as controls in the training trials, which was also confirmed by their normal performance in the probe tests. However, when conditions of a well-trained task are changed in the reversal trials, knockouts experience more difficulty than controls in learning these changes in spatial information. At this point, when the position of the platform is changed, the animals have mastered nonspatial aspects

of the task (coping with learning stress, connecting the platform to escape, employing a motor praxis strategy, etc.) as well as spatial aspects (locating the platform with the use of distal cues). When the position of the platform is changed, mice are still able to use much of their knowledge of the nonspatial aspects of the task, and this may explain why normal mice display a much shorter latency during the first reversal trial as compared with the first training trial. Since knockouts reach similar levels of performance as controls at the end of the training session, they seem to be as able as their normal littermates in mastering the nonspatial and spatial aspects of the task. Hence, knockouts do not seem to be impaired in the retrieval of spatial and nonspatial information in training and reversal trials once this information has been learned, but they are significantly impaired in their acquisition of the reversal task. This does not appear to be caused by impairment in their ability to change their nonspatial information scheme (e.g., changing their praxis strategy), since there was no difference between knockouts and controls in the visible condition of the task. The latter also suggests that the increased latency of knockouts in the hidden platform condition is not caused by some underlying motivational, motor, or sensory deficit. Hence, the observed impairment appears to be limited to the spatial abilities of the mouse. This might be connected to the observed hyperactivity and increased exploratory behavior that might be responsible for excessive early search behavior or interfering intertrial hyperactivity. Further research is warranted to learn more about the nature of the observed impairment and about the strategy knockout mice use to locate the hidden platform. In conclusion, the knockout mice provide a good model to study the complex combination of cognitive and noncognitive effects of the fragile X syndrome.

It is not clear what might be the underlying mechanism of this impaired acquisition of novel spatial information in knockout mice. Impaired synaptic plasticity, especially in hippocampus, could be one such possible mechanism. The hidden platform condition of the Morris water maze task is dependent upon hippocampal functions as well as on N-methyl-D-aspartate (NMDA) receptor-mediated long-term potentiation (LTP) of synaptic transmission (Morris et al., 1982, 1986). More recently, two independent groups reported that mice with specifically those genes knocked out that code for kinases supposedly involved in the induction of the LTP process display impaired hippocampal LTP as well as impaired Morris water maze performance (Silva et al., 1992a, 1992b; Grant et al., 1992). It must be pointed out, however, that the latency curve profile of our knockout mice is qualitatively different from that of rodents with hippocampal lesions or impaired NMDA receptor functions. These animals show normal initial learning but remain clearly above the lower asymptote of the normal latency curve. Our *Fmr1* knockout mice, however, show relatively unimpaired initial spatial learning and retrieval of learned spatial information. Prominent difficulties only become apparent when conditions in a well-trained task are changed. To investigate whether *Fmr1* is involved in LTP, it is necessary to perform electrophysiological studies on hippocampal slices.

Learning, memory, and behavior are complicated processes involving different brain regions and many specific proteins. The biological analysis of cognitive functioning therefore requires identification of each of these proteins, before their overall interaction and functioning can be understood. Targeted disruption of specific genes is a powerful tool in the elucidation of the specific role of these genes in brain functioning. The knockout model for the *FMR1* gene presented here is a potentially valid model to provide insight into the physiological function of *FMR1* and the pathophysiology of the fragile X syndrome, as the transgenic mice lack Fmr1 protein and show abnormalities comparable to human fragile X patients. Therefore, these mutant mice offer a good animal model to study the mechanisms leading to macroorchidism, abnormal behavior, and mental retardation. Furthermore, experimental designs can now be made to introduce foreign *FMR1* into the knockout mice in a first step towards gene therapy for fragile X syndrome in humans.

Experimental Procedures

Construction of pMG5, ES Cells, and Transfection

The targeting vector pMG5 was composed of pBluescript KS(−) and a 5.7 kb mouse genomic DNA SalI–SpeI fragment containing exons 4–9 of the *Fmr1* gene. This fragment was obtained from a 129-derived ES cell phage library (provided by Dr. G. Grosveld) after probing with a human *FMR1* cDNA sequence. The vector was interrupted with a *neo* resistance cassette, a 1645 bp blunted XhoI fragment obtained from pPGKneobpA (Soriano et al., 1991), in a blunted unique SnaBI site in exon 5. The *neo* cassette is antisense orientated compared with genomic orientation. A 2.9 kb blunted SalI fragment of the *tk* cassette (pHA140) (Clarke et al., 1992) was inserted 3′ of exon 9 in the blunted NotI site of the vector. The *tk* cassette is also antisense orientated. Vector pMG5 was digested with PvuI to eliminate as much bacterial plasmid sequences as possible, and 15–20 μg of this DNA was used to transfect 1×10^7 E14 ES cells in 400 μl PBS using a Progenetor II Gene Pulser (1200 μF and 117 V during 10 ms). The treated cells were seeded onto 100 mm culture dishes coated with gelatine in medium composed of 60% BRL-conditioned medium, 40% DMEM supplemented with 1 U/ml LIF (GIBCO BRL). The medium was replaced by medium containing 200 μg/ml G418 (Geneticin, GIBCO) 24 hr after transfection. The medium was refreshed every day and during days 3 and 4 was also supplemented with 2 μM gancyclovir (Cymevene). After 7–10 days, clones became visible, could be picked, treated with TE (0.05% trypsine, 0.02% EDTA in PBS), and seeded onto gelatine-coated 24-well plates, one clone per well. After growing to confluency, clones were treated with TE again, and 9/10 of the clone was frozen down slowly and stored in liquid nitrogen in medium containing 10% DMSO. One-tenth of each clone was spun down and resuspended in 30 μl water, and 20 μl 20% Chelex-100 beats (Bio-Rad) was added. The cells were boiled at 95°C for 10 min to free the DNA. This solution (20 μl) was used for PCR analysis. For Southern blot analysis, 1/10 of each clone was grown to confluency again and harvested, and genomic DNA was isolated from the cells.

DNA Analysis

PCR screening for a homologous recombination event was performed using 100 pmol of the primer M2 (5′-ATCTAGTCATGCTATGGATATCAGC-3′) and N2 (5′-GTGGGCTCTATGGCTTCTGAGG-3′) in a PCR buffer containing 10 mM Tris, 10 mM $MgCl_2$, 0.2 mM dNTPs, and 2.5 U Taq DNA polymerase (Bethesda Research Laboratories). The samples were preheated at 94°C for 5 min. Thirty PCR cycles were performed composed of 1.5 min denaturation at 94°C, 1.5 min annealing at 65°C, and 2.5 min extension at 72°C. The products (15 μl) were electrophoresed on a 1.5% agarose gel.

Generation and Analysis of Chimeric and Knockout Mice

The positive ES clone CB5.2 was injected into C57BL/6J blastocysts,

and the blastocysts were transferred to pseudopregnant female mice. Highly chimeric males were crossed with C57BL/6J wild-type females, and the offspring was tailed for DNA analysis after 2–3 weeks. Tail DNA was isolated according to Hogan et al. (1986), and 1 μl was used in the PCR with primer M2 and N2 (see above). Screening for the presence or absence of the wild-type allele was performed using primer S1 (5'-GTGGTTAGCTAAAGTGAGGATGAT-3') and S2 (5'-CAGGTTT-GTTGGGATTAACAGATC-3') (Figures 1A and 1B) in the PCR program described before with an annealing temperature of 63°C instead of 65°C.

RT-PCR

Total RNA was isolated from testes of mutant and control mice using the LiCl method (Verkerk et al., 1993). RNA (5 μg) was reverse transcribed (Pieretti et al., 1991), and 2 μl of the reverse transcribed reaction was directly subjected to PCR using combinations of the primers S1–N2 and S1–S2 according to the program described before with an annealing temperature of 63°C. The PCR products (5 μl) were analyzed on a agarose gel (1.5% agarose and 1.5% low melting temperature agarose).

Western Blotting

Proteins were isolated from brain, testes, liver, and kidney from knockout and control mice. Tissue (200 mg) was homogenized in 750 μl of buffer (10 mM HEPES, 300 mM KCl, 5 mM $MgCl_2$, 100 μM $CaCl_2$, 0.45% Triton X-100, 0.05% Tween [pH 7.4]) using a 8-type pestle (5 × 2 strokes). The homogenates were sonificated for 45 s and spun down for 30 min at 10,000 × g. Seven mg was used in an immunoprecipitation with polyclonal antibodies α734 raised against the middle part of FMR1 protein (Verheij et al., 1993). Immunoprecipitations, gelelectrophoresis, and Western blotting were performed as described by Verheij et al. (1993), with the exception that we used [^{125}I]protein A to detect the polypeptides.

Histochemistry

Twelve mice were killed by ether anaesthesia. Testes were fixed in Bouin's solution, and sections were stained with haematoxylin/azophloxine. Brains were fixed in 4% buffered formaldehyde, methacarn, and Bouin's solution. Two brains were snap frozen in liquid nitrogen chilled isopentane. Paraffin sections were examined blind with respect to the transgenic status of the mice. Sections were stained with cresyl violet, enolase, haematoxylin eosin, and Luxol fast blue. For immunohistochemistry, cryostat sections (7 μm) were fixed with 4% paraformaldehyde followed by a methanol step. Subsequently, sections were incubated with monoclonal antibodies against human neuron-specific enolase (EPOS/NSE, DAKO), neurofilament-M (provided by Dr. J. Q. Trojanowski), or rabbit polyclonal antibodies against L7 (provided by Dr. Morgan), followed by an indirect immunoperoxidase technique using 3,3'-diaminobenzidine.HCl (Serva) as a substrate. Endogenous peroxidases were inhibited by a 30 min incubation in PBS–hydrogen peroxide–sodium-azide solution (Li et al., 1987).

Passive Avoidance Learning

For passive avoidance learning, male mice were placed in the small (5 × 9 cm) brightly lit compartment of a step-through box. After 5 s, the door that led to the big (20 × 30 cm) dark compartment of the box was opened. Knockouts as well as littermates entered the dark compartment within 30 s. Upon entrance of the dark compartment, the sliding door was closed and the mice received a slight electric foot-shock (Coulbourn Instruments Small Animal Shocker, 0.2 mA, 1 s). After 24 hr, the mice were placed in the small compartment once more, and latency to enter the big compartment was noted up to a maximum of 300 s.

Exploratory Behavior Test

The apparatus used in this test consisted of an open box (55 × 75 cm), divided into an illuminated (45 × 75 cm) and a dark (10 × 75 cm) compartment. Transitions between these areas are enabled by means of four semicircular openings (4 cm diameter), evenly spaced (15 cm) in the dividing wall. Three infrared beams are aligned to the dividing wall. One of these is situated in the dark compartment (1 cm distance to the dividing wall), whereas the other two beams are in the illuminated compartment (1 and 7 cm distance to the dividing wall, respectively). Data from the three infrared detectors were continually read into a personal computer and analyzed off-line. Three measurements were derived from this: percentage of time spent in the lit compartment and number of crossings of each of the two beams in the lit compartment.

All tests were conducted between 1 and 5 p.m. in a quiet, dimly lit room. Animals were allowed to adapt to a reversed light–dark cycle (lights on at 5.30 p.m., lights off at 5.30 a.m.) for 8 days prior to testing. Ten minutes before testing, they stayed separately in a uniform white plastic box. Then, they were placed in the dark compartment, and their activity was monitored during their 10 min stay in the box.

Motor Activity Test

Locomotor activity was measured with a technique modified from Crawley and Goodwin (1980). The apparatus used to measure the activity of the mice is an empty transparant cage (16 × 22 cm). Three infrared beams (two perpendicular to the long axis and one perpendicular to the short axis) divide the cage in six compartments. A personal computer interfaced to the three infrared sensors was used to count the number of crossings of the beams after the mice were placed in the cage over 40 min. All measurements were performed with lights on during the day phase of the light–dark cycle of the animals.

Morris Water Maze Task

The water maze used here consisted of a circular grey plastic pool (150 cm in diameter, 30 cm high) filled with water made opaque by nontoxic opacified Lytron 621 and maintained at 18°C. A round perspex platform (15 cm in diameter) was placed inside the pool in a fixed position at the center of one of the four quadrants. The platform was placed 1 cm below the water surface in the hidden platform test, but was clearly visible in the visible platform test. It remained in that position during 12 training trials, whereupon its position was changed to the center of the opposite quadrant for four reversal trials. Two drawings were put on the wall behind the water maze as external cues, and the interior of the experimental room was not changed during the trials. Only males, both knockouts and control littermates, were used in this experiment. Albino animals were not tested in the Morris water task as their performance might be impaired by visual abnormalities. During a swimming trial, the animals were placed in the pool four times, each time at one of the north, south, east, and west starting positions in random order, and with a 15 min interval. If the animals could not find the platform within the maximum swimming time of 120 s, they were placed on the platform and the maximal escape latency of 120 s was noted. They had to stay on the platform for 15 s before removal. Two swimming trials were performed daily, with a 90 min interval between trials. Significance of the effects of trial number and genotype were determined by one- and two-factor analyses of variance (ANOVA). During probe trials, mice had to swim for 100 s in the pool without a platform. Probe trial 1 was performed after the first eight training trials and probe trial II after the four reversal trials of the hidden platform condition of the Morris water maze. A two-tailed Student's t test was used to assess the significance of differences between mutants and control littermates.

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4.2 Publication 2

Introduction of a *Fmr1* transgene in the fragile X knockout mouse

C.E. Bakker[1], R.F. Kooy[2], R. D'Hooge[3], F. Tamanini[1], R. Willemsen[1], I. Nieuwenhuizen[1], B.B.A. de Vries[1], E. Reyniers[2], A.T. Hoogeveen[1], P.J. Willems[3], P.P. De Deyn[3], B.A. Oostra[1]

[1]Dept. of Clinical Genetics, Erasmus University Rotterdam, The Netherlands
[2]Dept. of Medical Genetics, [3]Dept. of Neurochemistry and Behavior, University of Antwerp, Belgium

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INTRODUCTION OF A *FMR1* TRANSGENE IN THE FRAGILE X KNOCKOUT MOUSE.

C.E. Bakker[1], R. F. Kooy[2], R. D' Hooge[3], F. Tamanini[1], R. Willemsen[1], I. Nieuwenhuizen[1], B.B.A. De Vries[1], E. Reyniers[2], A.T Hoogeveen[1], P.J. Willems[3], P.P. De Deyn[3], B.A Oostra[1]

[1]Dept of Clin Genetics, Erasmus Univ, Rotterdam, The Netherlands, [2]Dept of Med Genetics, Univ of Antwerp, Belgium, [3]Lab of Neurochemistry and Behaviour, Born Bunge Foundation, Univ of Antwerp, Belgium

SUMMARY

Patients with fragile X syndrome show mental retardation, behavioural abnormalities, facial anomalies and macroorchidism due to the lack of the FMR1 protein (FMRP). Recently a knockout mouse model for fragile X syndrome has been made through homologous recombination of the murine *Fmr1* gene by an inactivated *Fmr1* gene construct in embryonic stem cells. The knockout mouse lacks Fmrp and shows symptoms similar to those found in fragile X patients. To answer the question whether reintroduction of Fmrp can restore the normal phenotype a transgenic mouse was generated expressing human FMRP. The *FMR1* transgene was under control of a CMV promoter to obtain ubiquitous FMRP expression. Transgenic mice were crossed with knockout mice to obtain a transgenic knockout mouse. The rescue mouse did express *FMR1* protein, but did not show a reversal of the phenotype, most likely because the level of FMRP expressed from the transgene is inadequate or not time or cell specific.

Key words: mental retardation, fragile X syndrome, mouse model, FMRP, transgenic mouse.

INTRODUCTION

Fragile X syndrome is the most common form of inherited mental retardation. Fragile X syndrome patients lack the fragile X mental retardation protein (FMRP). The gene involved is the Fragile X mental retardation gene (*FMR1*) located on the X chromosome (Xq27.3). The prevalence of the fragile X syndrome is 1 in 4000 to 6000 males [1, 2] and the phenotypic abnormalities include behavioural and cognitive deficits, ranging from moderate to severe mental retardation, macroorchidism and facial abnormalities (reviewed by [3]). In fragile X syndrome the *FMR1* gene is inactivated due to methylation of the promoter region and a CGG repeat located in the 3' UTR of *FMR1* [4]. Methylation occurs when the

CGG repeat increases in length to over 200 repeat units (full mutation) upon transmission to the next generation. The full mutation evolves from a premutation which is unstable and contains between 43 to 200 repeats. The carrier of a premutation has normal protein expression and shows a normal phenotype. Normal individuals have a stable repeat of 6 to 53 units [5].

FMRP is widely expressed during human embryonic development but expression decreases to become more localised in specific tissues in successive stages of embryonic development [6, 7]. In adults high expression of FMRP is found in brain in the neurons of the granular layer of the hippocampus and the cerebellum, in the grey matter of the cortex and in Purkinje cells [6-9]. In testis there is a high expression in early spermatogonia [8, 9].

Available tissues of fragile X patients, such as blood cells, cultured skin fibroblasts and (rarely) post-mortem material do not easily allow studies on the molecular pathogenesis of the disease. No naturally occurring animal models for the trinucleotide diseases have been described. To gain more insight in the pathology and physiology of trinucleotide diseases, several groups have tried to generate animal models for the different diseases. The development of an animal model has major advantages. First, the unlimited supply of tissues gives the opportunity to study the effects on the morphological and molecular level. Second, the phenotype and behaviour of mice can be studied in order to understand the development of the disease phenotype found in human patients.

Different animal models can be made. First, a knockout model in which the mouse homologue of the human gene of interest is modulated in such a way that this gene is inactived. Second, a transgenic model can be made in which a mutation is introduced in the mouse homologue of the human gene. Third, a copy of (part of) the human in which the human mutation is present gene can be randomly introduced in the mouse genome.

For FMR1 the knockout mice were generated by replacing the wildtype murine Fmr1 gene, also located on the X chromosome, by a non-functional Fmr1 gene in which a neomycin resistance cassette was placed in exon 5, using homologous recombination in embryonic stem cells employing conventional transgenic ES technology [10]. These ES cells were injected into blastocysts and transferred to pseudopregnant females. Highly chimeric male offspring was crossed with wildtype females to give birth to females heterozygous for the knockout mutation. Breeding those females with wild-type males resulted in knockout males. As a result of the integration of the neo cassette in the Fmr1 gene the mutant mice are no longer able to make normal Fmr1 mRNA. Although the knockout mutation in the animal model is

different from the mutation found in human fragile X patients, both mutations lead to an absence of the FMR1 protein. Phenotypic characteristics as the long face, prominent ears, high-arched palate, flat feet, hand calluses and hyperextensible finger joints have not been found in fragile X mice [11]. One of the most obvious phenotypic characteristics of fragile X patients is macroorchidism; sometimes this is manifested in childhood, but it is present in almost all fragile X patients after puberty. Like in fragile X males, macroorchidism is present in >90% of adult knockout mice [11].

The behaviour of the knockout mice was tested with the use of exploratory and motor activity tests as well as with the Morris water maze test [10, 11]. The tests revealed that the knockout mice had a higher motor activity than control and impaired in the acquisition of novel spatial information. In the Morris water maze test impairments in visual short-term memory and visual-spatial abilities can be measured in small rodents. In the water maze test the mice are placed in a large circular pool filled with opaque water and they have to swim to a platform that is visible or hidden. The knockout mice experienced more difficulty than normal littermates in learning the new position, which was apparent both in increased escape latency and path length. It appears to be due to a relative inability of knockouts to change a learned spatial strategy.

The question is whether the defect in knockout mice can be restored. To address this question the *FMR1* gene was reintroduced into the knockout background to create a rescue mouse. Reintroduction of the protein might tell more about the level of protein expression needed to restore the phenotype, about the importance of the moment of expression and the cell specificity.

MATERIAL AND METHODS

Construction of the transgene

To rescue the knockout phenotype a transgene construct was made. An EcoRI fragment containing the human *FMR1* cDNA [12] was cloned in the EcoRI site of expression vector pcDNA I/amp (Invitrogen)(Fig. 1A). The cDNA contains an CGG repeat of 29 triplets in the 5' UTR, the open reading frame, and approximately 1.6 kb 3' UTR, lacking exon 12 due to alternative splicing, and a 250 bp KpnI-fragment (base 3360 to 3610). The *FMR1* cDNA in this transgene construct is located downstream of the CMV promoter, which constitutively expresses the transgene in all cells. The transgene was linearized with PvuI-AvrII digestion, purified in agarose gel and dialysed over night. A solution of 0.3 ng/µl was microinjected into pronuclei of fertilised murine oocytes.

A second construct was made containing three endogenous introns of the human *FMR1* by introduction of intron 5, 6, and 7 into the transgene through replacement of the BglII fragment of the cDNA (pos. 535 to 875) with its genomic counterpart (Fig. 1B).

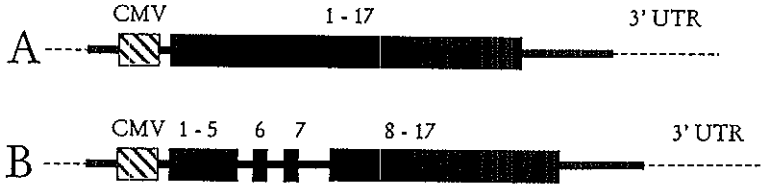

Fig 1. Schematic representation of the transgenic constructs introduced into fertilized murine oocytes. (A) Vector pcDNAI/amp containing the human *FMR1* cDNA, present in mouse line G6 and G8; (B) Vector pcDNAI/amp containing the human *FMR1* cDNA including intron 5, 6 and 7, present in mouse line K1, J22 and J26. Black boxes indicate exon sequences; lines in between black boxes indicate intron sequences.

DNA analysis

The transgenic state of the mice was tested by PCR with primer set S1 (5' GTG GTT AGC TAA AGT GAG GAT GAT 3') in exon 4 and S2 (5' CAG GTT TGT TGG GAT TAA CAG ATC 3') in exon 5. The primers fitted on both human and mouse DNA. This PCR produces an endogenous murine band of 465 bp in which intron 4 is included, and a 125 bp transgenic band derived from the human cDNA. With primer set S1 and K2 (5' AAA ATC CTT ATG TGC CGC CTC TTT 3') a distinction could be made between the construct with and without endogenous introns revealing 1363 and 213 bp respectively. PCR was performed using 100 pmol of the primers in a PCR buffer containing 10 mM Tris, 10 mM $MgCl_2$, 0.2 mM dNTPs, and 2.5 U Taq DNA polymerase (BRL). The samples were preheated at 95°C for 5 min. Thirty PCR cycles were performed composed of 30 sec denaturation at 95 °C, 30 sec annealing at 65 °C, and 1.5 min extension at 72 °C. The products (15 µl) were electrophoresed on a 2% agarose gel. The knockout allele could be detected by PCR with primers M2 and N2 giving rise to a 800 bp band [10]. Mice were tested for the presence of a normal Rd gene with primer set 73 + 82 giving a 580 bp mutated fragment and primer set 81 + 82 giving a 240 bp wild type fragment [13] . Mice homozygous for the mutated allel are visually impaired and skipped in the Morris water maze.

Western blotting and Immunocytochemistry

Brain and testis from rescue and control male mice were used on Western blot to detect the presence and the level of expression of the protein transcribed from the transgene. A biotinylated monoclonal antibody T1Ab directed against FMRP was used for detection [10].

For immunocytochemistry brain and testis samples were embedded in Tissue-Tek (Miles, Inc.) immediately after dissection and snap frozen in liquid nitrogen. Cryostat sections (8 µm) were fixed with 3% paraformaldehyde (10 min) followed by a methanol step (20 min). Endogenous peroxidase activity was inhibited by 30 min incubation in PBS-hydrogen peroxide-sodium azide solution (100 ml 0.1 M PBS + 2 ml 30% H_2O_2 + 1ml sodiumazide)[14]. Free biotin was blocked by an Avidin-Biotin blocking system (DAKO). Subsequently sections were incubated with the monoclonal antibody T1Ab (1:400) for 1 hr. Subsequently, a 45 min incubation with a streptavidin-biotinylated peroxidase complex was performed. Peroxidase activity was demonstrated in a substrate solution containing 3,3'-diamino-benzidine.HCl (Serva). Finally, sections were counterstained with Hematoxilin followed by dehydration in ethanol and

mounting with Entellan.

Testicular weight, and Behavioural and Cognitive tests

Mice were killed in ether and both testicles were carefully prepared free, dried, and weighted. Testicular weight data represent the combined weight of both testes.

Animal selection: The knockout mice, having a FVB/129 genetic background, were screened for the presence of a normal Rd gene, coming form the 129 background, as described before. Mice homozygous for the retinal degeneration mutation (Rd), segregating with the FVB background, were excluded from behavioural and cognitive tests. In addition, albino mice, with little eye pigment, were excluded from the Morris water maze test.

Open field: Mouse movements were registered in a transparent Plexiglas square cage (50 x 50 cm), positioned in an artificially lit room different from the one the mice are kept in. Movements were registered using a video camera. Registration was performed with lights on during the dark phase of the light/dark cycle of the mice. The mice were always released from the same corner of the cage. Registration started 30 sec after release of the mouse, and lasted 10 min. All testswere conducted between 1 and 5.30 p.m. Registered parameters include entries in the centre of the field, entries in the corners of the field, percentage of time spend in the centre, path length, and velocity.

Morris water-maze: The mice were trained to find a submerged platform (Ø 15 cm) in a round basin (Ø 150 cm) filled with opaque water, using exactly the same conditions and protocol as described [15]. Briefly, an experiment consists of 12 learning trials followed by 4 reversal trials, with the platform moved to the centre of the opposite quadrant. Each trial consists of 4 separate releases from each side of the pool. Cutoff time is 120 sec. Total escape latency, total path length and average swim velocity are registered per trial.

Statistical analysis: Statistical analysis was performed using the SPSS program. Groups in the behavioural tests and the probe trials of the Morris water maze test were compared by an independent samples *t*- test with unequal variance. Learning and reversal curves in the Morris water maze were compared by two-way ANOVA.

RESULTS

Construction and analysis of the rescue mouse

Human *FMR1* cDNA constructs under the control of the CMV promoter were injected into fertilised oocytes from wild type FVB mice, establishing five transgenic founder mice. These founder mice were crossed with fragile X knockout mice (genetic background FVB/129) to obtain five lines of transgenic knockout mice, which we called "rescue mice". Mouse lines G6 and G8 contain the *FMR1* cDNA construct without introns (Fig. 1A), while lines K1, J22 and J26 contain the cDNA construct with three endogenous introns (Fig. 1B). In the offspring the presence of the transgene and the knockout status of the animal could be detected by PCR. Southern blotting of mouse tail DNA of first generation offspring showed that there

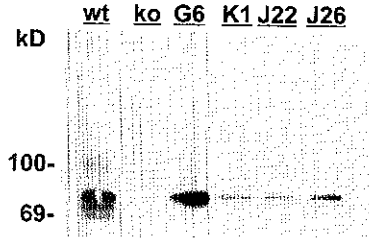

FIG. 2.: Western blot analysis of FMRP expression in brain of a wild type (wt), knockout (ko), G6, K1, J22 and J26 mouse

was one chromosomal site of transgene integration in all mouse lines (data not shown). The pattern of inheritance after crossing showed that the transgene had integrated in an autosome in all five lines.

For an initial characterisation brain and testis tissues of these rescue lines were tested for FMRP expression by Western blotting and by immunocytochemistry. Lines G6, K1, J22 and J26 showed FMRP expression in brain on Western blots (Fig. 2), whereas G8 was found not to express the transgene. Since line G6 had the highest level of expression in brain, although still at a level of approximately 50% of the wild type level (Fig. 2) and similar levels of FMRP expression in testes as seen in wild type (data not shown), we performed further studies with this mouse line.

Successive crossings of G6 mice showed that it was not possible to obtain mice with two transgene alleles, the so-called homozygous transgenic mice. Litter sizes indicated that embryonic lethality is very likely to have occurred in G6 (data not shown). The other lines could be crossed to transgenic homozygosity.

In order to determine in individual cells the localisation of FMRP expressed from the transgene a biotinylated monoclonal antibody directed against both FMRP and Fmrp was used in cryostat sections of the brain. Sections of wild type mouse brain showed staining in neurons of the cortex, hippocampus and brainstem and in the cerebellar Purkinje cells, while in the knockout mice no Fmrp could be detected (data not shown). In G6 mice intense staining was found in the brain in some neurons of the cortex, but not in all. In neurons of the hippocampus, those located in the brainstem and in Purkinje cells intense staining was visible. There was an overall light staining in non-neuronal cells of the brain, such as glia cells, astrocytes and oligodendrocytes, which was not seen in wild type brain sections. Sections of knockout brain were negative in all cells.

Immunocytochemistry on testis sections was not possible due to high background labelling in wild type, G6 and also in knockout mice. The intense background was caused by aspecific binding of the streptavidin to free biotin groups in interstitial cells.

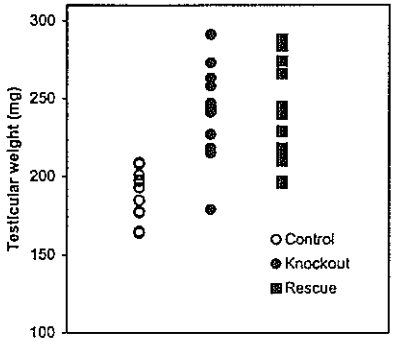

FIG. 3.: Combined testicular weight (mg) of individual wild type (control), knockout, and G6 rescue mice compared.

Testicular weight

The testicular weight (expressed as combined weight of two testes) of the three different genotypes was determined at 16-20 weeks of age. The difference between the mean (± SD) from wild type mice (189 ± 14 mg, N = 14) and knockouts (243 ± 27, N = 15) bred in an FVB background was highly significant ($P << 0.001$), and in the same range as previously described for wild type versus knockouts bred in a C57BL/6J background (Fig. 4). The G6 rescue mouse had a mean testicular weight of 238 ± 33 mg (N = 12), not significantly different from the testicular weight of the knockout mouse. Introduction of the transgene in the knockout mouse had thus no effect on the testicular weight.

Behavioural tests

An open field test was performed to detect possible abnormal spontaneous behaviour of the rescue mice compared to their wild type and knockout littermates. In the open field test, the behaviour of the mouse in a new, empty environment was analysed for 10 min. No significant differences between wild type (N = 13), knockout (N = 14) and G6 rescue mice (N = 9) were observed for entries in the centre of the field, entries in the corners of the field, percentage of time spend in the centre, path length, or velocity (data not shown).

Cognitive functioning of G6 rescue, wild type and knockout mice was compared in the Morris water

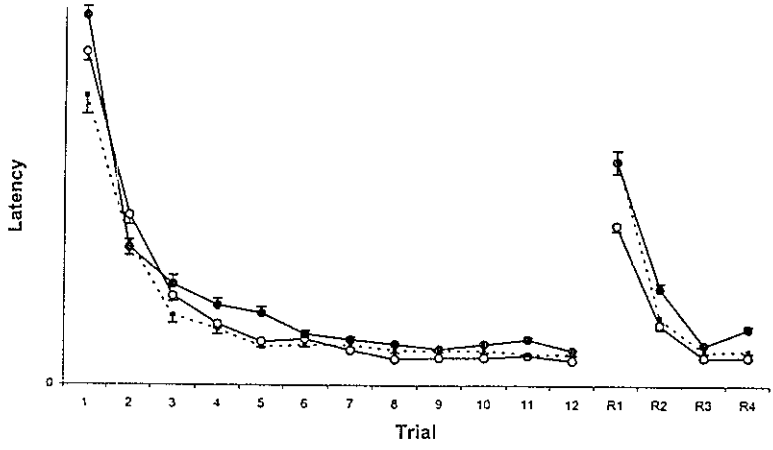

FIG. 4.: Escape latency (s) in the Morris water maze test of 7 G6 rescue (dashed line), 12 wild type (o), and 13 knockout ( ) mice compared. Error beams indicate SEM. The first group of trials (1-12) are learning trials, the second (R1-4) reversal trials. The differences in reversal performance between knockout and wild type mice ($P < 0.01$) and between G6 rescue and wild type mice ($P < 0.05$) are significant.

maze task. Albino mice and mice homozygous for a mutated Rd gene were skipped from this experiment, since they suffer from impaired vision.

In the Morris water maze, mice have to find an invisible escape platform in a circular pool depending on distal cues. Previous experiments with *Fmr1* knockout mice bred in a C57BL/6J background demonstrated that knockout mice show impaired performance in the Morris water maze task, most notable during the reversal trials [10, 11, 15].

Twelve acquisition trial blocks were followed by 4 reversal trial blocks during which the escape platform was moved to the opposite quadrant of the pool. Analysis of the results of the wild type, knockout and rescue mice during the acquisition trials revealed no significant effect of genotype on escape latency (two-way ANOVA for factors genotype and trial block on escape latency; Fig. 4). During the reversal trials, however, two-way ANOVA did show a significant effect of genotype on escape latency. Post-hoc comparison showed that both in the knockout and rescue groups, escape latency was significantly longer

compared to the wild type group ($p<0.05$)(Fig. 4). It is important to note that genotype had a significant effect both on path length ($p<0.004$), and on swimming velocity ($p<0.001$). Post-hoc comparison showed significant differences between wild type and knockout mice in path length, and between wild type and rescue mice in swimming velocity, but not between knockout and rescue mice.

DISCUSSION

We generated five rescue mouse lines by introducing the *FMR1* cDNA into wild type mice and inbreeding them with fragile X knockout mice. As a vector we used pcDNAI/amp containing a CMV promotor and a SV40 intron at the 5'end of the polylinker.

As one of the factors influencing the expression level of the transgene is the presence of introns in the transgene construct, and intron sequences in the transgene are reported to increase the transcriptional efficiency [16], we made in addition to the construct containing the *FMR1* cDNA, a second construct containing the cDNA plus three endogenous introns (intron 5, 6 and 7 of the human gene) (Fig. 1). Nevertheless, of the protein expressing mouse lines (G6, K1, J22 and J26) the line G6, without intron sequences, showed the highest level of expression in brain in a quantitative protein test (Fig. 2). An expression level of about 50% of the wild type FMRP expression was seen in G6 rescue mice. Expression in the testes of G6 rescue mice was at wild type level.

Testicular weight

A nearly 30% increase in testicular weight is a consistent feature of *Fmr1* knockout mice. Hardly any overlap in testicular weight between knockouts and wild type mice was observed in the current (Fig. 4) or previous experiments [10, 11]. The increase in testicular weight of the knockout mice was found to be the result of an increased rate of Sertoli cell division within the normal period of cell proliferation, between embryonic day 12 and day 15 postnatally [17]. During this developmental period, FMRP is expressed in the primordial germ cells of normal testes [9], but it is not clear whether the increased testicular weight of the knockout is the result of absence of FMRP expression locally in the testes or in the brain.

Introduction of the transgene in the knockout background had no significant effect on testicular weight. In foetal testes, FMRP is expressed in the primordial germ cells, while in adult testes, FMRP is expressed in the spermatogonia. *FMR1* expression in normal testes is thus under strict spatial and temporal

control. *FMR1* expression in the G6-rescue mouse is under control of the CMV promoter, which may be less time/place specific than the natural *FMR1* promoter. Therefore, while G6-rescue mice had a similar level of expression as controls as shown on Western blots, it may lack the specificity to rescue the phenotype of the knockout with regard to testicular weight.

Behavioural tests

Previous studies using the Morris water maze protocol have consistently shown slightly, but significantly increased escape latencies in *Fmr1* knockout mice during the reversal trials of the protocol [10, 15]. In one of the previous studies, the difference was shown to be due to increased path length in knockouts during the reversal trials, but reduced swimming velocity was also found in knockout mice during reversal trials, using the visible-platform condition of the task [15]. The differences might be due to impaired reversal learning abilities in knockout mice, but other reversal learning protocols failed to support this hypothesis [11]. As recent findings by Paradee et al (personal communication) show significantly reduced contextual fear conditioning in knockouts, the differences in water maze reversal learning might be due to differences in escape motivation, rather than to impaired learning and memory abilities as such.

We have shown in this study that, both in knockout and rescue mice, escape latency during the reversal trials was significantly longer than in wild type mice. This appeared to be due to increased path length in knockout mice, whereas in rescue mice this was mainly due to decreased swimming velocity. However, since the origin of the reversal learning impairment in knockouts is not clear, and since increased path length as well as reduced swimming velocity have been observed in these mice, the present findings do not provide sufficient evidence to conclude that the behavioural phenotype has been entirely or even partly restored in the rescue mice.

Also, no differences in spontaneous behaviour were observed between knockouts and wild type mice in the open field test, allowing the conclusion that gross motor or behavioural abnormalities are not introduced by transgene expression in the G6-rescue mouse.

Despite the introduction of the transgene the results of the tests performed show that the phenotype of the fragile X knockout mouse is not restored by the introduction of the G6 transgene. Several hypotheses might explain that the phenotype of the G6 rescue mouse is not restored.

First, the level of protein expression might not be high enough in the G6 rescue mouse. A factor

influencing the level of protein expression is the number of copies of the transgene integrated into the mouse genome. Immunocytochemistry showed that the transgene is expressed at a level comparable to wild type levels in some neurons, but not in all neurons. It has been shown [18] that this variegated pattern of expression might be the result of repeat-induced-silencing. The number of copies in an transgene-array is frequently manifested as a decrease in the proportion of cells that express the transgene.

To obtain transgenic mice, in first instance microinjections were performed using transgene concentrations of 6 ng/µl, a concentration normally used in experiments with other genes, but no transgenics were obtained. We lowered the concentration of transgene to 0.3 ng/µl. This was more successful and transgenics were born. An explanation can be that high concentrations of transgene, coinciding with integration of many transgene copies, leads to high expression levels of protein followed by embryonic lethality. The finding that too many copies of the transgene might be lethal in mice is in line with what was noticed in Hela cells. When *FMR1* is present in many copies, the Hela cells die (Steve Warren, personal communication).

A second explanation why the phenotype is not restored can be the expression level per cell. The CMV promoter constitutively expresses the transgene, thus not only in cells which naturally express Fmrp but in all the cells. In a quantitative protein test a homogenate of brain tissue shows half as much protein in G6 rescue mice as is seen in wild type mice. In wild type mice the highest expression is in specific cells, while in G6 rescue mice all the cells express FMRP. This implicates that in G6 rescue mice the expression level per cell is much lower than 50%. Thus the cells that normally do express Fmrp also have a low expression level of FMRP which is not adequate to restore the phenotype. That low levels of FMRP expression are not sufficient for a normal phenotype is also seen in the human situation where affected males appear to be mosaic for a full mutation and a premutation, with this premutation in only a few percentages of their cells.

The use of the CMV promoter might also be involved in the embryonic lethality of homozygous transgenic G6 mice, since this promoter expresses FMRP in the developing transgenic mouse embryo from the time of gestation onwards. In wild type mouse embryos Fmrp expression can be detected on day 10 after gestation, but not before day 7 [7]. Expression of the FMRP during early embryonic development, even at lower levels than normally found in Fmrp expressing cells, might be lethal. Also the expression of the transgenic protein in all cells, so also those cells that normally do not express Fmrp, might contribute to lethality. G6 rescue mice with one transgenic allel will survive since they express only half of the

amount of protein of homozygous transgenic mice. The absence of homozygous transgenic G6 mice could also be caused by integration of the transgene in an endogenous mouse gene that is needed for life in two copies. Last possibility is that all isoforms of FMRP are needed for complete rescue of the fragile X phenotype. In G6 mice only one isoform of FMRP is expressed. We can not role out the possibility that this isoform alone is not sufficient to restore the phenotype, although in humans and mice this isoform is the most abundant.

To elevate the level of protein expression in G6 mice by little steps, G6 mice are being crossed with J26 mice. J26 mice show much less protein expression on western blot and in cryosections, but can be crossed to transgenic homozygozity. In this way we hope to answer the questions whether the lethality of homozygous transgenic G6 mice is due to a too high expression of the *FMR1* gene, especially during early embryogenesis, and to answer the question whether we can create a mouse that shows phenotypic rescue.

If rescue of the phenotype is possible, we will introduce the *FMR1* gene under the control of tissue and time specific promoters to illucidate more about the function of FMRP in the mouse.

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4.3 Publication 3

Immunocytochemical and biochemical characterization of FMRP, FXR1P and FXR2P in the mouse

Cathy E. Bakker, Yolanda de Diego Otero, Carola Bontekoe, Prawien Raghoe, Tanya Luteijn, André T. Hoogeveen, Ben A. Oostra, and Rob Willemsen

Dept. of Clinical Genetics, Erasmus University Rotterdam, The Netherlands

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Immunocytochemical and Biochemical Characterization of FMRP, FXR1P, and FXR2P in the Mouse

Cathy E. Bakker, Yolanda de Diego Otero, Carola Bontekoe, Prawien Raghoe, Tanya Luteijn, André T. Hoogeveen, Ben A. Oostra, and Rob Willemsen[1]

Department of Clinical Genetics, Erasmus University, 3000 DR Rotterdam, The Netherlands

Fragile X syndrome is caused by the absence of expression of the *FMR1* gene. Both *FXR1* and *FXR2* are autosomal gene homologues of *FMR1*. The products of the three genes are belonging to a family of RNA-binding proteins, called FMRP, FXR1P, and FXR2P, respectively, and are associated with polyribosomes as cytoplasmic mRNP particles. The aim of the present study is to obtain more knowledge about the cellular function of the three proteins (Fxr proteins) and their interrelationships *in vivo*. We have utilized monospecific antibodies raised against each of these proteins and performed Western blotting and immunolabeling at the light-microscopic level on tissues of wild-type and *Fmr1* knockout adult mice. In addition, we have performed immunoelectron microscopy on hippocampal neurons of wild-type mice to study the subcellular distribution of the Fxr proteins. A high expression was found in brain and gonads for all three proteins. Skeletal muscle tissue showed only a high expression for Fxr1p. In the brain the three proteins were colocalized in the cytoplasm of the neurons; however, in specific neurons Fxr1p was also found in the nucleolus. Immunoelectronmicrsocopy on hippocampal neurons demonstrated the majority of the three proteins in association with ribosomes and a minority in the nucleus. The colocalization of the Fxr proteins in neurons is consistent with similar cellular functions in those specific cells. The presence of the three proteins in the nucleus of hippocampal neurons suggests a nucleocytoplasmic shuttling for the Fxr proteins. In maturing and adult testis a differential expression was observed for the three proteins in the spermatogenic cells. The similarities and differences between the distribution of the Fxr proteins have implications with respect to their normal function and the pathogenesis of the fragile X syndrome. © 2000 Academic Press

Key Words: **fragile X syndrome; Fmrp; Fxr1p; Fxr2p; localization; mouse tissues.**

INTRODUCTION

The fragile X mental retardation protein (FMRP) is the gene product of the fragile X mental retardation gene 1 (*FMR1*). The gene located at Xq27.3 is characterized by an CGG repeat in the 5′ UTR [1–3]. In normal individuals this repeat contains 6 to 50 CGG units [3]. In nonaffected carriers the repeat is between 50 and 200 units (premutation) and can become expanded upon maternal transmission to the next generation [3, 4]. Repeats expansions above 200 units (full mutation) usually coincides with methylation and inactivation of the *FMR1* gene. Absence of the *FMR1* gene product results in the fragile X syndrome [5–7]. The syndrome is characterized clinically by mental retardation, mild facial abnormalities, and macro-orchidism [8, 9]. Absence of FMRP in neurons is the cause of the mental retardation. The incidence of the disorder is 1 in 4000 males and 1 in 6000 females [10].

The *FMR1* gene is highly conserved among species [11] and is expressed in different isoforms due to alternative splicing [12]. Early studies in mice, using *in situ* hybridization, indicate widespread and strong expression during early embryogenesis and decreasing levels in later stages of embryonic development, whereas in adult mice high expression was found in the brain and the testes [13–15]. Surprisingly, the highest expression was observed in the epithelium of the esophagus and the cortex of the thymus [15]. In whole normal human embryos (3–7 weeks) and fetuses (16 and 25 weeks), *FMR1* mRNA was distributed in the nervous system and in several non-nervous system tissues [16, 17].

The distribution of FMRP in human tissues, using immunohistochemistry, is ubiquitous, albeit at different quantities. High levels are found in most neurons of the central nervous system and spermatogonia in the testes, which is consistent with the phenotype of the syndrome [7, 18–20]. Despite the high levels of *FMR1* mRNA in brain from human embryos, in the same study the quantity of FMRP in the brain was very limited [19], whereas other studies showed in human fetuses an intense labeling of FMRP in neurons [16, 17]. At the subcellular level, FMRP is mainly localized

[1] To whom correspondence and reprint requests should be addressed at Department of Clinical Genetics, Erasmus University Rotterdam, P.O. BOX 1738, 3000 DR Rotterdam, The Netherlands. Fax.: 31-104089489. E-mail: willemsen@ch1.fgg.eur.nl.

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in the cytoplasm either associated with polyribosomes or ribosomes attached to the endoplasmatic reticulum. Additionally in some studies a minority of FMRP is also found in the nucleus either at the nucleopore or in association with the nucleolus [21–23].

Two autosomal homologues of *FMR1* were identified, named *FXR1* and *FXR2* [24, 25]. The three homologues form a small family of proteins, named FXR proteins, and are thought to be related in their physiological function. Their amino acid sequence homology in the N-terminal and central region to FMRP is 86 and 70%, respectively [25]. The two protein isoforms encoded by the *FXR1 gene* (FXR1P) have a molecular mass of 70 and 78 kDa, while recently two novel isoforms of 81 and 84 kDa were identified, specific for skeletal muscle, heart, and differentiated cultured myoblasts [20]. The *FXR2* gene encodes a protein, FXR2P, of 95 kDa [25]. Both FXR1P and FXR2P are coexpressed with FMRP in adult human brain in the cytoplasm of neurons [19]. However, in human fetal brain FXR1P is in a substantial number of neurons also present in the nucleus [19]. In human fetal testes, FMRP and FXR1P are high expressed in all primordial germ cells. Additionally, FXR1P could also be localized to a lesser extent in the nonspermatogenic cells. In contrast, FXR2P was present only in the interstitial cells [19]. Also, in human adult testes, FMRP, FXR1P, and FXR2P are differentially expressed with high expression of FXR1P and FXR2P in the more maturing spermatogenic cells [19, 20].

All three proteins have conserved regions for nuclear localization (NLS) and nuclear export (NES), which suggests a function in shuttling between cytoplasm and nucleus [22, 24–26].They are involved in RNA binding by their two KH domains and an RGG box [24, 25, 27]. The three proteins can form homo- and heterodimers *in vitro* [25]. However, recent studies by Tamanini *et al.* illustrated the preferential presence of homomultimers *in vivo* [28].

Thus far a clear overview of the localization of the Fxr proteins in mouse tissues within one systematic study is missing. Such a descriptive study in combination with biochemistry might elucidate more about the role of the three proteins and their possible interactions. The use of *Fmr1* knockout tissues gives us the opportunity to study changes in Fxr1p and Fxr2p distribution as a result of the lack of Fmrp and might indicate whether they are able to compensate or whether they have a more regulatory function.

In the study presented here, we describe the localization of Fmrp, Fxr1p, and Fxr2p in several adult tissues from wild-type and *Fmr1* knockout mice with special emphasis to the central nervous system and the developing neonatal testis. In addition, we have performed immunoelectron microscopy to study the subcellular localization of the Fxr proteins in hippocampal neurons and Western blotting to identify the different molecular forms.

MATERIALS AND METHODS

Western blotting and antibodies. Wild-type mice were sacrificed and tissues (brain, testis, and skeletal muscle) were immediately dissected and frozen in liquid nitrogen. Western blotting and immunodetection of Fmrp, Fxr1p, and Fxr2p were performed as described previously by Tamanini *et al.* [19] using either Ab1C3 [7] or Ab734 (a rabbit polyclonal antibody against a GST *FMR1* fusion protein covering amino acid position 248 to 320 [21]), Ab2107 (a rabbit polyclonal antibody against the long isoform of FXR1P [19]), and Ab1937 (a rabbit polyclonal antibody against synthetic peptides covering amino acid position 625 to 641 of *FXR2* [19]), respectively.

Immunohistochemistry. Mice were sacrificed by cervical dislocation and organs were dissected and immediately embedded in Tissue-Tek (Miles, Inc.) and snap-frozen in liquid nitrogen. Cryostat sections (8 μm) were fixed with 3% paraformaldehyde (10 min) followed by a methanol step (20 min). Endogenous peroxidase activity was inhibited by a 30-min incubation in PBS–hydrogen peroxide–sodium azide solution (100 ml 0.1 M PBS + 2 ml 30% H_2O_2 + 1 ml 12.5% sodium azide). Sections were incubated with Ab734 at room temperature for 1 h followed by a 45-min incubation with a peroxidase-conjugated secondary antibody. Antigen–antibody complexes were visualized by incubation in substrate solution, containing hydrogen peroxide and 3,3′-diaminobenzidine · HCl (Serva). Finally, the sections were counterstained with hematoxylin.

For studies on paraffin sections, mice were perfused with 3% paraformaldehyde and organs dissected and postfixed overnight in 3% paraformaldehyde. Organs were embedded in paraffin according to standard protocols. Sections (5 μm) were deparaffinized, followed by microwave treatment in 0.01 M sodium citrate solution [7]. Endogenous peroxidase activity was blocked as described for cryostat sections. Sections were incubated with either monoclonal antibody Ab1C3 against FMRP, Ab2107 against FXR1P, or Ab1937 against FXR2P for 1 h at room temperature. For secondary antibodies, visualization and counterstaining were performed as describe above for cryostat sections.

Fmr1 knockout brain was used as a control for the labeling specificity of the antibodies against Fmrp. The labeling specificity of the antibodies against Fxr1p and Fxr2p was checked by performing an immunolabeling using preimmune serum as primary antibody.

Immunoelectron microscopy. Mice were perfused with paraformaldehyde and subsequently the hippocampus was dissected and stored in 2% paraformaldehyde/1 M sucrose for 48 h. The hippocampus was trimmed in small blocks and embedded in Lowicryl K4M according to a standard protocol. Ultrathin sections were cut with a Reichert Ultracut S and immunolabeled for the three proteins using Ab734, Ab2107, and Ab1937 for Fmrp, Fxr1p, and Fxr2p, respectively. Subsequently an incubation with secondary antibodies conjugated with 10 nm colloidal gold was performed. Finally, sections were stained with uranylacetate and lead nitrate and examined in a Philips CM100 at 80 kV. The specificity of the labeling procedure was tested by omitting the primary antibody or substitution of the primary antibody with preimmune serum.

RESULTS

In our study we have tested a number of mouse organs, including brain, spleen, esophagus, liver, gonads, skeletal muscle, intestine, thymus, and kidney. Here we describe only those organs that showed a high expression of the Fxr proteins. Organs displaying dif-

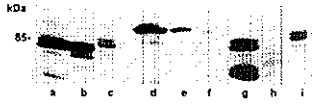


FIG. 1. Western blot analysis and immunodetection of protein samples from adult mouse brain (lanes b, e, and h), skeletal muscle (lanes c, f, and i), and testis (lanes a, d, and g). Immunodetection is performed with antibodies against FMRP (lanes a, b, and c), FXR2P (lanes d, e, and f), and FXR1P (lanes g, h, and i).

ferences in protein localization or expression levels between the different Fxr proteins are also described.

Western Blotting

The Fxr proteins were isolated from brain, testis, and skeletal muscle tissue of mice. In these organs the monospecific antibodies against human FMRP recognized isoforms of 70 to 80 kDa (Figs. 1a–1c). Fxr2p was predominantly present in brain, testis, and skeletal muscle as a 95-kDa form (Figs. 1d–1f). Antibodies against human Fxr1p detected isoforms of 70 and 78 kDa in brain and testis and of approximately 81–84 kDa in skeletal muscle (Figs. 1g–1i).

Immunohistochemistry

Figure 2 illustrates the light-microscopic expression pattern of the Fxr proteins in the cerebellum, the ovaries, and skeletal muscle tissue. Fmrp was highly expressed in the cytoplasm of most neurons in the central nervous system, including Purkinje cells located in the cerebellum (Fig. 2a). Glia cells were not labeled. In ovaries, Fmrp was expressed in the cytoplasm of the ovum, zona pellucida, antrum, and follicular cells of growing follicles (Fig. 2d), whereas the protein was virtually absent in skeletal muscle (Fig. 2g).

Immunolabeling of the central nervous system for Fxr1p results in an expression pattern similar to that of Fmrp with a clear presence in the dendrites. Additionally Fxr1p could be detected in the nucleolus of motorneurons (brainstem) and Purkinje cells (Fig. 2b).

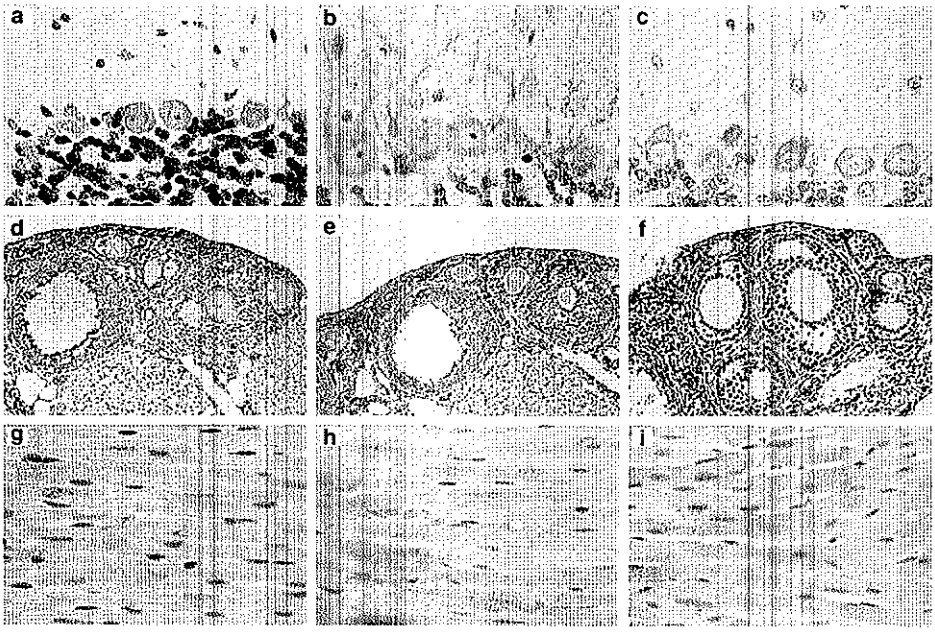


FIG. 2. Immunohistochemistry on paraffin sections of different adult mouse organs using antibodies directed against FMRP (a, d, g), FXR1P (b, e, h), and FXR2P (c, f, i). The brown staining depicts the localization of the different proteins in the cerebellum (a, b, and c), ovary (d, e, and f), and skeletal muscle (g, h, and i).

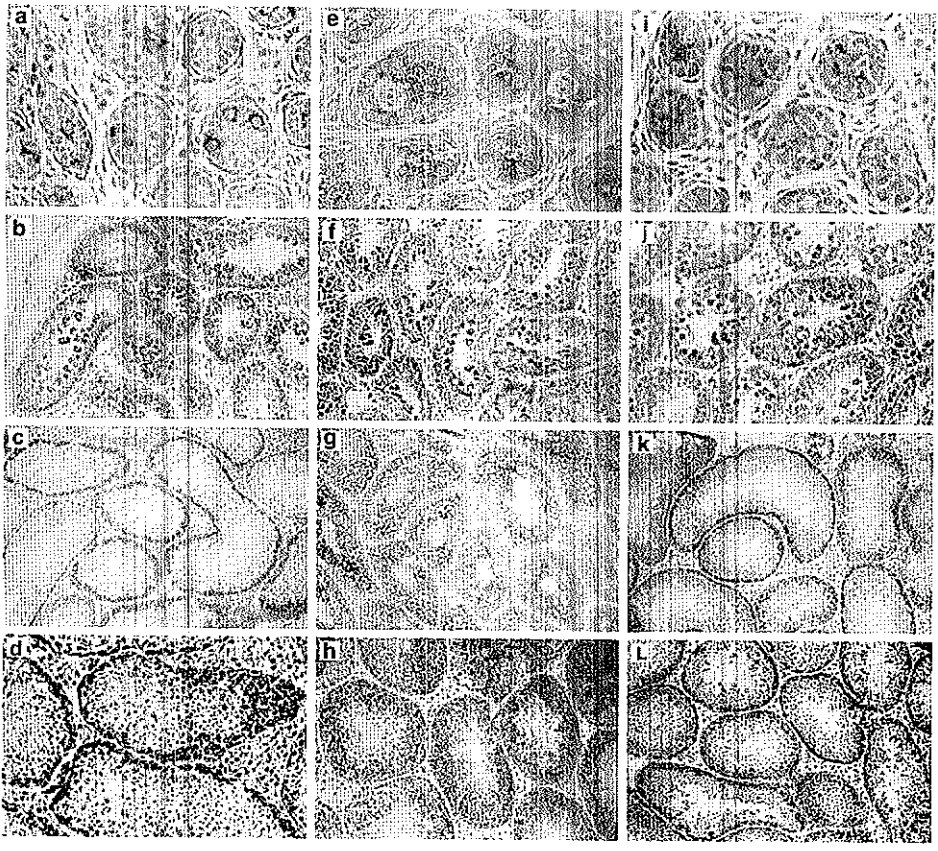


FIG. 3. Immunohistochemistry on cryostat sections of the mouse testis during different developmental stages, using antibodies against FMRP (a, b, c, d), FXR1P (e, f, g, h), and FXR2P (i, j, k, l). The brown staining depicts the localization of the different proteins in a neonatal testis at Day 3 (a, e, and i), at Day 14 (b, f, and j), at Day 28 (c, g, and k), and adult testis (Day 49; d, h, and l).

Also in ovaries Fxr1p showed a labeling pattern similar to that of Fmrp with the exclusion of the antrum; however, the overall intensity of the labeling in ovaries was much higher compared to Fmrp (Fig. 2e). In particular, the contractile bands of skeletal muscle tissue showed a strong labeling for Fxr1p (Fig. 2h).

The labeling pattern for Fxr2p in brain, ovaries, and skeletal muscle (Figs. 2c, 2f, and 2i) was similar to that of Fmrp (Figs. 2a, 2d, and 2g); however, the overall labeling intensity was less compared to Fmrp.

In mouse testis the Fxr proteins were differentially expressed. The results for the developing neonatal and adult testis are depicted in Fig. 3. Three days after birth Fmrp was highly expressed in the cytoplasm of primordial germ cells and to a lesser extent in the cytoplasm of Sertoli cells and interstitial cells (Fig. 3a). At Postnatal Day 7 the labeling pattern was similar to that of 3 days; however, the overall architecture of the tubules was changing (Fig. 3b). At Postnatal Day 14 the Sertoli cells were virtually devoid of labeling, whereas the spermatogonia, which had differentiated from the primordial cells, showed presence of Fmrp in the cytoplasm (Fig. 3c). In adult testis Fmrp was located in the cytoplasm of spermatogonia and in the

cytoplasm of very early primary spermatocytes. No labeling was seen in the later maturing sperm cells (Fig. 3d).

In contrast, Fxr1p showed a completely different labeling pattern. At Postnatal Day 3 the protein was present in high quantities in the cytoplasm of Sertoli cells and in the nucleoli of primordial germ cells, but not in the cytoplasm of primordial germ cells. During later stages Fxr1p was highly expressed in primordial germ cells in the cytoplasm, while the labeling in the Sertoli cells was gradually reduced (Fig. 3f). At Postnatal Day 14 as well as in adult testis Fxr1p was predominantly present in the cytoplasm of maturing sperm cells, which were located throughout the seminiferous tubules (Figs. 3g and 3h).

At Postnatal Day 3 Fxr2p showed a strong labeling in the cytoplasm of Sertoli cells and in the cytoplasm of primordial germ cells (Fig. 3i). In later stages the labeling in Sertoli cells disappeared and the presence in maturing sperm cells became more obvious (Figs. 3j and 3k). In adult testis Fxr2p was clearly present in the cytoplasm of spermatogonia and in the cytoplasm of spermatocytes, whereas spermatids and spermatozoa were almost unlabeled (Fig. 3l).

Low or no expression of the Fxr proteins was found in esophagus, thymus, intestine, liver, kidney, and spleen (data not shown).

Tissues of the *Fmr1* knockout mouse were used to determine the specificity of the labeling procedure for Fmrp and to investigate the localization of Fxr1p and Fxr2p in the absence of Fmrp. Tissues of the *Fmr1* knockout mouse were absolutely unlabeled for Fmrp, but both Fxr1p and Fxr2p were expressed in a pattern and at levels similar to those in tissues from wild-type mouse (data not shown).

Immunoelectron Microscopy

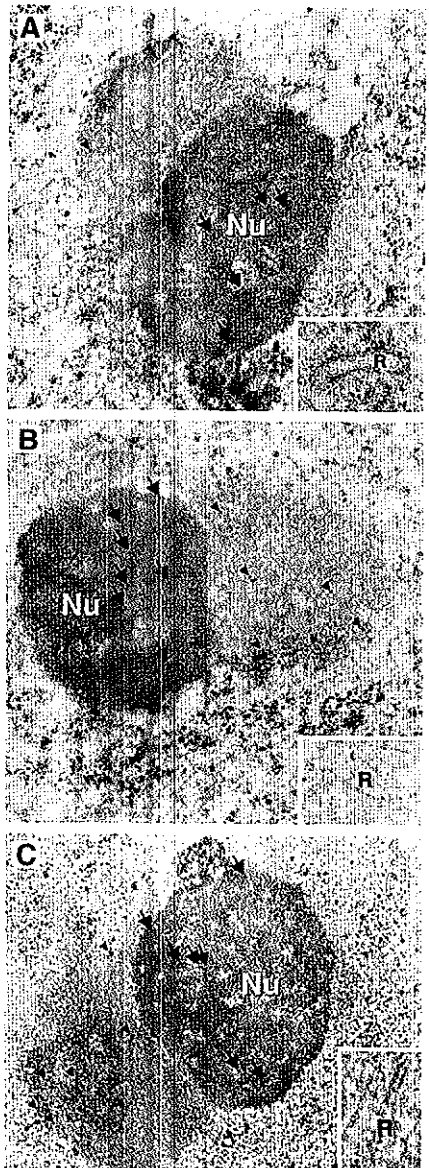
An indirect immunogold procedure was applied to study in more detail the subcellular localization of the Fxr proteins in hippocampal neurons of the mouse. All three proteins were predominantly found in the cytoplasm in association with polyribosomes and ribosomes attached to the endoplasmatic reticulum (Figs. 4a–4c; insets). In addition, we could detect a fraction of Fmrp, Fxr1p, and Fxr2p in the nucleus of hippocampal neurons. Besides a labeling in the nucleoplasm, often in association with heterochromatin, we also observed the presence of the three proteins in the nucleolus (Figs. 4a–4c). Background labeling using preimmune serum or without primary antibody step was negligible.

DISCUSSION

Since the discovery of the involvement of the *FMR1* gene in the fragile X syndrome, many studies have been focused on the unravelment of the physiological function of the gene product, FMRP. Resolving the function might lead to a better understanding of the pathogenesis of the disease. In particular, the cause of the mental retardation has been the major goal of such studies. The presence of two homologues, named FXR1P and FXR2P [25, 29], has complicated these studies because the three proteins are capable of forming *in vitro* heteromers with each other or homomers with themselves [25]. However, recent studies in cultured cells have shown *in vivo* the presence of mainly homo-multimers, which suggests an independent role for each individual Fxr protein under physiological conditions [28]. Here we describe for the first time a systematic study of Fmrp, Fxr1p, and Fxr2p distribution in the neonatal and adult mouse. The results of this study contribute to a better understanding of the physiological function of the Fxr proteins and their interrelationships *in vivo* [13, 15]. The results of our systematic study are discussed on the basis of the organs that showed the highest expression for the Fxr proteins. We compare the distribution of the individual proteins between several organs, whereby the labeling intensity of the neurons in the brain is used as a reference (high labeling). The immunocytochemical methods that were used in this study do not allow comparison between the intensities/quantities of the different Fxr proteins.

Central Nervous System Distribution

At the cellular level we demonstrated a similar labeling pattern of the three proteins in the neurons of the central nervous system with the most abundant labeling in the cytoplasm. Additionally, Fxr1p was also present in the nucleolus of Purkinje cells and motorneurons of the brainstem. At the subcellular level, using a highly sensitive immunogold technique, we demonstrated the presence of all three proteins in the cytoplasm of hippocampal neurons in association with active ribosomes. Thus, the Fxr proteins share not only functional domains, but are also *in vivo* all three associated with ribosomes, suggesting similar cellular functions. A minor amount of the Fxr proteins was present in the nucleus, often in association with heterochromatin in the periphery of the nucleolus and in the nucleolus itself. We were not able to demonstrate this nuclear localization of the Fxr proteins at the light microscopic level, with the exclusion of Fxr1p distribution in the nucleolus of Purkinje cells, motor neurons, and primordial germ cells at Day 3. This discrepancy is probably caused by the higher sensitivity of the immunogold method compared to the indirect alkaline phosphatase method. The picture emerging from these ultrastructural *in vivo* studies in combination with the RNA-binding capacities of the three proteins suggests



a role of the Fxr proteins in interaction with mRNP complexes and translation of specific mRNA's [30]. The role of the nucleolar localization is unclear; however, overexpression studies of FMRP in COS cells have demonstrated the presence of FMRP in the nucleolus too [23]. Whether the Fxr proteins play a role in ribosomal assembly or are involved in mRNA export out of the nucleus remains to be elucidated. Interestingly, a recent study using epitope-tagged FMRP demonstrated the presence of FMRP in a RNP complex together with mRNA, nucleolin, FXR1P, and FXR2P [31]. The subcellular localization of nucleolin has been extensively studied and there is general agreement that nucleolin is predominantly found in the nucleolus, although nucleolin itself does not contain a nucleolar targeting signal [32]. Since targeting of nucleolin to the nucleolus requires RNA, it is tempting to suggest a role for one of the other proteins in the RNP complex for targeting the complex to the nucleolus. Such a targeting signal has recently been identified in FXR2P (A. Hoogeveen, unpublished results). Treatment of transfected COS cells with leptomycin B (LMB), an inhibitor of the exportin1-mediated nuclear export pathway, resulted in accumulation of FXR2P in the nucleolus and both FMRP and FXR1P were retained in the nucleoplasm [33]. The differences between the localization of the FXR proteins after LMB treatment are probably caused by the relatively short transfection time (24–48 h) before treatment of LMB, since the initial experiments on *FMR1*-transfected COS cells resulted only in labeling of FMRP in the nucleolus after 72 h after transfection [23]. Another study using a two-hybrid assay in yeast described the identification of a novel protein interacting with FMRP, called NUFIP [34]. At the subcellular level also NUFIP was demonstrated in the nucleus. Alternatively, recently it has been suggested that the perinucleolar compartment that is localized in the vicinity of the nucleolus may play a role in mRNA export or degradation, which corresponds with the presence of the Fxr proteins in the periphery of the nucleolus [35, 36]. In conclusion, our results confirm the nucleocytoplasmic shuttling hypothesis for all three proteins.

We were able to demonstrate the lack of cross-reactive material with our Fmrp antibodies in tissues from *Fmr1* knockout mice, which was to be expected. In addition, our immunohistochemical study on *Fmr1* knockout brain showed that absence of Fmrp in neu-

FIG. 4. Immunoelectron microscopy on sections of Lowicryl-embedded hippocampal neurons from the mouse, using antibodies against FMRP (A), FXR1P (B), and FXR2P (C). Nu, nucleolus. The arrows indicate the gold particles located within the nucleolus. The arrowheads indicate the gold particles located in the nucleoplasm, associated with heterochromatin. Insets show association of the three protein with the ribosomes attached to the endoplasmic reticulum.

rons does not result in abnormal or significant increased or decreased Fxr1p and Fxr2p neuronal distribution. However, despite the normal distribution of Frx1p and Fxr2p in affected brain, it is possible that both proteins compensate (partially) for the absence of Fmrp. Alternatively, the absence of Fmrp in neurons and thus changes in oligomerization may lead to dysfunction of Fxr1p and Fxr2p, leading to mental retardation. The generation of double knockout mice (*Fmr1/Fxr1* or *Fmr1/Fxr2*) might help to determine possible compensatory functions for the FXR proteins, and the generation of such mice is in progress (B.A. Oostra, unpublished results).

In mouse brain we observed isoforms of the Fxr proteins, which were also described with these antibodies in human brain [19], suggesting the absence of specific isoforms in the brain of the mouse compared to human brain.

Male and Female Reproductive System Distribution

The general picture emerging from our immunohistochemical study on mouse testis confirms earlier studies on adult and fetal human testis (13–20 weeks) showing a differential expression of the FXR proteins in the spermatogenic cells. In this study we have extended our research and focused on the distribution of the Fxr proteins from developing neonatal testis to adult testis. In Sertoli cells of the early neonatal testis all three proteins are highly expressed, but are lost during further development to the adult testis. Sertoli cells are supportive cells during germ cell differentiation and maturation, and they produce many proteins necessary for this function. Perhaps the Fxr proteins play an important role in specific mRNA transport in Sertoli cells during very early spermatogenesis. The sole presence of Fxr1p in the nucleolus of primordial germ cells in 3-day neonatal testis, in contrast to the solely cytoplasmic localization of both Fmrp and Fxr2p, suggests a specific role for Fxr1p in this cell organelle during very early spermatogenesis. In later stages, when primordial germ cells have differentiated into spermatogonia, spermatocytes, spermatids, and spermatozoa, a clear differential expression of the Fxr proteins can be observed. In the adult testis, Fmrp is only localized in spermatogonia, which can be explained by the process of X-inactivation [37], and thus also *Fmr1* inactivation, that occurs in the early spermatocytes. The two autosomal genes, *Fxr1* and *Fxr2*, are not inactivated in this stage and can be transcribed normally, resulting in normal protein production. However, Fxr1p distribution in the more mature spermatogenic cells (spermatids) suggests a more important role in late spermatogenesis for Fxr1p than Fxr2p. Like in the central nervous system, the distribution of both Fxr1p and Fxr2p is not changed in the testis of *Fmr1* knockout mice. However, it should be noted that the methods used in this study do not have a quantitative character but are more semiquantitative. Thus subtle differences in quantities cannot be detected with this technique. The presence of Fmrp in Sertoli cells during early spermatogenesis and in later stages in spermatogonia is not essential for normal spermatogenesis, since male *Fmr1* knockout mice are fertile. The function of the Fxr proteins in the testis remains unclear, including the cause of macroorchidism in fragile X patients and *Fmr1* knockout mice in relation to the absence of Fmrp. Interestingly, it has recently been proposed that increased Sertoli cell proliferation during testis development is the cause of macroorchidism in *Fmr1* knockout mice [38]. In this respect, the high expression of Fmrp in Sertoli cells during very early development justifies further research on development of the testis in neonatal mice, with special emphasis on Sertoli cells.

Western blotting experiments for the Fxr proteins in testis showed the presence of similar isoforms compared to mouse brain, illustrating the absence of specific testicular isoforms.

The distribution of the Fxr in the ovaries has never been described and suggests a function of the Fxr proteins in oogenesis similar to that described for the sperm cells. In this respect it is intriguing that females carrying a premutation are considered to have a significantly higher risk for premature ovarian failure (POF) [39]. Only very recently, a comparative study has shown that POF in fragile X premutation carriers is inherited paternally and might be caused by paternal genomic imprinting [40]. A role for FMRP in maturation of the follicles might be the basis for this phenomenon. The high labeling for Fmrp in the follicular cells, which divide actively in the process of maturation, and the antrum is not completely understood. Although high FMRP expression has been shown before to occur in mitotic active cells, including dividing cells in the process of wound healing [7].

Skeletal Muscle Distribution

Using immunocytochemistry, both Fmrp and Fxr2p are weakly detectable in skeletal muscle tissue, whereas Fxr1p is highly expressed in skeletal muscle tissue. Fxr1p is mainly localized within the muscle contractile bands, which can best be observed in longitudinal sections. In mouse muscle we could detect, using Western blotting, instead of the brain-specific isoforms of 70–78 kDa, two Fxr1p isoforms of 81–84 kDa. These results are in line with a recent study on both Fmrp and Fxr1p characterization in mouse muscle and murine myoblastic cell lines [20]. From these studies it was suggested that these novel isoforms might target only specific mRNA's that are present in

myoblasts and not in differentiated muscle. This would implicate two different functions for these isoforms in muscle cells, depending on the stage of differentitation. Immunoelectron microscopic studies of the specific isoforms of Fxr1p in muscle are in progress and should shed some light on this specific function in the contractile bands of skeletal muscle tissue. The presence of cross-reactive material in muscle extracts for Fmrp and Fxr2p in Western blotting on the one hand and the almost absence of labeling for both proteins in the same tissue probably reflects differences in sensitivity between the two techniques. Also, the denaturing conditions in the Western blot might influence the recognition of the antibodies and thus the efficacy of the antigen–antibody reaction.

In this study we have given an overview of the distribution of Fmrp, Fxr1p, and Fxr2p in several mouse organs. Although this systematic study is more descriptive, it will help further studies in elucidating the role of the Fxr proteins in molecular mechanisms. In particular, immunohistochemical and biochemical studies on double knockout mice (*Fmr1/Fxr1* and *Fmr1/Fxr2*) should give insight and information about a possible role for *in vivo* oligomerization, their interrelationships, and possible compensatory functions of the FXR proteins. In addition, the generation in an *Fmr1* knockout background of transgenic mice that have tissue-specific or cell-specific Fmrp expression will enable us to perform rescue studies for the fragile X phenotype. These mice should provide a means to test novel therapeutic strategies. Our study presents the normal distribution of the Fxr proteins and may comply as a reference for above-mentioned studies.

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4.4 Publication 4

Immunocytochemical characterization of FMRP, FXR1P and FXR2P during embryonic development in the mouse

Yolanda de Diego Otero, Cathy E. Bakker, Prawien Raghoe, Lies-Anne W.F.M. Severijnen, André T. Hoogeveen, Ben A. Oostra, and Rob Willemsen

Dept. of Clinical Genetics, Erasmus University Rotterdam, The Netherlands

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Yolanda de Diego Otero[1]
Cathy E. Bakker[1]
Prawien Raghoe[1]
Lies-Anne W.F.M. Severijnen[1]
Andre Hoogeveen[1]
Ben A. Oostra[1]
Rob Willemsen*[1]

[1]CBG-Department of Clinical Genetics, Erasmus University, Rotterdam, The Netherlands.

Immunocytochemical characterization of FMRP, FXR1P and FXR2P during embryonic development in the mouse

The absence of the *FMR1* (fragile X mental retardation gene 1) gene product, protein FMRP (fragile X mental retardation protein) is causing the fragile X syndrome. FMRP, together with two homologues, called FXR1P and FXR2P, belongs to a small family of RNA-binding proteins (FXR proteins). The precise physiological function of the FXR proteins is unknown, but a role in mRNA transport has been suggested. In the present study, we have performed immunolocalization of these proteins during the embryonic development of the mouse to get more insight in their physiological function. All three proteins are expressed during mouse embryonic development, however, the pattern and intensity varies for each protein at the different developmental stages. During early development, the distribution of the Fxr proteins exhibits high similarities, however, during late development and in the neonate a more differential expression is observed especially in some non-neural tissues. The results of this descriptive study are discussed in relation to the pathogenesis of the fragile X syndrome.

Keywords: fragile X syndrome / *FMR1* / *FXR1* / *FXR2* / embryogenesis.

1 Introduction

The identification of the *FMR1* gene was a first and important step in the understanding of the molecular basis of the fragile X syndrome, the most frequent encountered form of inherited mental retardation in humans [1]. The prevalence is estimated to be 1:4000 males and 1:6000 females. The main characteristics in affected males are mental retardation, macroorchidism, and mild facial abnormalities [2]. The amplification of an CGG trinucleotide repeat upstream the coding region of *FMR1* is the most frequent mutation, which results in hypermethylation of the promoter and thus prevents the transcription of the *FMR1*. The absence or inactivity of the *FMR1* gene product (FMRP) is responsible for the mental retardation observed in fragile X patients.

Two autosomal human homologues of the *FMR1* gene, called *FXR1* and *FXR2*, have been identified. FMRP, FXR1P, and FXR2P form a small family of RNA-binding proteins (FXR protein family). All three proteins contain two KH domains and one RGG Box, which are characteristic for RNA-binding proteins [3–5], and are capable to form multiprotein complexes in vitro [6]. The amino acid sequence of FMRP (MW 70–80 kDa) is highly homologous to those of FXR1P (MW 70–80 kDa) and FXR2P (MW 95 kDa) along the amino-terminal and central regions (86% and 70%, respectively); however, the carboxy-terminal regions of these related proteins are divergent with only 6% similarity [6]. The presence of a nuclear localization signal in the N-terminal part of FMRP and a nuclear export signal within exon 14, suggests a shuttling between the nucleus and the cytoplasm. Similar signals are present in both, FXR1P and FXR2P [6–9]. Recently, such a nucleocytoplasmic shuttling has been demonstrated for the FXR proteins [9,10].

After cloning the *FMR1* gene, in situ hybridization was the first tool to study the expression pattern in a variety of human and murine tissues [11–13]. Later, when antibodies against FMRP became available, immunocytochemistry was used to study the (sub)cellular distribution. In human embryos and fetuses, high levels of *FMR1* mRNA were found in the nervous system and in several non-neural tissues [14,15]. In early stages of mouse embryos high expression of *Fmr1* mRNA is widespread. In later successive stages of mouse embryogenesis this general high expression disappears leading to a specific pattern of high *Fmr1* mRNA expression in the brain, testis, and other tissues [11–13]. The localization of high levels of FMRP, utilizing monoclonal antibodies, has been observed in most neurons of human and mouse adult brain and gonads [16–18]. In human fetal testis, FMRP was predominantly localized in all the primordial germ cells [17,19]. At the subcellular level, FMRP was mainly present in the cytoplasm in association with either polyribosomes or ribosomes attached to the endoplasmic reticulum [20–22], and a minority was found in the nucleus, either at the nucleopore or in association with the nucleolus [20,21].

In situ RNA hybridization studies of *FXR1* in human embryos have shown the presence of large amounts of mRNA in the nervous system and several non-neural tissues, including

Correspondence: Rob Willemsen Ph. D., Department of Clinical Genetics, Erasmus University, Rotterdam, P.O. BOX 1738, NL-3000 DR Rotterdam, The Netherlands.
Phone: +31-10-408-7152; FAX: +31-10-408-9489;
e-mail:willemsen@ch1.fgg.eur.nl

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cartilaginous structures, liver, intestine, skin, kidney, and gonads [15]. Mouse tissues (fetal and adult) showed the presence of *Fxr1* mRNA in skeletal muscle, central nervous system, and gonads [23]. Immunohistochemistry of human embryos and adult tissues showed a similar cytoplasmic labeling pattern of FMRP and FXR1P in human adult brain, whereas in fetal brain, FXR1P was also observed in the nucleus of a substantial number of neurons. In human adult testis, FXR1P was localized in spermatids and in human fetal testis, FXR1P was present in both primordial germ cells and non-spermatogenic cells [17]. In mouse muscle tissue specific isoforms (81–84 kDa; long isoform) of FXR1P were detected within the muscle contractile bands [18].

Thus far, the information about FXR2P distribution in tissues is limited. In human embryos and fetal brain (25 weeks), *FXR2* mRNA expression is similar to *FMR1* and *FXR1* mRNA expression [15]. Immunohistochemistry of FXR2P in human adult brain showed a similar cytoplasmic expression pattern as FMRP and FXR1P, whereas in human adult testis, FXR2P was the only FXR protein detectable in all the cells of the seminiferous tubules. In human fetal testis, FXR2P was only strongly expressed in interstitial cells [17].

Despite all these individual studies, no systematic study has been performed for the distribution of the Fxr proteins during embryonic development of the mouse. Such a study might be of great value to understand the biological function of the three proteins and possible relationships between the FXR proteins. An immunocytochemical study, using monospecific antibodies against the FXR proteins, is more informative than *in situ* hybridization studies, because it will allow detection of the protein instead of mRNA. Furthermore, the high homology between the Fxr genes and the choice of the probes in earlier studies does not exclude the detection of transcripts of the homologous genes. The use of embryonic *Fmr1* knockout tissues will allow studies to compare Fxr1p and Fxr2p distribution in the presence and absence of Fmrp and may tell more about possible compensatory mechanisms of Fxr1p and Fxr2p in the absence of Fmrp. In addition, knowledge about the expression pattern of the three proteins during embryonic development is essential as a reference for studies analyzing transgenic (rescue) mice. Here, we report the distribution of the Fxr proteins in tissues from wild type and *Fmr1* knockout mice from early embryonic stages until neonatal stage, with special attention to the central nervous system and gonads.

2 Materials and methods

2.1 Antibodies

Mouse monoclonal antibody 1a, which recognizes the N-terminal part of the protein, was used to detect FMRP [16]. Rabbit polyclonal antibodies against FXR1P (2107, which recognizes the long and short isoforms) and FXR2P (1937; C-terminal part) were used [17].

2.2 Mouse tissues

Unfertilized oocytes after ovulation, embryos from 0 until 17 days old, and neonates were obtained from wildtype (WT) and *Fmr1* knockout mice (KO). Day 0 of the embryos was determined by checking for the presence of a plug. Initial immunohistochemical experiments of all the different stages were performed on cryostat sections. To obtain a better morphology, similar studies on WT (all stages) and KO (9 and 11 days) tissues were performed on paraffin sections. Since no difference in labeling pattern was observed between both techniques, here only results from paraffin embedded tissues will be shown. Brain sections from adult WT and KO adult mice were used as positive and negative control for FMRP immunostaining.

2.3 Immunohistochemical studies

Mice were sacrificed by cervical dislocation and embryos were immediately dissected. Embryos were fixed overnight in 3% paraformaldehyde and embedded in paraffin according to standard procedures. Sagittal sections (5μm) of the whole embryos were made and sections were deparaffinized in xylene and rehydrated in water. Antigen retrieval was established by microwave treatment in 10 mM sodium citrate [16]. Endogenous peroxidase activity was blocked and subsequently, sections were washed in phosphate buffered saline (PBS) + (0.1 M PBS, pH 7.3, 0.5% Protifar, 0.15% glycine). Sections were incubated with the primary antibodies for 1 hour, followed by an incubation with either rabbit anti-mouse or goat anti-rabbit immunoglobulins, conjugated with peroxidase. Antigen-antibody complexes were visualized with 3',3' diaminobenzidine-tetrahydrocloride (DAB). The sections were counterstained with Gill's Haematoxilin, dehydrated, and mounted with Entellan. As a check for the labeling specificity of the rabbit polyclonal antibodies, sections were incubated with either rabbit pre-immune serum or without primary antibodies. *Fmr1* KO adult brain sections and KO embryos (9 and 11 days) were used as a control for the labeling specificity of the antibodies against Fmrp. Background labeling was negligible.

The signal observed under the microscope was scored according to the intensity of the labeling with: + (low intensity), ++ (medium intensity), +++ (high intensity) ++++ (very high intensity), and – (without signal).

3 Results

In this study, we present the expression pattern of the Fxr proteins during mouse embryonic development, utilizing monospecific antibodies on paraffin-embedded material. We have used an indirect immunoperoxidase technique to visualize the proteins. All stages throughout embryonic development were studied starting with unfertilized oocytes and fertilized eggs as earliest stages followed by 2, 7, 9, 11, 13, 15,

17 days old embryos and neonates. The indirect immunoperoxidase technique is not suitable for comparative quantitative analysis between the Fxr proteins. However, comparison between the different stages during embryonic development for the expression of each individual protein is possible. For this purpose, we have used the expression pattern for each protein in brain from adult WT mouse as a reference and scored this labeling intensity with three plusses. For this study, we have divided the stages of embryonic development in three categories, i.e., early stages (0–10 days), middle stages (11–14 days), and late stages (15–19 days).

3.1 Expression of Fmrp

Fmrp is not detectable during the earliest stage of embryonic development (Figure 1a). The protein expression starts at day 2 of gestational age with the appearance of small deposits in the cytoplasm (Figure 1d, arrows). By day 7 overall weak signal is present in the embryonic tissue, whereas the extraembryonic tissue shows a high labeling intensity (Figure 1g). In 9 days old embryos, an uniform cytoplasmic signal is observed in most embryonic cells, including neural tube (Figure 1j). The expression pattern of Fmrp during the

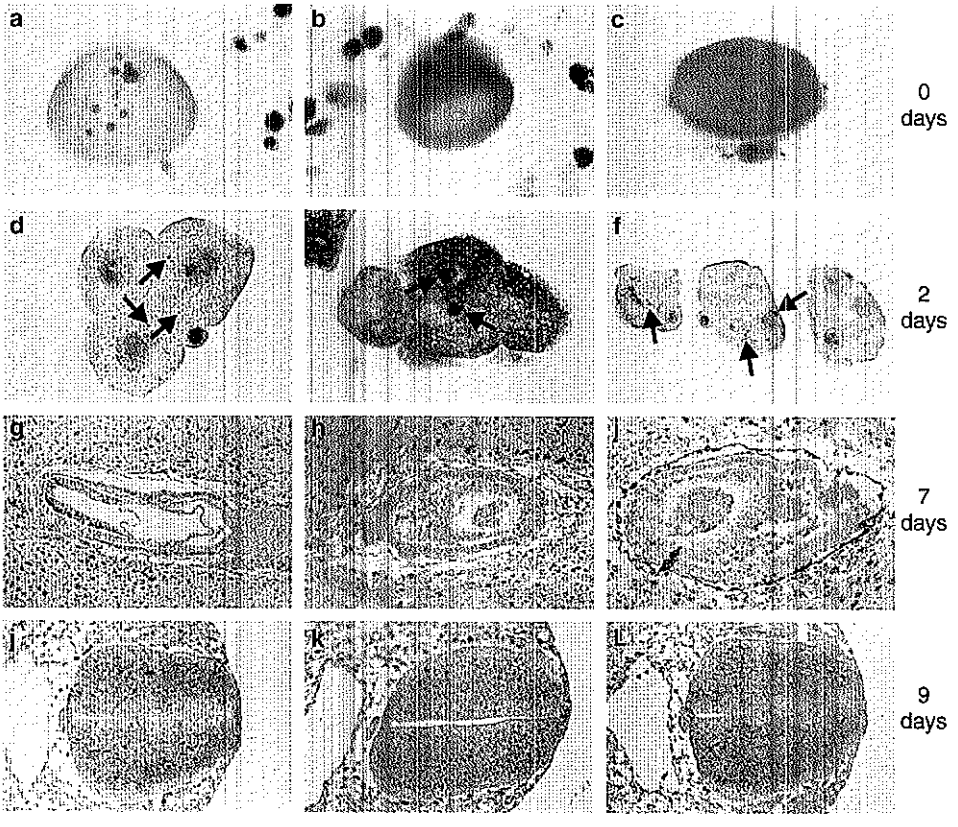

Figure 1. Immunohistochemistry on paraffin sections of early stages of embryonic development of the mouse. Antibodies directed against FMRP (a, d, g, j), FXR1P (b, e, h, k), and FXR2P (c, f, i, l) were used. The brown staining depicts the localization of the three different proteins in WT mouse embryos of 0 days (a, b, c), 2 days (d, e, f), 7 days (g, h, i), and 9 days (neural tube; j, k, l). Note the nucleolar labeling of Fxr1p (e; arrows).

Table 1. Expression of the Fxr proteins during early embryonic development of the mouse

	Fmrp	Fxr1p	Fxr2p
Unfertilized Oocytes	–	++	++
20 hours p.c.* (1 cell stage)	–	++ Cytoplasm	+ Cytoplasm (+++ some deposits)
2 days p.c.* (8 cell stage)	+/– Cytoplasm (+++ small deposits)	+++ Cytoplasm +++ Nucleolus	++ Cytoplasm (+++ some deposits)
7 days p.c.*	Cytoplasm ++ Embryonic tissue +++ Extra-embryonic	All cells ++ Cytoplasm +++ Nucleolus	Cytoplasm + Embryonic + Extraembryonic +++ Reichert's membrane
9 days p.c.*	++ Cytoplasm General signal	++ Cytoplasm General signal	++ Cytoplasm General signal

* p.c. = post conceptional

early embryonic development is summarized in the left column of Table 1.

After these early stages, the immunocytochemical signal increases until the embryos are 11–13 days old; especially, cells originating from the ectodermal germ layer show high labeling intensities in their cytoplasm, including ganglia (Figure 2a), neurons in the brain, and sensory cells. Gonads (Figure 2d) that originate from the mesodermal germ layer are the only exception from the ectodermal germ layer showing a strong labeling, too. Interestingly, the female primordial cells exhibit a much higher expression than male primordial germ cells. Looking at 15 days old embryos, the labeling intensity decreases in several tissues, especially in those that originate from the mesodermal and endodermal germ layer (Table 2). During late stages of embryonic develop-

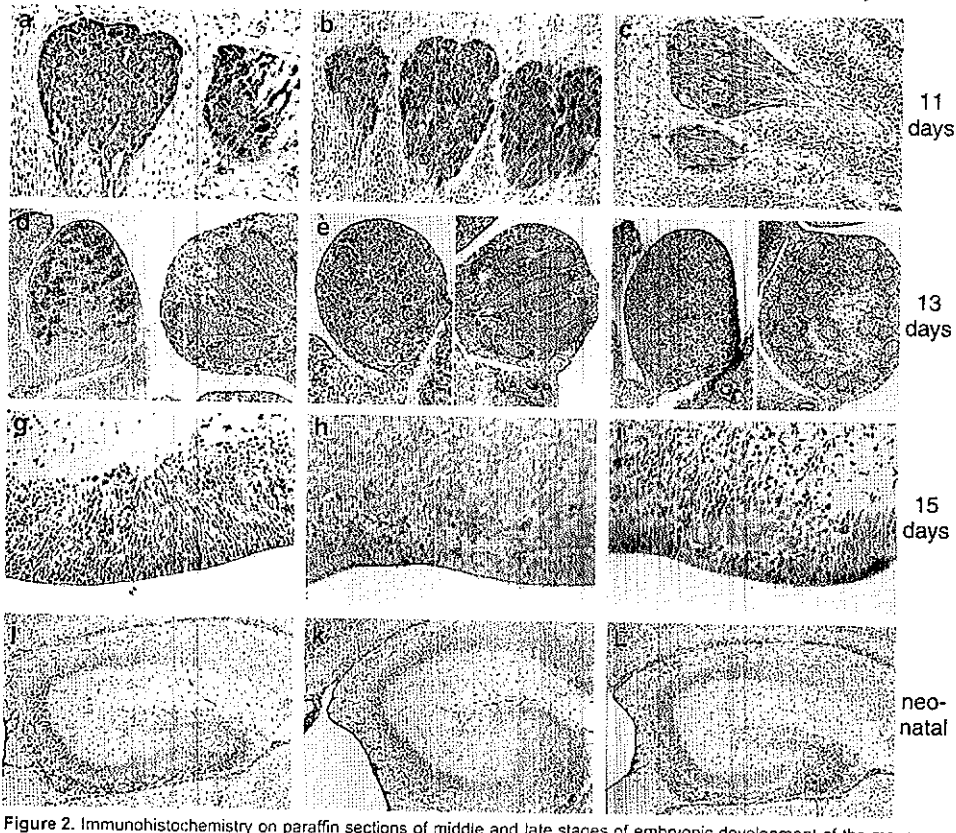

Figure 2. Immunohistochemistry on paraffin sections of middle and late stages of embryonic development of the mouse. Antibodies directed against FMRP (a, d, g, j), FXR1P (b, e, h, k), and FXR2P (c, f, i, l) were used. The brown staining depicts the localization of the three different proteins in the root ganglia of 11 days old WT mouse embryos (a, b, c), gonads of 13 days old WT mouse embryos (d, e, f; on the right site: ovary and on the left site: testis), prolific layer in the telencephalon of 15 days old WT mouse embryos (g, h, i), and hippocampus of WT neonatal mouse (j, k, l).

ment (15–19 days), the labeling intensity in neurons of the brain (Figure 2g) and gonads remains high. In neonates, many organs are virtually devoid of Fmrp (Table 2), as opposed to neurons of the brain (see Figure 2j for hippocampal neurons), gonads, and specific tissues that originate from ectodermal germ layer. Figure 3 illustrates the labeling patterns of the Fxr proteins in tissues with differential localization, including the absence of Fmrp in skeletal muscle (Figure 3a) and the presence in sensory cells of the sense organs, like olfactory epithelium (Figure 3d), otic cells, and receptor cells in the retina of the eyes (data not shown). In addition, ganglia, respiratory epithelium (Figure 3d), choroidal plexus (Figure 3g), chromaffin cells in the adrenal medulla (Figure 3j), and gonads in the neonate show a high Fmrp expression. Tissues of the *Fmr1* KO embryos were devoid of Fmrp (data not shown).

Table 2. Fmrp expression during mouse embryonic development

	Middle stages 11–14 days	Late stages 15–19 days	Neonate
Heart, Vessels, Dermis Skeletal muscle *Pancreas, Thymus* *Digestive epithelium* *Lung, Liver*	++	+	+/–
Thyroid *Kidney* *Adrenal cortex* **Pituitary, Epidermis**	++	++	++
Respiratory epithelium **Ganglions, Spinal cord** **Neurons, Hair follicle** **Choroidal plexus** **Adrenal medulla** **Sensory Cells**	+++	+++	+++
Gonads	+++	++++	++++

Endodermal origin *Mesodermal origin* Ectodermal origin

3.2 Expression of Fxr1p

Fxr1p expression is generally high during all the stages of embryonic development (Table 1 and 3). Expression starts already in the unfertilized and fertilized oocyte (Figure 1b) and continues during later stages. In 2 and 7 days old embryos, Fxr1p is present both in the cytoplasm and the nucleolus (Figure 1e, arrows, and 1 h, respectively). In middle stages the nucleolar labeling disappears and only a high cytoplasmic labeling remains, including neural tube (Figure 2k). In later the stages (15–19 days), Fxr1p is highly expressed in ganglia (Figure 2b) and neurons in the brain (Figure 2 h). Gonads show an intermediate labeling intensity (Figure 2e).

Some tissues show low expression at the late stages, including exocrine pancreas, thymus, kidney, adrenal gland, and blood vessels (Table 3). In neonates, the overall labeling pattern for Fxr1p did not change compared to the late embryonic stages (see Figure 2k for hippocampal neurons).

Figure 3 shows the Fxr1p distribution in the contractile bands of skeletal muscle tissue from the tongue of a neonate (Figure 3b), olfactory sensory cells (Figure 3e), respiratory cells (Figure 3e), and choroidal plexus (Figure 3 h). The absence of Fxr1p in chromaffin cells in the medulla and presence of Fxr1p in the cortex of the adrenal gland of the neonate is shown in Figure 3k. High levels of Fxr1p are also found in stratum spinosum of the epidermis (cytoplasmic and nucleolar; data not shown) and endocrine pancreatic tissue of the neonate. Tissues of the *Fmr1* KO mouse showed similar Fxr1p labeling patterns and levels compared to the tissues of WT mouse (data not shown).

Table 3. Fxr1p expression during mouse embryonic development

	Middle stages 11–14 days	Late stages 15–19 days	Neonate
Exocrine pancreas **Thymus** *Kidney* *Vessels* **Adrenal medulla**	++	+	+/–
Digestive epithelium Liver, Lung *Gonads* *Adrenal cortex* **Hair follicle** **Pituitary**	++	++	++
Thyroid Endocrine pancreas Respiratory epithelium *Heart* *Skeletal muscle* **Neurons Sensory Cells** **Spinal cord, Ganglions** **Choroidal plexus** **Epidermis**	+++	+++	+++

Endodermal origin *Mesodermal origin* Ectodermal origin

3.3 Expression of Fxr2P

Fxr2p distribution during early embryonic development starts in the unfertilized and fertilized oocyte with a clear cytoplasmic labeling (Table 1 and Figure 1c). In 2 days old embryos, Fxr2p, like Fmrp, is present in small deposits located in the cytoplasm (Figure 1f, arrows). At day 7, Fxr2p expression is very high in the Reichert's membrane (Figure 1i, ar-

row) and only weak in the embryonic and extraembryonic tissues (Figure 1i). A general signal is found in the cytoplasm of all the cells in 9 days old embryos, with a strong labeling in the cytoplasm of the cells of the neural tube (Figure 1 l).

Fxr2p labeling intensity does not increase in middle stages, as is illustrated for ganglia (Figure 2c), gonads (Figure 2f), and neurons of the brain (Figure 2i). Table 4 summarizes the results found for this protein during the middle and late stages of embryonic development.

In the neonate, a strong labeling was present in neurons of the brain, including the hippocampus (Figure 2l), and sensory cells (Figure 3f). Some tissues were not overlapping with the labeling of the other two proteins presented in this work, including thyroid gland (see Table 2, Table 3, Table 4), skeletal muscle tissue (Figure 3c), respiratory epithelium (Figure 3f), choroidal plexus (Figure 3i), and adrenal gland (Figure 3 l). In case of Fxr2p, similar results were obtained for *Fmr1* KO tissues compared to WT tissues (data not shown).

Table 4. Fxr2p expression during mouse embryonic development

	Middle stages 11–14 days	Late stages 15–19 days	Neonate
Choroidal plexus			
Digistive epithelium			
Respiratory epithelium			
Liver, Thyroid			
Exocrine pancreas			
Kidney, Dermis	++/+	+/–	–
Adrenal cortex			
Vessels, Heart			
Skeletal muscle			
Epidermis			
Adrenal medulla			
Gonads	++	++	++
Pituitary			
Endocrine pancreas			
Sensory Cells			
Ganglions	+++	+++	+++
Spinal cord			
Neurons			

Endodermal origin *Mesodermal origin* **Ectodermal origin**

4 Discussion

In the last decade, many studies have been performed to understand the pathophysiology of the fragile X syndrome. In particular, the unravelment of the physiological function of FMRP has been a major topic. Despite all the efforts thus far, the specific function of FMRP in relation to the mental retardation in fragile X patients is still unknown. Our investigation is the first systematic study dealing with the expression pattern of the Fxr proteins during all the different stages of the embryonic development of the mouse. We have used the expression level of the individual Fxr protein in the brain of adult WT mice as a reference (high labeling) for the intensity qualification in the embryonic tissues. However, it should be noted that the techniques used in this study do not allow quantification statements between the different Fxr proteins.

4.1 Fmrp expression pattern

During early and middle embryonic development (0–14 days), Fmrp is ubiquitously expressed, albeit at different quantities. During late embryonic development (15–19 days) and in the neonate, Fmrp is not generally expressed, but shows a more specific pattern, mainly in tissues from ectodermal origin, like brain, ganglia, hair follicles, sensory cells, and adrenal medulla. Only gonads, which originate from the mesoderm, are an exception and show a high expression too, especially the primordial cells in the ovaries. The observed differential gene expression during late embryonic development and in the neonate is consistent with studies using footprinting assays, showing the requirement of neuron specific transcription factors (Sp1 and AP2) for *FMR1* gene promoter activity [24]. On the other hand, the low expression of Fmrp in the other tissues suggests a dual function of *Fmr1*. First, a "housekeeping" function in most cell types that originate from the endoderm and mesoderm, illustrated by low levels of expression and, secondly, a more cell-type dependent function illustrated by high levels of expression in cells that originate from the ectoderm. Interestingly, the tissue-specific *Fmr1* gene expression coincides with the organ involvement in the fragile X phenotype. The high expression of Fmrp in female primordial germ cells is of special interest with respect to the significant higher risk for premature ovarian failure (POF) in females with a premutation [25]. A recent study has suggested a role for paternal genomic imprinting as the cause of POF in females carrying a premutation [26]. The precise cellular mechanism for this phenomenon is unknown, but a role for Fmrp in maturation of the follicles might be the basis, however, it can not be excluded that the presence of two active X chromosomes in female primordial cells is the solely reason for the high expression. Our immunocytochemical data are not in line with earlier *in situ* hybridization studies where high levels of *Fmr1* mRNA were not reported in late embryonic stages for ganglia, respiratory epithelium, choroid plexus, sensory cells, and adrenal medulla. In contrast, during late embryonic stages high *Fmr1* mRNA levels were described for thymus, intestine, submandibular gland, and eye [11]. Apparently, the probes used in this *in situ* hybridization study also detect *Fxr1* and *Fxr2* transcripts as a result of the high homology between the Fxr genes. The occurrence of these homolo-

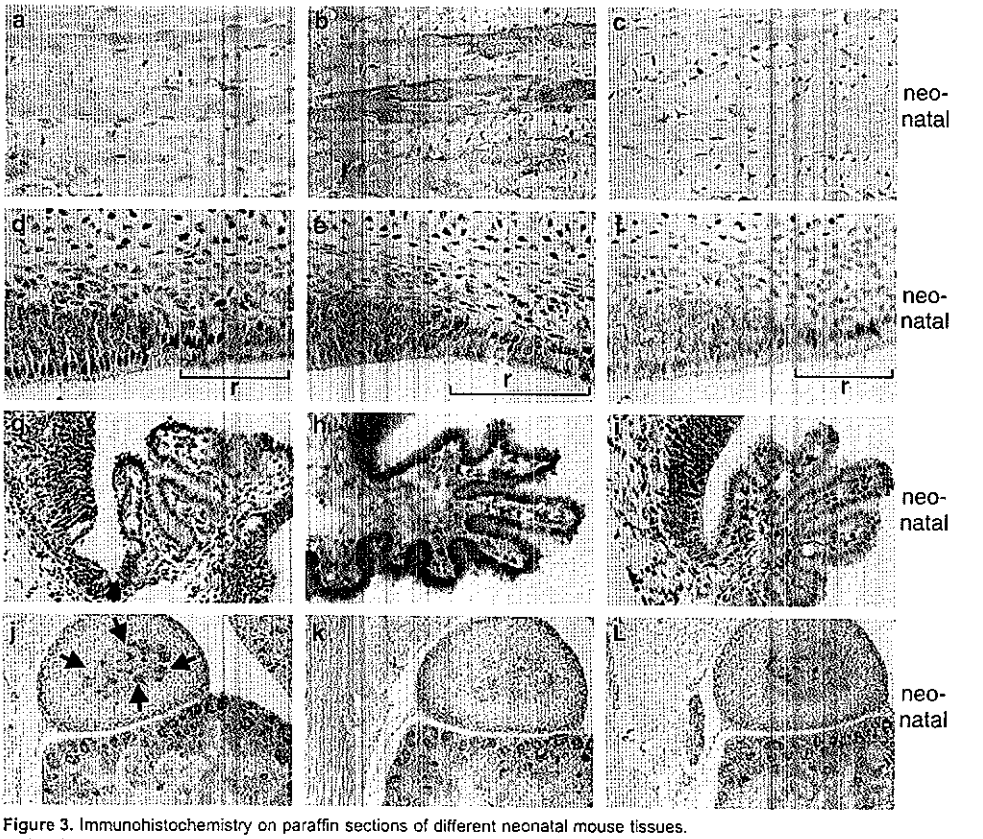

Figure 3. Immunohistochemistry on paraffin sections of different neonatal mouse tissues.
Antibodies directed against FMRP (a, d, g, j), FXR1P (b, e, h, k), and FXR2P (c, f, i, l) were used. Fmrp and Fxr2p are virtually absent in skeletal muscle tissue from the tongue (a and c, respectively), whereas Fxr1p shows a strong labeling. The olfactory sensory cells are labeled for all three proteins (d, e, f), whereas the respiratory cells (r) are only labeled for Fmrp and Fxr1p (d and e, respectively). A similar pattern is observed for the choroidal plexus (g, h, i). The medulla of the adrenal gland shows a positive Fmrp labeling in the chromaffin cells (j, arrows), whereas Fxr1p and Fxr2p are not detectable within these cells (k and l).

gous genes was not known at the time these studies were performed. Alternatively, cross-hybridization with unknown transcripts sharing a high homology with the *Fmr1* gene can be the cause of these conflicting results. *In situ* hybridization studies of *FMR1* mRNA and FMRP expression in early human embryos (3–7 weeks) are consistent with our immunocytochemical study in mice [14,15]. Thus far, late stages of human embryonic development only included brain for *FMR1* mRNA (25 weeks) and FMRP (18 and 25 weeks) expression [14,15,17]. Thus, no information is available about the FMRP distribution or *FMR1* mRNA expression during late human embryogenesis in other tissues than brain. The generation of transgenic mice using a reporter gene (*β*-galactosidase) linked to the *FMR1* gene promoter region revealed high expression of the reporter gene at embryonic day 11.5 in similar cells, as described in this study, including telencephalon, the genital ridge, and the notochord. However, no staining could be detected in cells of the neural tube, the neural crest, and the spinal ganglia [27], whereas we found a clear Fmrp expression. Unfortunately, in this study only one stage of embryonic development (day 11.5) was studied. Apparently, *Fmr1* gene expression during this particular stage of embryonic development is regulated by cis-acting sequences not included in the putative 5'-regulatory region used in this study. The high Fmrp distribution in cells that originate from the ectoderm is intriguing. The current working-hypothesis for the physiological function of FMRP in relation to the observed mental retardation in fra-

gile X patients focuses on a role in mRNA transport in neurons, based on the high expression in neurons and its RNA-binding capacities. In this way, FMRP might influence the translation of neuron-specific mRNA's and the reduced translation efficacy of these neuron-specific mRNA's is thought to be the cause of the observed mental retardation in fragile X patients. The observed altered dendritic spine morphology in the cortex of *Fmr1* KO mice and fragile X patients, suggesting a reduced maturation for spines, has been proposed as a result of the absence of FMRP [28]. Our immunohistochemical data on adrenal gland and sensory cells indicate a role for Fmrp in cellular processes of sensory cells and adrenal gland cells, too. Many patients with fragile X syndrome have sensory and perceptual processing problems which distort the way they receive information and their ability to use it, to learn, and behave appropriately. Recent literature suggests an essential function of corticosteroid hormones, secreted by the adrenal gland, for cognitive performance, via glucocorticoid and mineralcorticoid receptors in hippocampal neurons [28]. Therefore, our data justify that further research should not only be focused on the role of FMRP in cellular processes of neurons located in the central nervous system, but also on cellular processes in sensory cells and the adrenal gland.

Our results demonstrate the total absence of Fmrp in tissues of *Fmr1* knockout embryos, which is in accordance with our expectations. In addition, we could not detect gross morphologic abnormalities in the tissues of KO embryos, which is in line with studies on adult KO mice [30].

4.2 Fxr1p and Fxr2p expression pattern

A general and high expression is found for Fxr1p during all the stages of the embryonic development, which suggests a "housekeeping" function for this gene. The nucleolar localization of Fxr1p during early development indicates a specific role for Fxr1p in this specific cell organelle at this stage of the mouse embryonic development. At the subcellular level, a minority of all three Fxr proteins have been demonstrated to be present in the nucleolus of mouse hippocampal neurons, too (C. Bakker, unpublished results). In addition, FXR1P has been spotted in a RNP complex among other with RNA and nucleolin, a protein known to be predominantly localized in the nucleolus [31]. Recently, also components of another ribonucleoprotein involved in translation of mRNA's, the signal recognition particle, was detected in the nucleolus and suggests a role for the nucleolus in mRNA export [32–34]. The importance of the overall high expression of Fxr1p during mouse embryonic development is further illustrated by the early demise (within 24 hours after birth) of *Fxr1* KO mice (Siomi, personal communication). The strong labeling of Fxr1p in embryonic skeletal muscle within the contractile bands has also been observed in adult mouse skeletal muscle and represents the presence of specific isoforms of 81–84 kDa [18]. It has been suggested that these specific isoforms are involved in targeting specific mRNA's in this particular cell type [35]. The solely strong labeling of Fxr1p during late embryonic development and in the neonate in muscle, epidermis, and endocrine pancreatic tissue (islets of Langerhans) suggests a specific function within these tissues, which is not shared by Fmrp and Fxr2p.

In general, the Fxr2p distribution is similar to Fmrp, however, the overall intensity of the labeling in all the tissues is weaker. The presence of Fxr2p in exocrine pancreatic tissue (somatostatin cells) during late embryonic development and absence of Fmrp and Fxr1p in these cells suggests a specific role for Fxr2p in this cell type.

In embryonic and neonatal mouse brain, we observed similar distribution patterns for the Fxr proteins. In the absence of Fmrp, using *Fmr1* KO brain, we could not detect abnormal or significant increased or decreased Fxr1p and Fxr2p neuronal localization. However, despite this normal distribution of the other two Fxr proteins we cannot exclude (partially) compensatory effects for the absence of Fmrp. The generation of double KO mice (*Fmr1/Fxr1* and *Fmr1/Fxr2*) might be useful to elucidate the contribution of (partially) compensatory effects of Fxr1p and Fxr2p in the phenotype of the *Fmr1* KO mouse. The general picture emerging from our immunohistochemical study on embryonic and neonatal mouse tissues shows nearly similar distribution patterns for the Fxr proteins, albeit with some minor differences regarding intensity and (sub)cellular localization, during early embryonic development of the mouse. At later stages and in the neonate, a more differentiated expression pattern is observed, especially between Fmrp and Fxr2p on the one hand and Fxr1p on the other hand. Further research should be focused on those tissues, involved in fragile X syndrome, showing only expression of one of the Fxr proteins because the effect of compensatory mechanisms is minimal. This overview of the distribution of the Fxr proteins during mouse embryonic development may comply as a reference for future studies using transgenic mice, including transgenic mice with cell specific Fmrp expression in a *Fmr1* KO background to perform rescue studies for the fragile X phenotype.

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4.5 Publication 5

Instability of a $(CGG)_{98}$ repeat in the *Fmr1* promoter

C.J.M. Bontekoe, C.E. Bakker, I.M. Nieuwenhuizen, H. van der Linde, H. Lans, D. de Lange, M.C. Hirst[1], and B.A. Oostra

Dept. of Clinical Genetics, Erasmus University Rotterdam, The Netherlands
[1]Dept. of Biological Sciences, the Open University, Milton Keynes, UK

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Instability of a $(CGG)_{98}$ repeat in the *Fmr1* promoter

C. J. M. Bontekoe, C. E. Bakker, I. M. Nieuwenhuizen, H. van der Linde, H. Lans, D. de Lange, M. C. Hirst[1] and B. A. Oostra*

CBG Department of Clinical Genetics, Erasmus University Rotterdam, PO Box 1738, 3000DR Rotterdam, The Netherlands and [1]Department of Biological Sciences, The Open University, Milton Keynes, UK

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Fragile X syndrome is one of 14 trinucleotide repeat diseases. It arises due to expansion of a CGG repeat which is present in the 5′-untranslated region of the *FMR1* gene, disruption of which leads to mental retardation. The mechanisms involved in trinucleotide repeat expansion are poorly understood and to date, transgenic mouse models containing transgenic expanded CGG repeats have failed to reproduce the instability seen in humans. As both *cis*-acting factors and the genomic context of the CGG repeat are thought to play a role in expansion, we have now generated a knock-in mouse *Fmr1* gene in which the murine $(CGG)_8$ repeat has been exchanged with a human $(CGG)_{98}$ repeat. Unlike other CGG transgenic models, this model shows moderate CGG repeat instability upon both in maternal and paternal transmission. This model will now enable us to study the timing and the mechanism of repeat expansion in mice.

INTRODUCTION

The fragile X syndrome is one of over 14 human diseases associated with expanded trinucleotide repeats. Although much has been elucidated about the genetics of the trinucleotide repeat diseases, little is known about the mechanism(s) that cause the repeat instability. The highly polymorphic CGG trinucleotide repeat which is located in the 5′-untranslated region (5′-UTR) of the fragile X mental retardation gene (*FMR1*), is associated with the disease phenotype when the allele carries more than 200 triplets (1–3). Such alleles undergo methylation which extends across both the CpG island promoter region and the CGG repeat itself. This methylation blocks transcription of the gene which is normally translated into the fragile X mental retardation protein (FMRP) (4,5). This absence of FMRP results in the fragile X phenotype. The main characteristics of the fragile X syndrome are mental retardation and macroorchidism (6), with adult male mental retardation ranging from profound to borderline, with an average IQ in the moderate range. Macroorchidism is a common finding in post-pubescent affected males. With an incidence of 1:4000 males and 1:6000 females, this X-linked disorder is the most common form of inherited mental retardation (7,8).

FMR1 CGG repeat alleles can be divided into three groups: normal, premutation and full mutation alleles. Normal alleles range between 5 and 50 triplets. These alleles are stable upon transmission between generations. Premutations alleles, of between 50 to 200 triplets (9,10), are unstable upon transmission between generations with both expansions and contractions occurring. Since they allow FMRP expression (5,11) they do not result in the development of the fragile X phenotype, but they are prone to expand to full mutations of more than 200 triplets in the next generation. As described above, full mutation alleles with over 200 triplets undergo methylation and result in the absence of FMRP and thus the fragile X phenotype (5,11). These full mutations only arise upon transmission through the female germline, and males never transmit a full mutation to their daughters.

The exact timing of the repeat expansion is still under debate. Repeat expansions must occur during meiosis or early embryonic development (12,13). The most accepted model assumes that full mutations are already present in the oocyte and, thus, all cells in the resulting embryo will also have a full mutation. One, or several, mitotic contraction events to a premutation-length repeat could explain the mosaic pattern which is quite often observed in fragile X patients. The observation that oocytes of full mutation female fetuses also carry full mutation alleles appears to confirm this model, although it cannot be ruled out that the expansion from a premutation to a full mutation occurs during early embryogenesis (12,13). The repeat length(s) present in oocytes of premutation females is not known. In the male germline some kind of selection mechanism has to be assumed (14), as patients with a full mutation have only premutation alleles in their sperm. This mechanism appears, therefore, to protect the male germline against transmission of full mutations. The basis of such a selection mechanism is not known.

Examination of the mode of inheritance of premutations in fragile X families has shown that the risk of expansion to full mutation depends upon the size of the premutation. Small premutations give rise to both expansions and contractions, whereas premutations of over 90 triplets almost always expand to a full mutation in the next generation. The risk of this expansion to a full mutation increases with the length of the CGG repeat (15). This variation in risk accounts for the Sherman paradox (10,16). A more detailed insight into the repeat length and its behaviour upon transmission was gained by sequencing a large number normal and premutation alleles. It was found that most normal alleles are interspersed with

*To whom correspondence should be addressed. Tel: +31 10 4087198; Fax: +31 10 4089489; Email: oostra@kgen.fgg.eur.nl

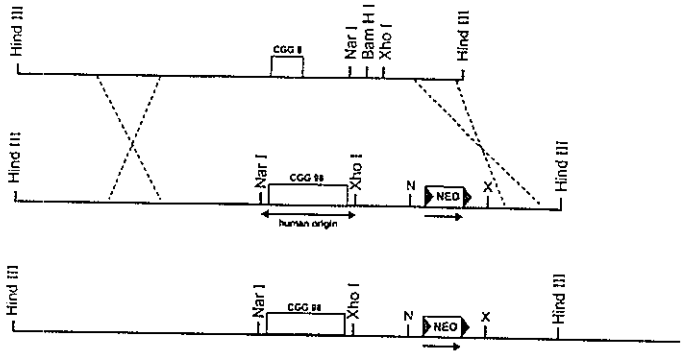

Figure 1. The targeting construct pCB66. The mouse endogenous *Fmr1* $(CGG)_8$ is exchanged for a $(CGG)_{98}$ repeat of human origin. X, removed *Xho*I site, N, removed *Nar*I site.

AGG triplets (17). The most common alleles of between 29 and 32 triplets are interspersed with two AGG interruptions. These AGG interruptions are normally found downstream of shorter tracts of 9 or 10 CGG triplets. In premutations, fewer AGG interruptions are present compared to normal alleles, variation in repeat length is polar and instability occurs always at the 3′ end of the repeat; the region where no or fewer AGG interruptions are present (17,18).

The most 3′ uninterrupted CGG tract appears to be the most important element of the CGG repeat; a pure CGG tract of more than 34–38 CGG triplets is enough to cause instability (19). Most premutation alleles contain only one or two AGG repeats, and the 3′ CGG tract is greater than 35 CGG triplets. Since the longest pure CGG tract is always found at the 3′ end of the CGG repeat and this is also the region where expansion occurs in fragile X families, this might give some insight into the mechanism of instability.

To study the timing and the mechanism of the CGG repeat expansion observed in the fragile X syndrome, it is, for obvious reasons, necessary to have an animal model. With such a model it would be possible to study the behaviour of the repeat through both the female and male germline and during (early) embryonic development. We have reported previously that a $[(CGG)_{11}AGG(CGG)_{60}CAG(CGG)_8]$ repeat was transmitted without any detectable length change through several generations of transgenic mice (20). Similar results were obtained in mice with a $[(CGG)_{22}TGG(CGG)_{43}TGG(CGG)_{21}]$ tract and with tracts having a pure 3′ CGG tract up to 97 triplets (21,22). Several hypotheses have been put forward to explain the stability of these repeats in mice. Firstly, the interruptions present in the first two transgenes discussed above may be acting as stabilizers. However, in the mice carrying a 97 repeat CGG tract at the 3′ end, other factors might be involved. Amongst these, the chromosomal or genomic context might be an important factor.

The *FMR1* gene is highly conserved among vertebrates. The murine homologue, *Fmr1*, is 97% identical in amino acid sequence to the human gene and exhibits an expression pattern very similar to that observed in humans (23,24). This homology extends to the repeat region and also across the promoter. We therefore generated a mouse model in which the endogenous mouse CGG repeat was replaced by a human CGG repeat carrying 98 CGG triplets. This was done using a homologous recombination strategy with a mouse promoter construct where the mouse endogenous $(CGG)_8$ repeat was exchanged for a $(CGG)_{98}$ repeat of human origin. We describe the first generation of such a 'knock-in' CGG triplet mouse and report the behaviour of the premutation allele in the *Fmr1* gene.

RESULTS

Construct *FMR1* promoter region

The mouse homologue of human *FMR1* was isolated from an E14 ES cell phage library (kindly provided by D.Meijer, Department of Genetics, Erasmus University, Rotterdam). A *neo* cassette, flanked by *lox*P sites, was cloned in the *Bam*HI site. The endogenous $(CGG)_8$ present in the mouse promoter was replaced by a $(CGG)_{98}$ of human origin. To allow cloning of the human CGG repeat minimal changes were made to the mouse promoter region (Fig. 1). Cloning of the CGG repeat was the last cloning step, because deletions were often found in plasmids containing the expanded CGG repeat.

Cloning of the mouse promoter region revealed that the mouse promoter region cloned into a vector and propagated in bacteria was, in itself, prone to deletions. Even in the absence of the CGG triplet repeat, deletions were often observed after a simple digestion, re-ligation and transformation into bacteria (different strains were used but in most cases we used the strain *E.coli* DH5α). The frequency of these deletions appeared to be dependent on the restriction enzyme being used or the localization of the restriction sites in the construct, as well as the ligation buffer used (data not shown). Since for most DNA constructs digestion, re-ligation and transformation is a very straightforward experiment, this could indicate that *cis*-acting factors important for instability might be present in the mouse promoter region. Comparison of the promoter sequence of *FMR1* and *Fmr1* showed that all the identified regulatory

elements were conserved. Whether or not all these elements are functional is not known.

Generation of the mice

Homologous recombinants were recognized by the absence of the endogenous mouse $(CGG)_8$ allele, and the presence of the expanded $(CGG)_{98}$ allele. Of the 1200 screened ES cell clones, 13 were identified as possible homologous recombinants. The length of the observed expanded CGG repeat differed between the clones. These different lengths most likely represent deletions of the repeat and/or flanking sequences in the plasmid DNA.

Clone 651 contained exactly the same length as the cloned $(CGG)_{98}$ repeat in the plasmid. This clone was therefore selected for blastocyst injection from which three chimeras with the same repeat length were identified. These animals were crossed onto an FVB background and the repeat length was determined in the next generation. The first heterozygous females were crossed either with a wild-type male or a male expressing *Cre*-recombinase. Expression of *Cre*-recombinase allows deletion of the *neo* cassette present between the *lox*P sites, minimizing the changes to the mouse genomic flanking sequence in the resultant transgenic animal. $(CGG)_{98}$ mice with [$(CGG)_{98}$/*neo*] and without [$(CGG)_{98}$/*neo*$^-$] the inserted *neo* marker were subsequently crossed and the offspring examined for repeat length.

In total, 155 $(CGG)_{98}$ transmissions were studied. $(CGG)_{98}$/*neo* was transmitted 34 times, 15 male transmissions and 19 female transmissions. $(CGG)_{98}$/*neo*$^-$ was transmitted 121 times, 80 maternal transmissions and 41 paternal transmissions. Offspring homozygous for the expanded repeat were included for paternal (13) and maternal (13) transmission. In total, 15 instabilities were found using a radioactive PCR assay to detect CGG length (Fig. 2). Confirmation and exact sizing of the repeat was carried out using the CGG expansion method of Perkin-Elmer. An example is shown in Figure 3 and a summary is presented in Table 1. In total, we have observed 15 alterations in length of the CGG repeat: two contractions and 13 expansions. Both contractions (of 11 triplets) were observed after (different) paternal transmission, but male transmission also led to six expansions. Five expansions were observed in transmission via the female germline. Two instabilities were observed in homozygous $(CGG)_{98}$ female offspring and in these cases the origin of the instability could be either paternal or maternal. The shortest CGG repeat present in the descendent mice is 87 CGGs and after passages through several generations the longest repeat is 108 CGGs. While the number of paternal changes (at least eight) outweigh maternal ones (at least five), the number of maternal transmissions (at least 86) appears to be greater than paternal ones (at least 43). Thus, the rate of change in paternal transmission of unstable repeats would appear to be 3-fold that of maternal transmission.

DISCUSSION

The mechanism underlying fragile X repeat instability in humans is still unknown, although many pathways and intermediates have been implicated. It has been postulated that during DNA replication, slippage synthesis can occur within the repeat due to the repetitive nature of the sequence. Through

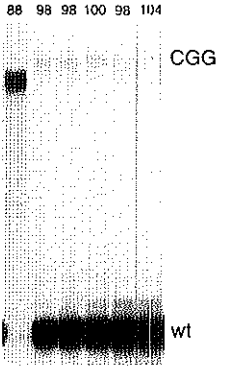

Figure 2. CGG repeat instabilities detected by radioactive PCR. On top of the lanes are the sizes as determined with the Fragile X polymorphism assay.

this and the formation of single-stranded breaks, both expansion and contraction can occur (25,26). It has also been suggested that secondary structures such as hairpins and tetraplex DNA might also play a role in repeat expansion, but the existence of these remains to be established *in vivo* (27). From *in vitro* studies, as well as studies in bacteria and yeast, it is also clear that repeat stability is dependent on the length of the repeat and the number of AGG interruptions, reminiscent of the situation in fragile X families (28,29). Furthermore, for yeast and bacteria it has been observed that host cell genotypes, the orientation of the repeat with respect to DNA replication and the position of the cloned repeat within the vector can all influence instability (30).

In humans carrying expanded CGG repeats, their length increases over generations. Once beyond a specific threshold the repeat becomes dramatically unstable, expanding rapidly up to a few thousand triplets. This phenomenon has given rise to the term 'dynamic mutation' (31). Although for some trinucleotide repeat mouse models small expansions or contractions have been observed, dynamic mutations such as those in human disease have never been observed. Before this study, transgenic mouse models have carried non-targeted autosomal CGG repeats and all have failed to show any evidence of instability (20–22). The absence of important *cis*-acting factors and the random integration of the transgene on one of the autosomes instead of the X chromosome are both possible explanations for the relative stability of the CGG repeats in the earlier mouse models (20).

To circumvent this, we generated a mouse with an expanded CGG repeat within the endogenous *Fmr1* promoter by using a homologous recombination technique. In this way, the behaviour of the expanded CGG repeat can be studied in its endogenous genomic localization. In total, 155 transmissions of the expanded $(CGG)_{98}$ allele were studied and 15 instabilities were observed: two contraction events and 13 expansions. As detection was performed using a radioactive PCR technique (where small changes are difficult to detect) this equates to a rate of instability of at least 10%. Both contractions occurred

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Table 1. Total numbers of male and female transmissions for both $(CGG)_{98}/neo$ and $(CGG)_{98}/neo^-$

	Female/male transmissions	Instabilities
$(CGG)_{98}/neo$	Female 19	0
$(CGG)_{98}/neo$	Male 15	4 (+2, +6, +4, +4)
$(CGG)_{98}/neo^-$	Female 67	5 (+2, +2, +1, +1, +1)
$(CGG)_{98}/neo^-$	Male 28	4 (−11, −11, +5, +3)
$(CGG)_{98}/neo^-$	Female/Male 13/13	2 (+2, +3)

Most instabilities are found upon male transmission. Only for a limited number the size of the instabilities is determined. Length changes are depicted in parentheses.

by paternal transmission, as did six of the expansions. Two expansions could be of either paternal or maternal origin. Given the total number of maternal and paternal transmissions, these results are surprising. Although the numbers are still limited, there appears to be a tendency of higher instability (both expansion and contraction) upon paternal transmission. In human fragile X premutations, small changes in repeat length of this magnitude are observed upon both male and female transmission. In contrast, dynamic mutations in humans are only observed upon female transmission. However, in this $(CGG)_{98}$ mouse model, no dynamic mutations were observed. In contrast to fragile X syndrome, most other trinucleotide repeat disorders show preferential instability upon male transmission. For one transgenic mouse model containing an expanded CAG repeat, it was found that the gender of the offspring determined the degree of instability (32). Whether the gender of the offspring in our CGG repeat mouse model plays a role in instability could not be determined.

The human and mouse FMR1 gene promoter regions are very homologous. With the human promoter, four 'footprints' have been identified reflecting positions of various DNA–protein interactions. These footprints correspond to consensus binding sites of various transcription factors and are absent in fragile X patients, indicating that they reflect functional regulatory elements (33). These regulatory elements are also present in the mouse promoter region. It is not known whether these sequences in the promoter region might be influencing the behaviour of the repeat instability. Despite the overall homology between the mouse and human promoters, it does not necessarily mean that DNA elements which influence repeat instability are conserved between mouse and human. Theoretically, there is still a possibility that the mouse promoter region does not contain the *cis*-acting elements involved in (large/dynamic) repeat instability which is seen in the human *FMR1* gene.

Interestingly, cloning of the mouse *Fmr1* promoter showed that this region was prone to deletions when maintained in plasmids in bacteria. These deletions occurred after digestion of the DNA, re-ligation and transformation into bacteria. Most, although not all, deletions occurred in the region directly down and upstream of the $(CGG)_8$ repeat. These results indicate that the *Fmr1* promoter region itself is unstable in bacteria. The reason for this instability might be the high GC content of the region flanking the CGG repeat. Although there is no direct evidence that a DNA region which is highly unstable in bacteria might influence instability in the mouse genome, this may well contribute to the instability of the CGG repeat, although we hypothesize that factors other than *cis*-acting factors alone play a role in determining instability.

Length variation found in fragile X alleles appears to be polar, instability always occurring at the 3′ end. This might be influenced by the direction of DNA replication through the repeat (29,30). The direction of replication is important in determining repeat instability in both *Escherichia coli* and yeast. The position of the origin of replication in artificial DNA constructs determines whether the 5′-CGG-3′ or the 5′-CCG-3′ strand is the leading or the lagging strand during DNA synthesis. For the mouse and human genomic *FMR1* loci the direction of replication is not known. It may well be that the direction of replication in the human situation might favour the occurrence of expansions, but that the situation might be different for the mouse *Fmr1* locus.

Flap endonuclease 1 (FEN1), a protein involved in DNA replication and long-patch base excision repair, is thought to play a role in trinucleotide repeat instability (34). In yeast, loss of flap endonuclease activity (*rad27* mutants) increases instabilities throughout the whole genome, including trinucleotide repeats (35–37). For normal and premutation size CGG repeats, a 10-fold elevated frequency of expansion in *rad27* yeast strain has been found (38). This suggests that FEN1 could play a role in CGG trinucleotide repeat instability.

The endogenous mouse *Fmr1* 5′-UTR contains a CGG repeat of between 8 and12 triplets. In the ES cells used in this study, the $(CGG)_8$ repeat was exchanged with a $(CGG)_{98}$ repeat in the expectation that this length of repeat exceeds a threshold of instability in the mouse. In humans, repeat instability occurs when the number of CGG triplets is greater than 50; the threshold for repeat instability in mice is as yet unknown. In mice containing an expanded Huntington CAG repeat, it has been found that the rate of instability is less than that observed for similar sized repeats in humans (39). Mice heterozygous for the CAG expansion show intergenerational repeat instability (+2 to −6) at a much higher frequency in maternal transmission than in paternal transmission. The majority of changes transmitted through the female germline were small contractions, as in humans, whereas small expansions occurred more frequently in paternal transmission. (40). The mouse Hdh gene with a knock-in of 90 and 109 units produced a graded increase in the mutation frequency to >70%, with instability being more evident in female transmissions. No large jumps in CAG length were detected in either male or female transmissions. Instead, size changes were modest increases and decreases, with expansions typically emanating from males and contractions from females. (41). For the Fmr1 mouse model described here, the rate of change in paternal transmission of unstable repeats would appear to be 3-fold that of maternal transmission. Extrapolation of the data obtained from the expanded trinucleotide mice suggests that, if there is a threshold for instability in mice, the threshold might be higher in mice than for humans.

Results of this CGG repeat expanded mouse model, together with the studies of other trinucleotide repeats, suggest that mouse might not be a perfect model in which to study repeat instability. This might be due to the fact that the mechanism involved in repeat instability in humans might be absent in mice, or perhaps that the environment leading to the instability

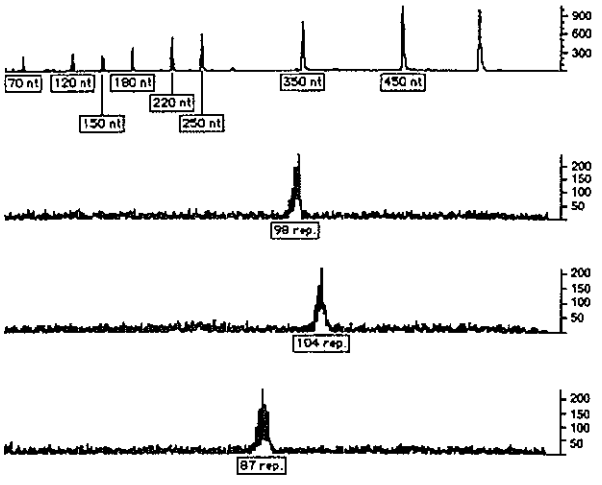

Figure 3. The Fragile X polymorphism assay is used to determine the exact repeat length of the observed instabilities.

in humans might be absent in mice. The mechanisms which have been proposed to play a role in repeat instability involve meiosis as well as DNA replication, recombination and repair. During all these processes, duplication of the DNA occurs, allowing an opportunity for DNA mutations and secondary structures like hairpins or tetraplex to form, errors which must be removed by proof-reading and repair processes. These processes, which aim to keep mistakes in the copying of DNA to a minimum, are balanced by processes in meiosis such as recombination which generate maximum genetic variation for a species. Although these processes are known to occur in humans as well as in mice, the balance between them might be different between the two species, giving rise to repeat instability in humans, but not in mice.

The involvement of DNA repair processes in repeat instability might be studied by crossing mice with expanded CGG repeats with mice deficient for different repair pathways. In yeast and *E.coli* it has been shown that the rate of instability changed in certain repair-deficient strains (38,42,43). Crossings between trinucleotide repeat mice and mice with certain repair deficiencies might give more insight into repeat instability in mice as well as in humans. Furthermore, it might be worthwhile to study the timing of the small repeat instabilities that occur as this might give more insight in the difference observed between male and female transmission.

Our transgenic $(CGG)_{98}$ repeat might also eventually also be used to study inactivation of FMRP expression. In the $(CGG)_{98}$ mice described in this study, FMRP expression was present, suggesting that the promoter region was not inactivated (data not shown). As small expansions are observed, it might be possible that expansion over several generations will generate a larger expanded allele which will inactivate the FMR1 gene and lead to loss of FMRP expression. Subsequently, both repeat expansion and FMRP expression could be studied in descendent generations. It has been reported recently that premutation alleles result in elevated mRNA levels (44) in human fragile X carriers. In contrast to this, FMRP expression in lymphocytes of premutation carriers with over 100 CGG triplets was reduced. This mouse model might, therefore, also be used to study the mRNA levels in premutation alleles, the translation of Fmr1 mRNA containing expanded CGG repeats, and maybe the mechanistic switch, which occurs when the *Fmr1* gene is silenced.

MATERIALS AND METHODS

Construction of pCB66 and ES cell electroporation

The construct pCB66 contained a 7 kb *Hin*dIII fragment of the mouse *Fmr1* promoter region inserted in a pBR322 derived vector (pBR322 – *Bam*HI 375–*Nar*I 1205). In this fragment, a *neo* cassette flanked by *lox*P sites was cloned in the *Bam*HI site present in intron 1 (Fig. 1). The mouse $(CGG)_8$ repeat was exchanged for a $(CGG)_{98}$ repeat of human origin. The human-derived $(CGG)_{98}$ repeat was isolated as an *Sfo*I–*Xho*I fragment from a cloned expanded CGG repeat. This cloned repeat was isolated from a yeast strain carrying a transgenic human premutation as described in references 28 and 38. To clone the expanded human CGG repeat in the murine *Fmr1* promoter, minimal changes were made to the flanking sequence. These changes involved a point mutation to abolish an *Nar*I site in intron 1 and the substitution of 4 bp (TCGA) to abolish an *Xho*I site in intron 1. The *Xho*I site flanking the CGG repeat in the human situation was not present in the mouse promoter. To facilitate the cloning of the expanded $(CGG)_{98}$ repeat an *Xho*I site was generated by an A→G transition based on the

homology between the human and the mouse promoter. The integrity of the cloned fragment was determined by sequencing and restriction enzyme digestion. Cloning was performed using standard procedures.

For electroporation of E14 ES cells to allow homologous recombination plasmid, pCB66 was linearized by an *Xbal* digest. After purification, linearized plasmid DNA was used to transfect ES cells. Electroporation was performed with 10^7 ES cells in 400 µl PBS using a Progenetor II Gene Pulser (1200 µF and 117 V during 10 ms). Using double selection with G418 (200 µg/ml) and Fiau (2 µM) the cells were cultured to allow colony forming. Colonies were picked and cultured separately. for DNA isolation.

DNA analysis

ES cell clones were grown to confluency in a 24-well plate. The medium was removed and 300 µl (10 mM Tris–HCl, 400 mM NaCl, 2 mM EDTA pH 7.3–7.4) and 30 µl of 10 mg/ml Prot K was added to lyse the cells during overnight incubation at 55°C. An aliquot of 150 µl of 6 M NaCl was added and the suspension was centrifuged. To the supernatant, 2 vol of 96% ethanol were added to precipitate the DNA. DNA was dissolved in 50 µl H_2O. For radioactive PCR, 1 µl DNA was used.

Radioactive PCR was performed to determine the repeat length in the ES clones. Primers C (5′-GCTCAGCTCCGTTT-CGGTTTCACTTCCGGT-3′) and F (5′-AGCCCCGCACTT-CCACCACCAGCTCCTCCA-3′) were used. PCR conditions were as described by Deelen *et al.* (45). PCR products were run in a 6 % denaturing polyacrylamide gel.

Generation of knockout mice

ES clone 651 was used for injection into C57/BL6J blastocysts. These blastocysts were transferred to pseudopregnant female mice. Three chimeras were generated and crossed with wild-type FVB and C57/Bl6J females. Female offspring of these chimeras was tested for the presence of the expanded $(CGG)_{98}$ repeat. Mice containing the expanded CGG repeat were crossed with wild-type mice as well as with $(CGG)_{98}$ littermates. Repeat instability upon transmission to offspring was tested by radioactive PCR.

The *neo* cassette inserted into the *Bam*HI was flanked by *lox*P sites. Because the presence of the *neo* cassette might disturb the natural environment of the CGG repeat, knock-in mice were crossed with mice expressing *Cre* recombinase. In this way the changes made to the *Fmr1* gene were kept minimal. These $(CGG)_{98}$/*neo*$^-$ mice were also crossed with wild-type mice, and with $(CGG)_{98}$/*neo*$^-$ littermates.

Fragile X size polymorphism assay

Radioactive PCR as described above is informative to determine whether instability occurs, but it does not give the exact length changes observed. Also, small changes ± 1 CGG triplet might be missed. The fragile X size polymorphism assay (Perkin Elmer Biosystems) allows us to determine the exact length of the CGG repeat. This test was used to determine the exact size changes. PCR conditions were as described by the manufacturer. PCR samples were analysed using an ABI377 sequencer (PE Biosystems).

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Summary

The Fragile X syndrome is characterized by mental retardation, behavioural deficits, facial abnormalities and macroorchidism (enlargement of the testes). The molecular mechanism underlying the fragile X syndrome is the expansion of a polymorphic trinucleotide $(CGG)_n$ repeat in the 5' untranslated region of the fragile X gene, *FMR1*. A CGG repeat of over 200 repeat units is called a full mutation. The presence of a full mutation repeat coincides with methylation of this CGG repeat and of the promoter region of the *FMR1* gene, thereby inactivating the gene. This results in the absence of the fragile X protein FMRP and the presence of the fragile X phenotype.
In the normal population the CGG repeat has a length of 5 to 50 repeat units with an average of 30 repeat units. This repeat does not show instability, in contrast to the premutation CGG repeat of 50 to 200 repeat units that is instable upon transmission to the next generation. Individuals with a normal or premutation length CGG repeat have expression of FMRP in their cells.

To study the molecular mechanisms involved in CGG repeat instability and expansion, the physiological function of FMRP and the pathogenesis of the fragile X syndrome, model systems are needed. Immortalized cells of patients were studied and experiments with yeast did shed some light on the possible mechanisms involved in CGG repeat instability. Since the *FMR1* gene is highly conserved among different species, the gene has been isolated from organisms like the fruit fly *Drosophila melanogaster*, the zebra fish *Danio rerio* or the frog *Xenopus leavis*. But the mammal mostly resembling the human, that is convenient to use in an experimental setting, is the mouse.

The mouse *Fmr1* gene is very homologous to the human *FMR1* gene and via genetic modification the mouse gene was inactivated (Chapter 4.1). The resulting *Fmr1* knockout mouse is lacking Fmrp. It is showing macroorchidism, but no gross neuroanatomical abnormalities have been found in the knockout mouse. The knockout mouse shows increased activity and exploratory behaviour and behavioural tests measuring spatial learning and memory dependent on hippocampal function show abnormalities in the knockout mouse. Long-term potentiation, which is involved in the process of learning and memory, is not altered in knockout mice. On the other hand, long-term depression in the knockout hippocampus is enhanced, suggesting interference with the formation and maintenance of strong synapses required for normal brain function. Indeed, in the knockout mouse abnormalities in the dendritic spines have been described. Also the susceptibility to audiogenic seizures is increased in the knockout mouse.

To study the timing and mechanism of CGG repeat expansion several mouse models with long CGG repeats have been made. Like humans, mice have a polymorphic CGG repeat in their *Fmr1* gene. Introduction of a repeat sequence on an autosome in the mouse has not resulted in repeat instability. Replacement of the murine CGG repeat in the *Fmr1* gene by a human premutation repeat of 98 CGG units has revealed mild CGG repeat instability (Chapter 4.5). However, the instability is less than the instability seen in humans and no increase from the premutation repeat to a full mutation size has been observed. Further experiments with this mouse model are still in progress.

To understand the role of the FMRP and the effect of its absence on the development of mental retardation, it is important to determine in which cells the *Fmr1* gene is expressed and whether there are differences in expression during the stages of (embryonic) development. Descriptive studies on embryonic, neonatal and adult tissues of mice have been performed in order to localize the three Fxr proteins: Fmrp and its two homologs Fxr1p and Fxr2p (Chapter 4.3 and 4.4). During early embryonic development, the expression pattern of the Fxr proteins exhibits similarities, however, during late embryonic development and in the neonate a more differential expression pattern is observed, especially in some non-neural tissues. The high tissue-specific Fmrp expression during late embryonic and neonatal development, which coincides with the organ involvement in the fragile X phenotype, suggests an early cell-type dependent function for Fmrp. In the brain of adult mice the three Fxr proteins are associated with ribosomes in the cytoplasm and localized within nucleus of neurons. In addition, the results illustrate that the absence of Fmrp in neurons from *Fmr1* knockout mice does not result in an abnormal (sub)cellular distribution or significantly changed expression levels of Fxr1p or Fxr2p. In maturing and adult testis a differential expression pattern is observed for the Fxr proteins in spermatogenic cells. Skeletal muscle tissue shows only a high expression for Fxr1p. The similarities and differences between the distributions of the Fxr proteins have implications with respect to their normal function and the pathogenesis of the fragile X syndrome. It can be concluded that the three genes cannot complement each other, despite their high homology and their largely overlapping expression pattern.

The knockout mouse model for the fragile X syndrome has been used to reintroduce the *FMR1* gene in order to observe a rescue of the phenotype (Chapter 4.2). The rescue mouse shows FMRP expression in brain tissue, but is not showing a reversal of the phenotype. This is most likely because the level of FMRP expressed from the transgene is inadequate, or the timing of gene expression or the cell type specificity of expression is different from that in the wild type situation. These problems can be overcome by introduction of the gene with all its control elements. With this and other rescue studies

it has become clear that many problems need to be encountered before the fragile X phenotype will be rescued.

In conclusion, the studies using mouse models have demonstrated that mouse models are valuable tools to study the fragile X syndrome and mouse models have already elucidated many aspects of the physiological function of FMRP and the pathogenesis of the fragile X syndrome.

Samenvatting

Het fragiele X syndroom wordt gekarakteriseerd door mentale retardatie, abnormaal gedrag, afwijkingen aan het gezicht en macro-orchidisme (vergroting van de testes). Het moleculaire mechanisme dat ten grondslag ligt aan het fragiele X syndroom is de expansie van een polymorfe $(CGG)_n$ repeat in het 5' onvertaalde gebied van het fragiele X gen *FMR1*. Een CGG repeat met meer dan 200 repeat eenheden wordt een volledige mutatie genoemd. De aanwezigheid van een volledige mutatie gaat samen met methylering van deze CGG repeat en het promoter gebied van het *FMR1* gen, waardoor het gen wordt geïnactiveerd. Dit resulteert in de afwezigheid van het fragiele X eiwit FMRP en de aanwezigheid van het fragiele X fenotype.
In de normale populatie heeft de CGG repeat een lengte van 5 tot 50 repeat units, met een gemiddelde van 30 repeat units. Deze repeat vertoont geen instabiliteit, in tegenstelling tot de premutatie CGG repeat, die 50 tot 200 repeat units lang is, en instabiliteit vertoont bij overdracht naar de volgende generatie. Individuën met een normale of premutatie CGG repeat brengen FMRP tot expressie in hun cellen.

Om het moleculaire mechanisme betrokken bij CGG repeat instabiliteit en expansie, de functie van FMRP en het ziekteproces van het fragiele X syndroom te bestuderen, zijn modelsystemen noodzakelijk. Cellen van patiënten zijn bestudeerd en experimenten met gist hebben een tipje van de sluier opgelicht over de mechanismen die mogelijk betrokken zijn bij CGG repeat instabiliteit. Omdat het *FMR1* gen heel geconserveerd is in verschillende soorten organismen, heeft men het gen kunnen isoleren van organismen zoals de fruitvlieg *Drosophila melanogaster*, de zebravis *Dania rerio* en de klauwpad *Xenopus leavis*. Maar het gewervelde dier dat het meest op de mens lijkt en dat geschikt is om in een experimentele setting te gebruiken, is de muis.

Het muizen *Fmr1* gen is heel homoloog aan het humane *FMR1* gen en via genetische modificatie is het muizen gen geïnactiveerd (Hoofdstuk 4.1). De ontstane *Fmr1* knock-out muis mist het eiwit Fmrp. De muis vertoont macro-orchidisme, maar er zijn geen grote neuro-anatomische afwijkingen waargenomen. De knock-out muis laat verhoogde activiteit en verkenningsdrang zien. In gedragsproeven, die hippocampus afhankelijke leer- en geheugenfuncties bestuderen, vertoont de knock-out muis afwijkingen. Long-term potentiation, een proces dat is betrokken bij leer- en geheugenfuncties, is niet veranderd in de knock-out muis. Aan de andere kant is long-term depression in de knock-out hippocampus verhoogd, wat betrokkenheid suggereert bij de vorming en instandhouding van sterke synapsen, die nodig zijn voor normale hersenfunctie. Inderdaad zijn in de knock-out muis abnormaliteiten van de dendritische zenuwen

beschreven. Ook de gevoeligheid voor door geluid opgewekte toevallen is verhoogd in de knock-out muis.

Om de timing en het mechanisme betrokken bij de expansie van de CGG repeat te bestuderen zijn verschillende muismodellen gemaakt. Net als de mens heeft de muis een polymorfe CGG repeat in het *Fmr1* gen. Introductie van een CGG repeat sequentie op een autosoom in de muis heeft niet tot instabiliteit geleid. Vervanging van de muizen CGG repeat in het *Fmr1* gen, door een humane premutatie repeat van 98 CGG units, heeft tot een kleine CGG repeat instabiliteit geleid (Hoofdstuk 4.5). De instabiliteit is minder dan de instabiliteit die gezien wordt in de mens en een toename van de premutatie naar een volledige mutatie is niet waargenomen. Vervolgexperimenten zijn momenteel in volle gang.

Om de rol van FMRP en de gevolgen van de afwezigheid van FMRP op de ontwikkeling van mentale retardatie te bestuderen, is het belangrijk om te bepalen in welke cellen het *FMR1* gen tot expressie komt en of er verschillen in expressie zijn gedurende de stadia van (embryonale) ontwikkeling. Beschrijvende studies met embryonale, neonatale en volwassen weefsels van muizen zijn uitgevoerd om de drie Fxr-eiwitten, Fmrp en zijn twee homologen Fxr1p en Fxr2p, te lokaliseren (Hoofdstuk 4.3 en 4.4). Gedurende de vroege embryonale ontwikkeling vertoont het expressie patroon van de Fxr-eiwitten veel overeenkomsten, terwijl tijdens de late embryonale ontwikkeling en in de neonaat een meer afwijkend expressie patroon is waargenomen, vooral in enkele niet-neuronale weefsels. De hoge weefselspecifieke Fmrp expressie tijdens de late embryonale en neonatale ontwikkeling, die samengaat met de orgaanbetrokkenheid in het fragiele X fenotype, suggereert een vroege, celtype afhankelijke functie voor Fmrp. In de hersenen van volwassen muizen zijn de drie Fxr-eiwitten geassocieerd met ribosomen in het cytoplasma en bevinden ze zich in de kern van neuronen. Daarbij laten de resultaten zien dat de afwezigheid van Fmrp in neuronen van de *Fmr1* knock-out muis niet leidt tot een afwijkende (sub)cellulaire lokalisatie of een significant veranderde expressie van Fxr1p of Fxr2p. In de rijpende en volwassen testis is een verschillend expressiepatroon voor de Fxr-eiwitten waargenomen. Alleen skeletspierweefsel laat een hoge expressie zien voor Fxr1p. De verschillen en overeenkomsten tussen de distributie van de Fxr-eiwitten hebben implicaties voor wat betreft de normale functie en de betrokkenheid van de Fxr-eiwitten bij het fragiele X syndroom. Het kan geconcludeerd worden dat de drie genen elkaar niet kunnen complementeren, ondanks de hoge mate van homologie en de grote overlap in expressiepatroon van de Fxr-eiwitten.

De knock-out muis voor het fragiele X syndroom is gebruikt voor de herintroductie van het *FMR1* gen, om zo een herstel van het fenotype waar te nemen (Hoofdstuk 4.2). De zo ontstane rescue-muis laat FMRP expressie zien in hersenweefsel, maar het fenotype wordt niet hersteld. Dit wordt mogelijk veroorzaakt doordat het expressieniveau van het transgene FMRP niet voldoende hoog is, of omdat de timing van genexpressie of de celspecificiteit van de expressie verschillend is van die in de normale situatie. Deze problemen kunnen worden verholpen door het *FMR1* gen met al zijn regulerende sequenties te introduceren. Door deze en andere rescue-studies is het duidelijk geworden dat nog veel problemen verholpen moeten worden, voordat het fragiele X fenotype herstelt zal kunnen worden.

Tot slot hebben de studies met de muismodellen aangetoond dat muismodellen waardevol zijn in het onderzoek naar het fragiele X syndroom en hebben muismodellen al vele aspecten van de fysiologische functie van FMRP en het ziekteproces opgehelderd.

Abbreviations

AGS	audiogenic seizure susceptibility
Asn	asparagine
DNA	deoxyribonucleic acid
ES	embryonic stem cell
FBS	FMRP binding site
FMR1	fragile X mental retardation gene 1 (human)
Fmr1	fragile X mental retardation gene 1 (mouse)
FMRP	fragile X mental retardation protein (human)
Fmrp	fragile X mental retardation protein (mouse)
FSH	follicle-stimulating hormone
FXR1	FMR1-crossreacting relative gene 1 (human)
Fxr1	Fmr1-crossreacting relative gene 1 (mouse)
FXR1P	FMR1-crossreacting relative gene 1 protein (human)
Fxr1p	Fmr1-crossreacting relative gene 1 protein (mouse)
FXR2	FMR1-crossreacting relative gene 2 (human)
Fxr2	Fmr1-crossreacting relative gene 2 (mouse)
FXR2P	FMR1-crossreacting relative gene 2 protein (human)
Fxr2p	Fmr1-crossreacting relative gene 2 protein (mouse)
GluR	glutamine receptor
Ile	isoleucine
IRES	internal ribosome entry site
kb	kilobase
kD	kilo Dalton
KH	K-homology
LTD	long-term depression
LTP	long-term potentiation
MCH	mean corpuscular haemoglobin
MRI	magnetic resonance imaging
RNA	ribonucleic acid
mRNA	messenger ribonucleic acid
mRNP	messenger ribonucleoprotein
NES	nuclear export signal
NLS	nuclear localisation signal
PCR	polymerase chain reaction
RGG box	arginine/glycine-rich RNA-binding motif
UTR	untranslated region
YAC	yeast artificial chromosome

Curriculum Vitae

Naam	Cathy (Katharina Elisabeth) Bakker-van Kempen
Geboren	4 december 1966 te Leiden
1979-1984/86	HAVO/VWO, Bonaventura College te Leiden.
Juni 1990	Doctoraalexamen Biomedische Wetenschappen, Rijks Universiteit Leiden. Hoofdvakstage bij Pharming, Leiden: Klonering van het α2-caseïne gen.
Februari 1991	Aanstelling bij de afdeling Klinische Genetica van de EUR te Rotterdam in de onderzoeksgroep van Prof. Dr. H Galjaard onder supervisie van Prof. Dr. B.A. Oostra; Onderzoek naar het Fragiele X Syndroom.
Februari 1992	Aanvang promotieonderzoek met als doel het ontwikkelen van proefdiermodellen voor het Fragiele X Syndroom.
Oktober 1995	Cursus Proefdierkunde, waarbij de status van artikel 9 functionaris is verkregen.

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Dankwoord

"Er was eens..."
Nee, een sprookje is het niet geworden, maar wel een lang verhaal.
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Zo.
Het boekje is af en daarmee uit.

Stellingen behorende bij het proefschrift

Mouse models for the fragile X syndrome

De hoge FMRP expressie in Sertoli cellen gedurende de vroege postnatale fase suggereert een directe rol voor FMRP in de regulatie van Sertoli cel proliferatie, waarbij afwezigheid van FMRP leidt tot de ontwikkeling van macro-orchidisme bij fragiele X patiënten.

(Slegtenhorst-Eegdeman et al. (1998), Endocrinology 139: 156-162)
(Dit proefschrift)

Het hoge mRNA niveau en het (bijna) normale eiwit niveau bij mannelijke dragers van een grote premutatie wijst op een probleem in de translatie van het *FMR1* mRNA.

(Tassone et al. (2000), Am. J. Hum. Genet. 66: 6-15)
(Kenneson et al. (2001), Hum. Mol. Genet. 10(14): 1449-1454)
(Dit proefschrift)

De interlab verschillen, die gerapporteerd worden door Crabbe *et al.*, laten zien dat technieken die gedrag in muizen analyseren zich nog steeds aan het ontwikkelen zijn en dat communicatie tussen de wetenschappers onontbeerlijk is.

(Crabbe et al. (1999), Science 284: 1670-1672)
(Comments, Science 284: 1599/ Science 285: 2067-2070)
(Dit proefschrift)

De start van een therapeutische studie met antagonisten van de glutamaat receptor bij fragiele X patiënten is voorbarig.

(Huber et al. (2002), Proc. Natl. Acad. Sci. USA 99:7746-7750)
(Dit proefschrift)

De term 'normal transmitting male' voor mannelijke dragers van een premutatie is achterhaald.

(Hagerman et al. (2001), Neurology 57: 127-130)
(Greco et al. (2002), Brain 125: 1760-1771)

Zowel dFmrp als PERIOD2 shuttlen tussen het cytoplasma en de celkern, maar ze vervullen een functie op een verschillend niveau in het clock mechanisme.

(Dockendorff et al. (2002), Neuron 34: 973-984)
(Yagita et al. (2002), EMBO 21(6): 1301-1314)

Als een behoorlijk deel van de totale variantie in IQ wordt bepaald door seksgebonden genen, is het voor een jongen belangrijk een pientere moeder te hebben.

(Hogben 1932, quoted by Lehrke 1974)
(O'Donnell & Warren (2002), Ann. Rev. Neurosci. 25: 315-338)

Als de duur van het onderzoek naar de oorzaak van het Gilles de la Tourette syndroom een maat is voor de bijzonderheid van het betrokken mechanisme, schept dit hoge verwachtingen.

Hypospady bij zonen van vrouwen die *in utero* zijn blootgesteld aan diethylstilbestrol (DES) is het eerste transgeneratie effect van DES beschreven in de mens.
(H. Klip et al. (2002), Lancet 359: 1102-1107)

In onze gejaagde maatschappij is inbakeren voor enkele van de allerkleinsten een probaat hulpmiddel om het tij te doen keren van onrust naar rust en regelmaat.
(Lopend wetenschappelijk onderzoek in het Wilhelmina Kinderziekenhuis te Utrecht door B. Sleuwen en R. Blom)
(Persoonlijke ervaring)

De overlap van de biomedische wetenschap met de autotechniek beperkt zich tot de term "ontsteking". Zelfs de inhoud van de term is in beide vakgebieden geheel verschillend.

Rotterdam, 11 december 2002

Cathy Bakker