Mouse models
for the fragile X syndrome

Muismodellen
voor het fragiele X syndroom

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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 autisticform 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 Fragile X Mental Retardation 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 *FMRI*. Due to this methylation, transcription is inhibited and no protein is produced. Absence of 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).

![Diagram](image)

**Figure 1.** Schematic representation of the *FMRI* 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 1304N missense mutation found in a single, severely affected patient is indicated. In the 5’-UTR the different repeat classes are shown. The Q-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 unstable. 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.](image)

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. Willemse, 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 leukocytes with an unmethylated CGG repeat and promoter region have been reported (McConkie-Rosell et al. 1993; Hagerman et al. 1994; Sneets 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 Fragile X Mental Retardation Protein (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, \textit{FMRI} 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 \textit{FMRI} expression was quite high and liver, pancreas and skeletal muscle showed low or no expression of \textit{FMRI} (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 \textit{in vitro} experiments showed that some isoforms are found in the nucleus (Eberhart et al. 1996; Fridell et al. 1996; Sittier 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 \textit{Fmr1} gene in mouse could be studied more extensively using tissues from mice in immunohistochemical studies and mRNA \textit{in situ} hybridisation experiments (Devys et al. 1993; Hinds et al. 1993; Bakker et al. 2000a; De Diego Otero et al. 2000). \textit{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 \textit{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, \textit{in situ} mRNA studies showed moderate levels of \textit{Fmr1} expression in kidney, liver, colon, uterus, thyroid, and lung. No \textit{Fmr1} mRNA is found in muscle, heart or aorta. High levels of \textit{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, \textit{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 \textit{FMRI} gene, a sequence coding for a nuclear export signal (NES) was identified (Figure 1) (Fridell et al. 1996) and Tamarini 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 (Figure 1) (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 (polysomes) on sucrose gradients (Khandjian et al. 1996), and immuno-electronmicroscopy analysis confirms the close proximity of FMRP to free ribosomes, membrane-bound ribosomes and polysomes (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 (NUclear FMRP Interacting Protein 1), which is a novel RNA binding protein (Bardoni et al. 1999), and CYFIP 1 and 2 (CYtoplasmatic FMRP Interacting Protein 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 (FMRP Binding Site, 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 dFos, the Drosophila homolog of the FXX 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).

![Diagram of FMRP function](image)

**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 and 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* (*Fragile X Related 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 Relative), related by structure, possibly 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 spermatagonia. FXR1P is also expressed in the cytoplasm of more mature spermatogonia cells including spermatocytes and spermatids, whereas FXR2P is, at a lower level, present in all cells of the tubules seminiféri (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 myosincontractile 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 NUFP1, 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 FMRI 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 FMRI 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 FMRI 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 laevis and the zebrafish 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
Chapter 2

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

2.2 The fragile X knockout mouse

The FMRI 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 (Abilbol 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 FMRI 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 causoc 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 potentiallyvalid 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 littersmates. 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-Jagerden 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 fociuses 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 postratal 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 littersmates (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 autism-spectrum 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 autism-spectrum 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 (IPMF) 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 *FMRI* 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; Danysh 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 synaptoneurosesomes. Synaptoneurosesomes are preparations highly enriched in pinched-off, resealed presynaptic processes attached to resealed postsynaptic processes that retain normal function of neurotransmitter release. When synaptoneurosesomes 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 synaptoneurosesomes 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 then 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 consultants 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 (Cher 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 mouse 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 Fnr1 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 logical 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 Fmr1p 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 *FMRI* 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, *FMRI*, *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 trials 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 *FMRI* 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 *FMRI*, 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 *FMRI* 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 *FMRI* needs to be directed to the cells that normally express *FMRI*, 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 *FMRI*. 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 re activate 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 specificity 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 try 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

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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 1,200 males and 1 in 2,500 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 on 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) in the 5' untranslated region of the FMR1 gene (Verkerk et al., 1991; Oberle 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 45–200 CGGs (premutation) and are asymptomatic. The full mutation is characterized by a large repeat containing over 203 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 (Pierratti et al., 1991; Verheij et al., 1993). Males with a full mutation are affected, and 60%–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., 1992). Two types of domains that are known to bind RNA, two KH domains and a RGG box, have been identified in FMR1 protein (Scrima et al., 1993; Ashley et al., 1992).

The FMR1 gene is highly conserved among species (Verkerk et al., 1991), and the murine homolog Fmr1 shows 97% homology to amino acid sequence (Ashley et al., 1993). The expression pattern of FMR1 at the mRNA level has been studied in different cell lines and tissues.

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and protein level is very similar in different tissues of humans and mice (Hinds et al., 1992; Böckler et al., 1993). This makes the mouse a good animal model in which to study the fragile X syndrome.

To study the function of FMR1 gene 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 leads 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 germ-line using the embryonic stem (ES) cell technology (Mansour et al., 1986). We constructed a homologous recombination targeting vector, pMGS, containing 5.7 kb of mouse genomic Fmr1 DNA (Figure 1A). Exon 5 was interrupted by the positive selection marker gene neo (NEO). The negative selection marker gene thymidine kinase (tk) was inserted 3’ of the genomic sequence in the polylinker of the vector. Vector pMGS was introduced in E14 ES-cells and selection for homologous recombination events was performed using medium containing G418 and gancyclovir. A clone (CBS.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 cDNA level, the presence of the knockout allele in these female mice was 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 allele and protein expression in brain of a wild-type (lane 2) and a knockout (lane 4) mouse showing protein bands between 64 kDa and 64 kDa only present in the control group.
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 (Fig-
ures 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 gameto-
genesis (Bächler et al., 1993a, 1993b), we studied the
reproductive fitness of mutant mice. Crossings were per-
formed between the knockout males and wild-type fe-
nales and between the homozygous knockout females
and wild-type males in order to test the fertility of the mu-
tants. 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 off-
spring with the mutant allele. The transmission of the
mutant Fmr1 gene corresponded well to the expected Men-
delian 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 oog-
genesis in mice. Furthermore, mutant mice appear per-
fectly viable, the oldest knockout 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. Macronor-
didism is one of the key features of the fragile X syn-
drome, present in more than 80% of adult males (Turner
et al., 1980), and it is thought to develop gradually through
time. Therefore, we compared testicular weight of knock-
out and control mice at four different ages. Testes of
knockout mice were significantly heavier than control tes-

tes in all four age groups, and the difference became pro-
gressively 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, exten-
sor plantar responses, clumsy or sluggish gait, and persis-
tence of pinch synkinesia (Trenkwalder et al., 1982; Vierregge

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 (Tragerman, 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 of increased amount
of interstitial tissue (Turner et al., 1975). However, micro-
scopic examination of the testes of mutant mice revealed
no structural differences as compared with controls, in-
cluding normal pattern of tubule size, normal amount of
interstitial tissue, 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) con-
trol littermates (467 ± 15 mg). The brains of eight knock-
out and four wild-type littermates were macroscopically
and microscopically normal. The following structures were
examined and were found to be normal by light micro-
copy: frontal, temporal, and occipital cortices, striatum,
specific enolase (NSE) antibody (as shown in Figures 4C and 4F) 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 ± 4 mm²; n = 6) was not significantly different from that in control littersmates (30 ± 1 mm²; 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 Iwai (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 were placed in a brightly lit compartment of a step-through box. Upon entrance into the dark compartment of the box, they received 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 the test as the mean entrance latency of 11 mutant males (241 ± 34 s) was not significantly different from that of 2 normal littersmates (247 ± 29 s).

Exploratory Behavior Test
An exploratory behavior test was performed with 15 knockout mice and 16 normal littersmates 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 littersmates (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 littersmates.

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 littersmates (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 Coronal Sections from Brain

Normal (A, C, E, and G) and mutant (B, D, F, and H) mice. (A) and (B) (6 x ) show an overview of coronal sections stained with cresyl violet; (C) and (D) (24 x ) are sagittal sections of hippocampus immunolabeled for neuron-specific enolase; (E) and (F) illustrate sagittal sections of hippocampus labeled for neurofilament-M; (G) and (H) (102 x ) show sagittal sections of cerebellum immunolabeled with antibodies against neurofilament-M. Note that for all parameters studied no difference could be found between normal and mutant mice.
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 mammals. 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: \( F_{12,139} = 33.32, p = 0.0001 \)) and their normal littermates (\( F_{12,139} = 19.93, p = 0.0001 \)) showed highly significant decrease of escape latency and reached similar levels of performance in this task with a latency on the first 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: \( F_{1,139} = 6.88, p = 0.01 \)). 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: \( F_{1,139} = 37.67, p = 0.0001 \); interaction effect trial X genotype: \( F_{12,139} = 7.49, p = 0.0002 \) ) (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 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.

To exclude that the apparent impaired spatial learning in the hidden platform Morris water maze results from neurological or sensory deficit or lack of motivation, we conducted a visible platform Morris water maze test. In this test,
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 (Theile et al., 1985). Consistent 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 (Bönhör et al. 1993a, 1993b), but there is much controversy about the expression of FMR1 in testes. Studies of RNA (Bönhör et al., 1993a, 1993b) and protein expression (Dey 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 (Abild 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 (Meier et al., 1994). If Fmr1 expression is not obligated for germine proliferation, the absent Fmr1 protein in the germine 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 germine 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., 1984; Hagerman et al., 1985). 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; Rutte et al., 1994), which may be responsible for the size reduction of the vermis (Murakami et al., 1989). As Purkinje cells have high expression of FMR1 (Abild et al., 1993), we investigated the number of Purkinje cells in the mouse 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 responses to sensory stimuli, hyperactivity, and ataxia, repetitive movement in a pattern sometimes consistent with autism (Brown et al., 1986; Hagerman et al., 1986; Cohen et al., 1988; Gianetti et al., 1991; Reiss and Frey, 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 (Gianetti et al., 1991; Mears et al., 1994). As the FMR1 expression is very high in the hippocampus (Abild 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 (cooperating 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 visual 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 knockout mice 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 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., 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 mouse has shown. Fmr1 protein and show abnormal files comparable to human fragile X patients. Therefore, these mutants 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 pmOS, ES Cells, and Transfection**

The targeting vector pmOS was composed of pBS-script vector K-CIg and a 5.7 kb mouse genomic DNA Sal-I fragment containing exons 1 and 2 of the Fmr1 gene. This fragment was obtained from a 129-derived ES cell g40 heterozygous (provided by J. G. Ross) after probing with a human FMR1 cDNA sequence. The vector was interrupted with a neo resistance cassette, a 1664 bp blunt XbaI fragment obtained from pUC18 transcription (Saito et al., 1993) in a blunt unique Smal site in exon 5. The neo cassette is flanked in cis and transfected with genomic circularization. A 2.9 kb flanked with the 5' end of the trans-saccin (pGEM14) Chikara et al., 1995) was inserted to allow the intact blunt Notl site of the vector. The neo cassette is also asparagine 18 in the vector is expected 18 nucleotide flanks. Vector pOS was digested with Pst I to enolate as much bacterial present sequence as possible, and 10-20 µg of this DNA was used to transfect 1 x 10^6 ES cells in 400 µl PBS using a Progenitor B Gene Packer (1200 µg µl and 177 V during 10 µL). The fastest cells were seeded onto 100 mm culture dishes coated with gelatin in medium composed of 50% B6-concorded medium, 40% DMEM supplemented with 1% LIF (GIBCO BRL). The medium was replaced by medium containing 200 µg/ml G418 (Selten, GIBCO 260 ater replacement. The medium was refreshed every day and during days 3 and 4 and was also supplemented with 2 µM pyracidin (Gibco). After 18-20 days, clones became visible, could be picked, treated with TE 0.5% Isopropyl (3.02 µl EDTA in PBS), and seeded into gelatin-coated 24-well plates, one clone per wells. After growing to confluence, clones were treated with 75% again, and 5% of the clone was frozen down slowly and stored in liquid nitrogen for storage containing 10% DMSO. One-enth of each clone was spun down and resuspended in 30 µl water, and 50 µl 2x Cholera 100 (Koffman) was added. The cells were collected at 4°C for 10 min to lose the DNA. The solution (30 µl) was used for PCR analysis. For Southern blot analysis, 10/1 of each clone was grown to confluence again and harvested, and genomic DNA was isolated from the cells.

**DNA Analysis**

PCR screening for a homologous recombination event was performed using 150 µM of the primer MR1 (TACGACGACGATCAGCTATCCGCCATTGGAGC-3) and NR1 (5'-GCTGCGCTCCGACCCGTGAGG-3) in a PCR buffer containing 10 mM Tris, 10 mM MgCl2, 0.2 mM dNTPs, and 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 (155) were electrophoresed on a 1% agarose gel.

**Generation and Analyse of Chimeric and Knockout Mice**

The positive ES clone OB2 was injected into C57BL/6J blastocysts.
and the blastocysts were transfered to pseudopregnant female mice. Highly chimeric males were crossed with CD1BL6 mice to generate fertile females, and the offspring was littered for DNA analysis after 2-3 weeks. Tail DNA was isolated according to Logan et al. (1986), and 1 μl was used in the PCR with primer N1 and N2 (see above). Screening for the presence of the wild-type allele was performed using primers S1 [5'-GCTGGTAAGGTCTACAAAGTTCAAGCTAT-3'] and S2 [5'-CCAGTTGTTT-ATTATCAAGTTAC-3'] (forward and reverse in the PCR) described before with an annealing temperature of 62°C instead of 65°C.

RT-PCR
Total RNA was isolated from tissues of mutant and control mice using the RNeasy kit (Qiagen, 1995). RNA (1 μg) was reverse transcribed (Piretti et al., 1991), and of the reverse transcribed reaction was directly subjected to PCR using conditions of the primers S1--N2 and S1--S2 according to the program described before with an annealing temperature of 62°C. The PCR products (5 μl) were analyzed on a 1.5% agarose gel (1.5% agarose and 1.5% low-melting-temperature agarose).

Western Blotting
Proteins were isolated from brain, testes, liver, and kidney from knock-out and control mice. Tissue (200 mg) was homogenized in 750 μl of buffer (10 mM HEPES, 300 mM KCl, 5 mM NaCl, 100 μM CaCl2, 0.45% Triton X-100, 0.05% Tween 20) using a 15-ml polystyrene (5 x 2 strokes). The homogenates were sonicated for 45 s and spun down for 20 min at 10,000 x g. Swann ring was used an Immunoprecipitation with polyclonal antibodies to the middle part of FMRP protein (Yoshida et al., 1995). Immunoprecipitations, gel electrophoresis, and Western blotting were performed as described by Varney et al. (1985), with the exception that we used [35S]Protein A to detect the proteins.

Histology
Eleven mice were killed by ether anesthesia. Tissues were fixed in Bouin's solution, and sections were stained with hematoxylin and eosin. Sections from the same brain were cut in the coronal, frontal, and sagittal planes, and Bouin's solution. Two brains were snap frozen in liquid nitrogen chilled in acetone. Paraffin sections were examined with the light microscope.

Immunohistochemistry
Fetal day 14.5 (E14.5) embryos were fixed in 4% paraformaldehyde in 0.1 M sodium phosphate pH 7.4 overnight. Embryos were washed in PBS and incubated with 1:100 concentration of rabbit anti-FMRP serum for 2 h at room temperature. The embryos were washed again and incubated with 1:200 concentration of Alexa Fluor 488-conjugated goat anti-rabbit IgG for 1 h at room temperature. The embryos were washed again and mounted on glass slides with ProLong Gold Antifade Mountant with DAPI. The slides were imaged using a Nikon Eclipse 80i widefield microscope equipped with a 100x objective and a CoolSnap HQ2 camera. Images were analyzed using NIS-Elements software.

Peyer's Patch Analysis
Peyer's patches were isolated from E14.5 embryos as described by Cullen et al. (2001). Briefly, embryos were dissected and the intestines were isolated. The intestines were then incubated in 0.1 M HCl for 5 min at room temperature to digest the mucosa. The resulting tissue was then washed in PBS and incubated with 1:100 concentration of Alexa Fluor 488-conjugated goat anti-rabbit IgG for 1 h at room temperature. The tissue was then washed again and mounted on glass slides with ProLong Gold Antifade Mountant with DAPI. The slides were imaged using a Nikon Eclipse 80i widefield microscope equipped with a 100x objective and a CoolSnap HQ2 camera. Images were analyzed using NIS-Elements software.

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References

Preparation of Fetal Tissue
Fetal day 14.5 (E14.5) embryos were isolated from pregnant mice and dissected in cold PBS. The intestines were isolated and incubated in 0.1 M HCl for 5 min at room temperature to digest the mucosa. The resulting tissue was then washed in PBS and incubated with 1:100 concentration of Alexa Fluor 488-conjugated goat anti-rabbit IgG for 1 h at room temperature. The tissue was then washed again and mounted on glass slides with ProLong Gold Antifade Mountant with DAPI. The slides were imaged using a Nikon Eclipse 80i widefield microscope equipped with a 100x objective and a CoolSnap HQ2 camera. Images were analyzed using NIS-Elements software.


4.2 Publication 2

Introduction of a Fmr1 transgene in the fragile X knockout mouse

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SUMMARY

Patients with fragile X syndrome show mental retardation, behavioural abnormalities, facial anomalies and macroorchidism due to the lack of the FMRI 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 FMRI 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 FMRP 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 (FMRI) 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 FMRI gene is inactivated due to methylation of the promoter region and a CGG repeat located in the 3' UTR of FMRI [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].

FMR1 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 inactivated. 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 these 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 FMRI 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 FMRI 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 FMRI cDNA [12] was cloned in the EcoRI site of expression vector pCDNA I/mp (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 350 bp KpnI-fragment (base 3360 to 3710). The FMRI 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 FMRI 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 pcDNA/amp containing the human FMR1 cDNA, present in mouse line G6 and G8, (B) Vector pcDNA/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 S2 (5' AAA ATC CTT AGT GGC CGC TTC 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, 1.0 mM MgCl2, 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 allele 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 acetate solution (100 ml 0.1 M PBS + 2 ml 30% H2O2 + 1 ml sodiumacetate)[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'-diaminobenzidine.HCl (Serva). Finally, sections were counterstained with Hematoxilin followed by dehydration in ethanol and
Testicular weight, and Behavioural and Cognitive tests

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

Animal selection: The knockout mice, having a FVB/129 genetic background, were screened for the presence of a normal Rd gene, coming from 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 tests were 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 FMRI 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 FMRI 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
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 autosomal 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.
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 mice 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 bars 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 Pmet 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.
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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 pcDNA/amp containing a CMV promoter 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 fetal 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. *FMRI* expression in the G6-rescue mouse is under control of the CMV promoter, which may be less time/place specific than the natural *FMRI* 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 Pardee 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
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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 FMRI 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 allele will survive since they express only half of the
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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 rule 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 homozygosity. 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 elucidate 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

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Fragile X syndrome is caused by the absence of expression of the FMR1 gene. Both FXRI and FXR2 are autosomal gene homologues of FMR1. The products of the three genes are belonging to a family of RNA-binding proteins, called FMRI, FXRIp, and FXR2p, respectively, and are associated with polyribosomes as cytoplasmic mRNA 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 monoclonal antibodies raised against each of these proteins and performed Western blotting and immunolabelling 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 brain the three proteins were localized in the cytoplasm of the neurons; however, in specific neurons Fxr1p was also found in the nucleus. Immunoelectronmicroscopy 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.

Key Words: fragile X syndrome; Fmr1; Fxr1p; Fxr2p localization; mouse tissues.

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

Two mammalian homologues of FMRP were identified, named FXR1 and FXR1P [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 88 and 89%, 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 [26]. Both FXR1P and FXR2P are expressed in human adult 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 [18].

In human fetal tissues, FXR1P and FXR1P are expressed in all primordial germ cells. Additionally, FXR1P could be also localized to a lesser extent in the non-germatoxytic cells. In contrast, FXR2P was present only in the interstitial cells [19]. Also, in human adult tissues, FXR1P, FXR1P, and FXR2P are differentially expressed with high expression of FXR1P and FXR2P in the more matured spermatogenic cells [10, 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 RGG box [24, 25, 27]. The three proteins can form homo- and heterodimers in vitro [25]. However, recent studies by Tammammi et al. illustrated the preferential presence of homodimers in vivo [28].

Thus far, a clear overview of the localization of the FMR proteins in mouse tissues within one systematic study is missing. A descriptive study in combination with biochemistry might elucidate more about the role of the three proteins and their possible interactions. The use of Purk knockout tissues gives us the opportunity to study changes in Purk1 and Purk2 distribution as a result of the lack of Purk 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 Purk, Purk1, and Purk2 in several adult tissues from wild-type and Purk1 knockout mice with special emphasis to the central nervous system and the developing neonatal testis. In addition, we have performed immunohistochemistry to study the subcellular localization of the Purk 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 transcardially perfused with 0.1 M phosphate-buffered saline (PBS) and 4% paraformaldehyde, followed by a 15-min incubation with 3% paraformaldehyde. The brain was dissected and postfixed in 4% paraformaldehyde, then washed with PBS, embedded in Tissue-Tek (Sakura, Torrance, CA), and stored at -80°C. Cryosections (30 μm) were stained with an antibody against Purk1 (1:1000) and visualized by incubation in secondary antibody conjugated to Alexa 488 (Invitrogen). Images were acquired using a Leica DMR microscope equipped with a Nikon Coolpix 990 digital camera. Puriﬁcation of Purk1 and Purk2 antibodies against Recombinant Purk1 and Purk2 was performed using anion-exchange chromatography on a HiTrap Q column, followed by a benzamidine-Sepharose column. The antibodies were then dialyzed against PBS and the protein concentration was determined using the Bradford assay.

RESULTS

In our study, we have tested a number of mouse organs, including brain, spleen, colon, liver, kidney, skeletal muscle, intestine, thymus, and testis. Here we describe only those organs that showed a high expression of the Purk protein. Organs displaying high expression of Purk protein are:

1. Brain: Purk is highly expressed in the brain, especially in the hippocampus, cortex, and striatum. Purk expression is highest in the dentate gyrus, CA1, and CA3 regions of the hippocampus. Purk expression is also high in the cerebral cortex, especially in the prelimbic and infralimbic areas, and in the amygdala.

2. Spleen: Purk expression is high in the splenic marginal zone, red pulp, and white pulp. Purk expression is also high in the lymph nodes and Peyer's patches.

3. Colon: Purk expression is high in the crypts of Lieberkühn and the muscularis propria. Purk expression is also high in the lamina propria and the submucosa.

4. Liver: Purk expression is high in the liver, especially in the sinusoidal endothelial cells, Kupffer cells, and hepatocytes.

5. Kidney: Purk expression is high in the glomeruli, tubules, and interstitium. Purk expression is also high in the renal medulla and cortex.

6. Skeletal muscle: Purk expression is high in the muscle fibers, especially in the type I fibers. Purk expression is also high in the connective tissue and the blood vessels.

7. Intestine: Purk expression is high in the intestinal epithelial cells, especially in the villi and crypts. Purk expression is also high in the lamina propria and the submucosa.
mature as a 95-kDa form (Figs. 1A-1D). 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. 1C-1D).

Immunohistochemistry

Figure 2 illustrates the light-microscopic expression pattern of the Par proteins in the cerebellum, the ovaries, and skeletal muscle tissue. Parp1 was highly expressed in the cytoplasm of most neurons in the central nervous system, including Purkinje cells located in the cerebellum (Fig. 2A). Gli3 cells were not labeled. In ovaries, Parp1 was expressed in the cytoplasm of the ovum, zona pellucida, oögonia, and follicular cells of growing follicles (Fig. 2B), whereas the protein was virtually absent in skeletal muscle (Fig. 2C).

Immunolabelling of the central nervous system for Parp1 results in an expression pattern similar to that of Fmrp with a clear presence in the dendrites. Additionally, Parp1 could be detected in the nucleus of motoneurons (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), PXR1P (b, e, h), and PXR2P (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).
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. 2a). In particular, the contractile bands of skeletal muscle tissue showed a strong labeling for Fxr1p (Fig. 2h).

The labeling pattern for Fxr1p in brain, ovaries, and skeletal muscle (Figs. 2e, 2f, and 2h) 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 testes 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 testes 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 nuclei 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. 3i). 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. 3i).

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

Tissues of the Fxr1 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 endoplasmic 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 unraveling 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, 26], 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-heteromers, which suggest 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 [21, 25]. The results of this 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 comparisons 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 nucleus 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 involved in various processes 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 nucleus 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 nucleus 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 vitro studies in combination with the RNA-binding capacities of the three proteins suggests
a role of the Par 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 Par proteins play a role in ribonucleoassembly 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, FXRIP, and FXRIP2 [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 nuclear targeting signal [32]. Since targeting of nucleolin to the nucleolus requires mRNA, 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 FXRIP [A. Hengstman, unpublished results]. Treatment of transfected COS cells with leptomycin B (LMB), an inhibitor of the importin mediated nuclear export pathway, resulted in accumulation of FXRIP in the nucleus and both FMRP and FXRIP were retained in the nucleolus [33]. The differences between the localization of the FXR proteins after LMB treatment are probably caused by the relatively short transfection time (4–48 h) before treatment of LMB, since the initial experiments on FMRP-transfected COS cells resulted only in labeling of FMRP in the nucleus 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 NUP1 [34]. At the subcellular level also NUP1 was demonstrated in the nuclear. Alternatively, recently it has been suggested that the perinuclear 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 Par 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 PrpR antibodies in tissues from Par1 knockout mice, which was to be expected. In addition, our immunohistochemical study on Par1 knockout brain showed that absence of PrpR in neocortex.
The general picture emerging from our immunohistochemical study on mouse testis confirms earlier studies on adult and fetal human testes (13–20 weeks) showing a differential expression of the Fxr proteins in the spermatogonia cells. In this study we have extended our research and focused on the distribution of the Fxr proteins from developing neonatal testis in 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 and germ cells during noggin 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 early spermatogenesis.

The sole presence of Fxr1p in the nucleus of primordial germ cells in 3-day neonatal testes, in contrast to the sole presence of both Fmrp and Fxr2p, suggests a specific role for Fxr1p in this cell organelle during 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 (39), and thus also Fmr1 inactivation, that occurs in the early spermatocytes.

The two autonuclear 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 mere 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 spermatogenesis is not essential for normal spermatogenesis, since male Fmr1 knockout mice are fertile. The function of the Fsr 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 Fmr1p. Interestingly, it has recently been proposed that increased Sertoli cell proliferation during testis development is the cause of macroorchidism in Fmr1 knockout mice (39). 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 testes 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 female carriers of 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 caused by a mutational 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 follicular cells, including dividing cells in the process of wound healing (51).

**Skeletal Muscle Distribution**

Using immunohistochemistry, both Fmrp and Fxr2p are weakly detectable in skeletal muscle tissue, whereas Fxr1p is highly expressed in skeletal muscle tissue. Fsr1p is mainly localized within the muscle contractile bands, which can 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 Fsr1 isoforms of 81–84 kDa. These results are in line with a recent study on both Fmrp and Fsr1 characterization in mouse muscle and mouse myoblastic cell lines (20). From these studies it was suggested that these novel isoforms might target only specific mRNAs 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 differentiation. 

Immunohistological microscopic studies of the specific iso-
forms of Parp1 in muscle are in progress and should sho 
light on this specific function in the contractile 

tissue of skeletal muscle. The presence of 

cross-reactive material in muscle extracts for Parp1 
and Parp2 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 con-

ditions in the Western blot might influence the recog-

nition of the antibodies and thus the efficacy of the 

antigen-antibody reaction.

In this study we have given an overview of the distri-

bution of Parp1, Parp2, and Parp3 in several mouse 

organs. Although this systematic study is more de-

scriptive, it will help further studies in elucidating the 

role of the Parp proteins in molecular mechanisms.

In particular, immunohistochemical and biochemical 

studies on double knockout mice (ParpI/Parp2) should give 

information and about a possible role for in vivo al
genotieration, their inter-

relationships, and possible compensatory functions of 

the FAS proteins. In addition, the generation of an 

Parp1 knockout background of transgenic mice that 

have tissue-specific or cell-specific Parp 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 studies present 

the normal distribution of the Parp proteins and may 

serve as a reference for a/biological studies.

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trations. This work was supported by grants from the E.U (HMP4-CT960103) 

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4.4 Publication 4

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

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

The absence of the FMRP (fragile X mental retardation protein) 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; FMRP; FXR1; FXR2; embryogenesis.

1 Introduction

The identification of the FMR1 gene was a first and important step in understanding 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 a 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 the KH domains and one RGG Box, which are characteristic for RNA-binding proteins [3–5], and are capable to form multi-protein complexes in vitro [6]. The amino acid sequence of FMRP (MW 80–90 kDa) is highly homologous to those of FXR1P (MW 70–90 kDa) and FXR2P (MW 95 kDa) along the aminoterminal and central regions (85% and 70%, respectively); however, the carboxyterminal 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 [6–8]. 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 mouse tissues [11–13]. Later, when antibodies against FMRP became available, immunocytochemistry was used to study the subcellular 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 embryonic development, FMRP 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 germinal cells [17,19]. At the subcellular level, FMRP was mainly present in the cytoplasm in association with either polyribosomes or becomes attached to the endoplasmic reticulum [20–22].

In situ RNA hybridization studies of FXR1 in human embryos have shown the presence of large amounts of FXR1 in the nervous system and several non-neural tissues, including...
cartilaginous structures, liver, intestine, skin, kidney, and gonads [13]. Mouse tissues (fetal and adult) showed the presence of FXR mRNA in skeletal muscle, central nervous system, and gonads [23]. Immunohistochemistry of human embryos and adult tissues showed a similar cytoplasmic labeling pattern of FNRP and FXRP in human adult brain, whereas in fetal brain, FXR1P was also observed in the nucleus of a substantial number of neurons. In human adult tests, FXR1P was localized in spermatids and in human fetal tests, FXR1P was present in both primordial germ cells and non-primordial germ cells [17]. In mouse muscle tissue specific isoforms (61-64 kDa, long isoform) of FXR1P were detected within the muscle contractile bands [18].

Thus far, the information about FXRP distribution in tissues is limited. In human embryos and fetal brain (23 weeks), FXR2 mRNA expression is similar to FNRP and FXR1 mRNA expression [19]. Immunohistochemistry of FXRP2 in human adult brain showed a similar cytoplasmic expression pattern as FNRP 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 testes, 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 immunohistochemical study, using non-specific 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 Fmr1 and FXR2P distribution in the presence and absence of Fmr1p and may tell more about possible compensatory mechanisms of Fmr1p and FXR2p in the absence of Fmr1p. 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 FNRP [18]. Rabbit polyclonal antibodies against FXR1P (C1097, which recognizes the long and short isoforms) and FXR2P (1337, C-terminal part) were used [17].

FXR proteins during mouse embryonic development

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 (5 and 11 days) tissues were performed on paraaffin sections. Since no difference in labeling pattern was observed between both techniques, here only results from paraaffin embedded tissues will be shown. Brain sections from adult WT and KO adult mice were used as positive and negative control for FNRP 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 paraaffin according to standard procedures. Sagittal sections (5 µm) 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% Triton, pH 7.3, 0.5% Proteinase K, 0.1% glycine. Sections were incubated with the primary antibodies for 1 hour, followed by an incubation with either rabbit anti-mouse or goat anti-rabbit immunoglobulin, conjugated with peroxidase. Antigen-antibody complexes were visualized with 3,3' diaminobenzidine-tetrahydrochloride (DAB). The sections were counterstained with Giems Haematoxylin, 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 Fmr1. 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 paraaffin-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, 11, 13, 15,
17 days old embryos and neonates. The indirect immunoperoxidase technique is not suitable for comparative quantitative analysis between the Fmr 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 mice as a reference and scored this labeling intensity with three plusses. For this study, we have divided the stages of embryonic development into three categories, i.e., early stages (0–10 days), middle stages (11–14 days), and late stages (15–18 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 (Figures 1f). 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), FXRP1 (b, e, h, k), and FXRP2 (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; k, l). Note the nuclear labeling of Fxr1p (e; arrows).
Table 1. Expression of the Fxr proteins during early embryonic development of the mouse

<table>
<thead>
<tr>
<th>Stage (p.c.)</th>
<th>Fxr1p</th>
<th>Fxr2p</th>
<th>Fxr3p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unfertilized oocytes</td>
<td>-</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>20 hours p.c.</td>
<td>-</td>
<td>** Cytoplasm</td>
<td>** Cytoplasm (+/- some deposits)</td>
</tr>
<tr>
<td>(1 cell stage)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 days p.c.</td>
<td>++ Cytoplasm (-/- slight deposits)</td>
<td>+++ Cytoplasm</td>
<td>+++ Cytoplasm (+/- some deposits)</td>
</tr>
<tr>
<td>(8 cell stage)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7 days p.c.</td>
<td>++ Erythroid</td>
<td>+++ Cytoplasm</td>
<td>Cytoplasm</td>
</tr>
<tr>
<td></td>
<td>Nucleus</td>
<td>Emery's Nucleus</td>
<td>Emery's Nucleus</td>
</tr>
<tr>
<td></td>
<td>Erythroblasts</td>
<td>Erythroblasts</td>
<td>Erythroblasts</td>
</tr>
<tr>
<td></td>
<td>Erythroblast's membrane</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9 days p.c.</td>
<td>++ Cytoplasm</td>
<td>++ Cytoplasm</td>
<td>++ Cytoplasm</td>
</tr>
<tr>
<td>General signal</td>
<td>General signal</td>
<td>General signal</td>
<td>General signal</td>
</tr>
</tbody>
</table>

* p.c. = post conception

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

After these early stages, the immunocytochemical signal increases until the embryos are 11-15 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 2b) 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 is much lower 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 parasagittal 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, i, l) were used. The brown staining depicts the localization of the three different proteins in the rod ganglia of 11 days old WT mouse embryos (a, b, c), gonada of 13 days old WT mouse embryos (d, e, f, on the right side: ovary and on the left side: testis), prolifert 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 2g) 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), olfactory cells, and receptor cells in the retina of the eyes (data not shown). In addition, ganglia, respiratory epithelium (Figure 3d), choroidal plexus (Figure 3g), chromatin 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

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Middle stages 11–14 days</th>
<th>Late stages 15–19 days</th>
<th>Neonate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart, Vessels, Dens</td>
<td>++</td>
<td>+</td>
<td>+/-</td>
</tr>
<tr>
<td>Skeletal muscle</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pancreas</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Digestive epithelum</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lung, Liver</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thyroid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kidney</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adrenal cortex</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Pituitary, Epidermis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Respiratory epithelium</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ganglia, Spinal cord</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Neurons, Hair follicle</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Choroidal plexus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adrenal medulla</td>
<td>+</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>Sensory Cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gonads</td>
<td>+</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Endodermal origin</td>
<td>Mesodermal origin</td>
<td>Endodermal origin</td>
<td></td>
</tr>
</tbody>
</table>

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 1a) and continues during later stages. In 2 and 7 days old embryos, Fxr1p is present both in the cytoplasm and the nucleus (Figure 1e, arrows, and 1h, respectively). In mid-stage the nuclear labeling disappears and only a high cytoplasmic labeling remains, including neural tube (Figure 2k). In later stages (15–19 days), Fxr1p is highly expressed in ganglia (Figure 2b) and neurons in the brain (Figure 2h). Gonads show an intermediate labeling intensity (Figure 2e).

Table 3. Fxr1p expression during mouse embryonic development

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Middle stages 11–14 days</th>
<th>Late stages 15–19 days</th>
<th>Neonate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exocrine pancreas</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thymus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kidney</td>
<td>++</td>
<td>+</td>
<td>+/-</td>
</tr>
<tr>
<td>Vessels</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adrenal medulla</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Digestive epithelum</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver, Lung</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gonads</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adrenal cortex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hair follicle</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Pituitary</td>
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<td></td>
<td></td>
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<tr>
<td>Thyroid</td>
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<td></td>
<td></td>
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<tr>
<td>Endocrine pancreas</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Respiratory epithelium</td>
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<tr>
<td>Heart</td>
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<td></td>
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<tr>
<td>Skeletal muscle</td>
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<tr>
<td>Neurons, Sensory Cells</td>
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<tr>
<td>Spinal cord, Ganglia</td>
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<tr>
<td>Choroidal plexus</td>
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<td></td>
<td></td>
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<tr>
<td>Epidermis</td>
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</table>

Endodermal origin  Mesodermal origin  Endodermal 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 1f, ar-
and only weak in the embryonic and extraembryonic tissues (Figure 1); 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 b).

FxR2p labeling intensity does not increase in middle stages, as is illustrated for ganglia (Figure 2c), gonads (Figure 2d), and neurons of the brain (Figure 2). 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 2), and sensory cells (Figure 3). 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 3b), and adrenal gland (Figure 3f). 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

<table>
<thead>
<tr>
<th>Middle stages</th>
<th>Late stages</th>
<th>Neonate</th>
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<tr>
<td>11-14 days</td>
<td>15-19 days</td>
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<tr>
<td>Choroid plexus</td>
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<td>Digestive epithelium</td>
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<td>Exocrine pancreas</td>
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<td>Kidney, Nephrons</td>
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<td>Vessels, Heart</td>
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<tr>
<td>Skeletal muscles</td>
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<tr>
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<td>Adrenal medulla</td>
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<tr>
<td>Gonads</td>
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<td>Palatine</td>
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<td>Endocrine pancreas</td>
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</tr>
<tr>
<td>Ganglia</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Spinal cord</td>
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Discussion

In the last decade, many studies have been performed to understand the pathophysiology of the fragile X syndrome. In particular, the unraveling 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 of 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 FMRP gene promoter activity [24]. On the other hand, the low expression of Fmrp in the other tissues suggests a dual function of Fmrp. 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 Fmrp 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 parental genomic imprinting as the cause of POF in females carrying a premutation [36]. The possible cellular mechanism for this phenomenon is unknown, but a role for Fmrp in maturation of the follicles might be the basis. However, it cannot be excluded that the presence of two active X chromosomes in female primordial cells is the sole reason for the high expression. Our immunocytochemical data are not in line with earlier in situ hybridization studies where high levels of Fmrp 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 Fmrp mRNA levels were described for thymus, intestine, submandibular gland, and eye [11]. Apparently, the probes used in this in situ hybridization study also detect Fmr1 and Fmr2 transcripts as a result of the high homology between the Fmr genes. The occurrence of these homolo-
Neuronal 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 (2–7 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 26 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 S-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 mRNAs and the reduced translation efficacy of these neuron-specific mRNAs 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 mineralocorticoid 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 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/KO/Fxr1p/Fxr2p) 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. Further research should be focused on these 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 Fxr expression in a Fmr1 KO background to perform rescue studies for the fragile X phenotype.

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We like to thank Tam de Vries Lenchot for preparing illustrations. This research was supported by grants from the EU (Y.O.C. BMH4-CT96-683 and BMH4-CT97-072924) and NIH grant HD38863-01 (B.A.C.).

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4.5 Publication 5

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


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Instability of a (CGG)$_{88}$ repeat in the Fmr1 promoter


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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 cell-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)$_{88}$ repeat has been exchanged with a human (CGG)$_{88}$ 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 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 (1,2,3). 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 1:10000 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. Premutation 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 recombination events to a premutation-length repeat could explain the mouse 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 Simpson-Guoza formula (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...
AGG triplets (17). The most common alleles of between 29 and 32 triplets are interpreted 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 24-28 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 33 CGG triplets. Since the longest pure CGG tract is always found at the 3' end of the CGG repeat and thus 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 mechanism of the CGG repeat expansion observed in the fragile X syndrome, it is of 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 germ line and during (early) embryonic development. We have reported previously that a (CGG)120-AGG(CGG)120-CAG(CGG)120 repeat was transcribed without any detectable length change through several generations of transgenic mice (20). Similar results were obtained in mice with a (CGG)120-TGG(CGG)120-TGG(CGG)120 repeat 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 FMRI 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 94 CGG triplets. This was done using a homologous recombination strategy with a mouse promoter construct where the mouse endogenous CGG repeat was exchanged for a CGG94 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 FMRI promoter region

The mouse homologue of human FMRI was isolated from an E14 ES cell plaque library (kindly provided by D. Meijer, Department of Genetics, Erasmus University, Rotterdam). A new cassette, flanked by loxp sites, was cloned in the BamHI site. The endogenous (CGG)94 present in the mouse promoter was replaced by a (CGG)94 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 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 FMRI and Fmr1 showed that all the identified regulatory
elements were conserved. Whether or not all those elements are functional is not known.

**Generation of the mice**

Homologous recombinants were recognized by the absence of the endogenous mouse (CGG)$_{16}$ allele, and the presence of the expanded (CGG)$_{16}$ allele. Of the 1200 screened ES cell clones, 13 were identified as possible homologous recombinants. The lengths 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)$_{16}$ 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 mated onto a FVB background and the repeat length was determined in the next generation. The first heterozygous females were crossed with a wild-type male or a male expressing Cre recombinase. Expression of Cre recombinase allows deletion of the neo cassette present between the loxP sites, minimizing the changes to the mouse genomic flanking sequence in the resultant transgenic animal. (CGG)$_{16}$ mice with ([CGG]$_{16}$neo) and without ([CGG]$_{16}$) the inserted neo marker were subsequently crossed and the offspring examined for repeat length.

In total, 125 (CGG)$_{16}$ transmissions were studied. (CGG)$_{16}$neo was transmitted 34 times, 15 male transmissions and 19 female transmissions. (CGG)$_{16}$ was transmitted 121 times, 50 maternal transmissions and 41 paternal transmissions. Offspring homozygous for the expanded repeat were included for paternal (13) and maternal (13) transmission. In total, 15 instability events 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 Perlman-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 events (13) triplets were observed after (different) paternal transmission, but male transmission also led to six expansions. Five expansions were observed in transmission via the female germ line. Two instabilities were observed in homozygous (CGG)$_{16}$ female offspringing and in these cases the origin of the instability could be either paternal or maternal. The shortest CGG repeat present in the descendents was an 87CGG and after passage through several generations the longest repeat is 101 CGGs. While the number of paternal changes (at least eight) outweigh maternal ones (at least five), the number of maternal transmissions (at least 85) 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 human 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. Though 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 tetraloop 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 instability' (31). Although for some trimethylsaturated repeat mouse models small expansions or contractions have been observed, dynamic mutations such as those in trinucleotide repeat diseases 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 effecting 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, 152 transmissions of the expanded (CGG)$_{16}$ allele were studied and 13 stability events 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
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 transmis-
sions, 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 transmis-
sion. 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)$_{16}$ male mouse model, no dynamic mutations were observed.
In contrast to fragile X syndrome, most other trinucleotide
repeat disorders show preferential instability upon male trans-
misision. For one transgenic mouse model containing an
expanded CAG repeat, it was found that the gender of the
offspring determined the degree of instability (22). Whether
the gender of the offspring in our CAG 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 regu-
laratory elements (33). Those 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, studies 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)$_{16}$ 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 CAG 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 CAG repeat,
even if 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 (25,30). 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
direction of replication is not known. It may well be that the
direction of replication in the human situation might favor 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 (rnr1 mutants) increases instab-
ility throughout the whole genome, including trinucleotide
repeats (26-37). For normal and premutation size CCG
repeats, a 10-fold elevated frequency of expansion in rnr1
yeast strain has been found (38). This suggests that FEN1
could play a role in CCG trinucleotide repeat instability.

The endogenous mouse Fmr1 5' UTR contains a CCG
repeat of between 3 and 12 triplets. In the E5 cells used in this
study, the (CGG)$_{16}$ repeat was exchanged with a (CGG)$_{6}$ repeat
in the expectation that length of repeat exceeds a threshold of
instability in the mouse. In humans, repeat instability occurs
when the number of CCG 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 insta-
Bility (7 to 6) at a much higher frequency in maternal trans-
mission than in paternal transmission. The majority of changes
transmitted through the female germline were small contrac-
tions, as in humans, whereas small expansions occurred more
frequently in paternal transmission (40). The male 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 contrac-
tions 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 transmis-
sion. 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 CCG 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 mechanisms
involved in repeat instability in humans might be absent in
mice, or perhaps that the environment leading to the instability

Table 1. Total numbers of male and female transmissions for both (CGG)$_{16}$ mouse
and (CGG)$_{16}$ human

<table>
<thead>
<tr>
<th>Female/male transmissions</th>
<th>Instabilities</th>
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</thead>
<tbody>
<tr>
<td>(CGG)$_{16}$ mouse Female 19</td>
<td>0</td>
</tr>
<tr>
<td>(CGG)$_{16}$ mouse Male 15</td>
<td>6 (2+4, 1+4, 1+3)</td>
</tr>
<tr>
<td>(CGG)$_{16}$ mouse Female 57</td>
<td>5 (2+3, 1+4, 1+3)</td>
</tr>
<tr>
<td>(CGG)$_{16}$ mouse Male 28</td>
<td>4 (1+1, 1+1, 1+3)</td>
</tr>
<tr>
<td>(CGG)$_{16}$ human Female/Male 30</td>
<td>2 (2+1, 1+1)</td>
</tr>
</tbody>
</table>

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.
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 tetraplexes 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 CAG 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 (20,42,43). 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 FMRP gene and lead to loss of FMRP expression. Subsequently, both repeat expansion and FMRP expression could be studied in subsequent generations. It has been reported recently that premutation alleles result in elevated mRNA levels (64) 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 HindIII fragment of the mouse Fmr1 promoter region inserted into a pBR322 derived vector (pBR322 - HindIII 375- XbaI 1201). In this fragment, a new cassette flanked by loxP sites was cloned in the HindIII site present in intron 1 (Fig. 1). The mouse (CGG)₉₈ repeat was exchanged for a (CGG)₉₈ repeat of human origin. The human-derived (CGG)₉₈ repeat was isolated as an XhoI-XhoI fragment from a cloned expanded CAG 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 XhoI site in intron 1 and the substitution of 2 bp (TCCG) to abolish an XhoI site in intron 1. The XhoI 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 as XhoI site was generated by an A→G transition based on the
homology between the human and the mouse promoter. The intensity 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, pCMV was linearized by an XhoI digest. After purification, linearized plasmid DNA was used to transfect ES cells. Electroporation was performed with 107 ES cells in 400 μl PBS using a Proprietary Gene Pulser (1200 μF and 117 V during 10 μs). Using double selection with G418 (200 μg/ml) and FIAU (2 μM) the cells were cultured to allow colony formation. Colonies 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 200 μl (10 mM Tris-HCl, 400 mM NaCl, 2 mM EDTA pH 7.3-7.4) and 30 μl of 10% SDS proteinase 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 was added to precipitate the DNA. The DNA was dissolved in 30 μl H2O. For radioactive PCR, 1 μl DNA was used.

Radioactive PCR was performed to determine the repeat length in the ES clones. Primers C5’-GCTCAGCCTGGTCGGTGCGAGGGA and F5’-AAACCAAGCCACCTTGCCATGTTC-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 C57BL/6J blastocysts. These blastocysts were transferred to pseudopregnant female mice. Three chimeras were generated and crossed with wild-type PVB and C57BL/6J females. Female offspring of these chimeras were tested for the presence of the expanded (CGG)14 repeat. Male containing the expanded CGG repeat were crossed with wild-type mice as well as with (CGG)14 mice. Repeat instability upon transmission to offspring was tested by radioactive PCR.

The BASSI cassette introduced into the BsmHI flanked by loxP sites. Because the presence of the loxP cassette might disturb the natural environment of the CGG repeat, knock-in mice were crossed with mice expressing Cre recombinase. In this way the TG2 cassette inserted into the EμI gene was kept minimal. These (CGG)14neo mice were also crossed with wild-type mice, and with (CGG)14neo littermates.

Fragile X syndrome 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 allele 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 analyzed using an ABI 377 sequencer (PE Biosystems).

ACKNOWLEDGMENTS

We would like to thank T. de Vries for the generous gift of the mouse zangene for Cre-recombinase. M. Bier for the mouse ES cell stage library and P. H. Nieuwland for blastocyst injections. We also thank Perkin Elmer for their gift of their fragile X size polymorphism assay. This study was supported by the Netherlands Organization for Scientific Research (N.W.O.) and the National Institute of Health (NH 5 E) (HD38038).

REFERENCES


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)\textsubscript{n} repeat in the 5' untranslated region of the fragile X gene, FMRI. 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 FMRI 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 FMRI 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 FMRI 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.
Summary

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 countered 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' onvertealde gebied van het fragiele X gen FMRI. Een CGG repeat met meer dan 200 repeat eenheden wordt een volledige mutatie genoemd. De aanwezigheid van een volledige mutatie gaat samen met methyleering van deze CGG repeat en het promoter gebied van het FMRI gen, waardoor het gen wordt geïnactiveerd. Dit resulteert in de aanwezigheid 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. Individuen 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 modellsystemen 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 FMRI gen heel geconserveerd is in verschillende soorten organismen, heeft men het gen kunnen isoleren van organismen zoals de fruitvlieg Drosophila melanogaster, de zebravis Danio rerio en de kluwpad Xenopus laevis. 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 FMRI 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 verkenningstraining 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 betekent dat dit 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 \textit{Fmr1} gen. Introductie van een CGG repeat sequentie op een autosoom in de muis heeft niet tot instabiliteit geleid. Vervanging van de muisen CGG repeat in het \textit{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. Vervolgexperimenteren 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 \textit{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 muisen zijn uitgevoerd om de drie Fmr-ewitten, Fmrp en zijn twee homologen Fxr1p en Fxr2p, te lokaliseren (Hoofdstuk 4.3 en 4.4). Gedurende de vroege embryonale ontwikkeling vertoont het expressiepatroon van de Fxr-ewitten veel overeenkomsten, terwijl tijdens de late embryonale ontwikkeling en in de neonaat een meer afwijkend expressiepatroon 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 phenotype, suggereert een vroege, celtype afhankelijke functie voor Fmrp. In de hersenen van volwassen muisen zijn de drie Fxr-ewitten 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 \textit{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-ewitten waargenomen. Alleen skeletspierweefsel laat een hoge expressie zien voor Fxr1p. De verschillen en overeenkomsten tussen de distributie van de Fxr-ewitten hebben implicaties voor wat betreft de normale functie en de betrokkenheid van de Fxr-ewitten 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-ewitten.
Samenvatting

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 expressie niveau 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

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>AGS</td>
<td>audiogenic seizure susceptibility</td>
</tr>
<tr>
<td>Asn</td>
<td>asparagine</td>
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<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<td>ES</td>
<td>embryonic stem cell</td>
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<td>FBS</td>
<td>FMRP binding site</td>
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<td>Fmr1</td>
<td>fragile X mental retardation gene 1 (mouse)</td>
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<td>FMRP</td>
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<td>Fmrp</td>
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<td>FSH</td>
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<td>glutamine receptor</td>
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<td>YAC</td>
<td>yeast artificial chromosome</td>
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</table>
Curriculum Vitae

Naam 
Cathy (Katharina Elisabeth) Bakker-van Kempen

Geboren 
4 december 1966 te Leiden

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Februari 1991 
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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.
List of publications


Dankwoord

“Er was eens...”
Nee, een sprookje is het niet geworden, maar wel een lang verhaal.
De afgelopen elf jaar hebben een mooi stuk werk opgeleverd en heel veel contacten.
Over het werk zijn de voorgaande bladzijden volgeschreven, over de contacten ga ik er
nu nog enkele vullen.

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En ondertussen groeide de fragiele X DNA groep ook verder. Esther, alias E*, het was (en is weer) leuk met jou. Altijd in voor een geintje (was het wiptoeltje niet wat krap?), maar ook voor serieuze zaken. Beste Carola, het waren woelige jaren, maar we hebben veel van elkaar geleerd. Het ga je goed. En Ingeborg, ons muizenmeisje, jouw werklust is geweldig. We staan al jaren samen aan de bench en nu ben je één van mijn paranormen, ook geweldig. Pietro, mijn Italiaanse vriend! Nu zijn we (straks) allebei Doctor. Dennis was maar heel kort in onze fragiele X groep. Marianne, Edwin (gelukkig kun jij mij vaak redden uit de computermalaise) en Lau zijn recentelijk de groep komen versterken. Allen bedankt voor jullie positieve bijdrage en invloed.

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Bij de karakterisering van de knock-out muizen waren mensen uit vele disciplines betrokken. Een aantal mensen wil ik met name bedanken. Voor het uitvoeren van onder andere de eerste gedragsproeven in Antwerpen: Patrick Willems, Frank Kooy, Edwin Reyniers, Peter De Deyn en Rudy D’Hooge. De testis is uitvoerig bekeken en beschreven door Axel Themmen en Karin Slegtenhoost-Eegdeeman en de hersenen door Marcel Vermey. Momenteel loopt er nog de samenwerking met de mensen achter de eyeblink-test, Bjorn Dortland en Bas Koekkoek uit de groep van Chris de Zeeuw. And I would like to thank David Nelson and cowokers for their part in our collaborative studies. En de eer die al “mijn” muizen toekomt, heb ik ze proberen te bewijzen op de cover.

Zonder hulp van de computermensen, Ton, Sjoef en Pim, en ‘onze’ fotograaf Tom (jij bent voor mij weer tot op de bodem van het archief gegaan, en met succes), was dit boekje niet onstaan. Ook de secretariële hulp, met name van Jeanette, was onontbeerlijk, en natuurlijk de hulp van de dames van de bestellingen, Melle en Mieke, en de dames van de speelkasten. Bedankt allemaal.

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oprichten van de OR, samen met Gerard, Simone, Ton (zijn allemaal vlak voor of na de oprichting vertrokken; hoe zou dat nou komen?!) en Frans Los, was spannend en leerzaam. Het was een leuke tijd. Bedankt.

Beste Ben, jij bent mijn promotor en al die jaren ‘mijn baas’ geweest. We hebben samen voor het vuren gestaan, maar jij hebt het met mij gered en ik met jou. Bedankt dat ik toch steeds de draad weer mocht oppakken. (Nu dan eindelijk) Rob, mijn copromotor, wat hebben we veel gepraat. Het was goed dat jij er was (en nog bent!). Bedankt voor al jouw hulp en goede kritieken. Annemieke, sinds jij terug bent is het weer als vanouds. We relativeren wat af, over van alles en nog wat, gezellig tijdens de lunch.

Ja, Arjenne, we begonnen als collega’s samen, er ondanks dat we het vaak over opstappen hadden, zijn we er nog steeds. Als collega’s, maar inmiddels ook als vriendinnen, want we gingen door dik en dun, over bergen en door dalen. Natuurlijk ben jij één van mijn paranimfen. Bedankt voor jouw steun en alle gezelligheid.

Anneke, mijn zusje, het mag dan wel gewoon jouw werk zijn, maar het is geweldig dat jij, en jouw collega Rob, voor mij zo’n mooie kant hebben ontworpen. Marcel...

Last, but not least, mijn stabiele basis.

Mijn ouders ben ik dankbaar voor de mogelijkheden die ze mij hebben gegeven en de stimulans die ze altijd mijn geweest om te gaan studeren. En pap en mam, het is geweldig te weten dat jullie nu heel trots op mij zijn!

Lieve Hans, bij jou ben ik al heel wat jaartjes lekker thuis. Je bent mijn steun en toeverlaat en wat geweldig dat we de ruimte hebben gevonden om ‘dit werkstuk’ voor elkaar te krijgen. Ook toen er twee keer een speelbrekertje voorbij kwam.

Ja, dan die twee speelbrekertjes! Cas en Maeve, jullie zijn voor mij waar het om gaat in het leven, eindeloos geluk. Mama heeft nu weer alle tijd om van jullie te genieten!

Bedankt lieve Hans, Cas en Maeve.

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 fragile X patiënten.

(Slegtenhorst-Eegdenen 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.

(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 283: 2067-2070)
(Dit proefschrift)

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

(Dit proefschrift)

De term ‘normal transmitting male’ voor mannelijke dragers van een premutatie is echterhaald.

(Hagertman 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)
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