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The *FMR1* Gene and Fragile X-Associated Tremor/Ataxia Syndrome

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Abstract

The CGG-repeat present in the 5'UTR of the *FMR1* gene is unstable upon transmission to the next generation. The repeat is up to 55 CGGs long in the normal population. In fragile X patients, a repeat length exceeding 200 CGGs (full mutation: FM) generally leads to methylation of the repeat and the promoter region, which is accompanied by silencing of the *FMR1* gene. The gene product FMRP is involved in regulation of transport and translation of certain mRNA in the dendrite, thereby affecting synaptic plasticity. This is central to learning and memory processes. The absence of FMRP seen in FM is the cause of the mental retardation seen in fragile X patients. The premutation (PM) is defined as 55–200 CGGs. Female PM carriers are at risk of developing primary ovarian insufficiency. Recently it was discovered that elderly PM carriers might develop a progressive neurodegenerative disorder called fragile X-associated tremor/ataxia syndrome. Although arising from the mutations in the same gene, distinct mechanisms lead to fragile X syndrome (absence of FMRP) and FXTAS (toxic RNA gain of function). The pathogenic mechanisms thought to underlie these disorders are discussed, with a specific emphasis on FXTAS. This review gives insight on the implications of all possible repeat length categories seen in fragile X families.

Keywords

FMR1; FMRP; fragile X; FXTAS; CGG repeat instability; RNA gain-of-function

THE *FMR1* GENE AND THE UNSTABLE (CGG)_n-REPEAT

Fragile X syndrome (FXS) has been recognized as the most common inherited form of mental retardation [Glass, 1991]. Its name refers to a fragile site, which was discovered on the long arm of the X chromosome of four mentally retarded patients [Lubs, 1969]. This fragile site was later found to be inducible when culturing cells in folic acid-deficient medium. This facilitated the establishment of a link between expression of the fragile site (at Xq27) and the clinical phenotype [Sutherland, 1977; Turner et al., 1980]. Cloning of the gene responsible for FXS, *FMR1* (fragile X mental retardation 1), revealed the presence of an unstable expanded (CGG)_n in the 5' UTR. In FXS this repeat exceeds 200 trinucleotides (full mutation: FM) [Oberlé et al., 1991; Richards et al., 1991; Verkerk et al., 1991]. *FMR1*

consists of 17 exons, spanning a 38 kb region [Eichler et al., 1993]. The (CGG)_n in the *FMRI* gene is considered a “dynamic mutation;” the phenomenon that a trinucleotide repeat becomes dramatically unstable once it exceeds a certain threshold length [Richards and Sutherland, 1992]. Depending on the length of the (CGG)_n, different clinical outcomes can develop.

Analysis of repeat length variation in the normal population showed a range of allele sizes between 6 and 54 CGGs [Fu et al., 1991], with an average of 29–30 repeats [Dombrowski et al., 2002]. Alleles of between 45 and 54 CGGs form a distinct category. They were named gray zone alleles, when it was appreciated that these alleles are more likely to be unstable upon transmission, than are (CGG)_n shorter than 45 trinucleotides [Fu et al., 1991; Zhong et al., 1996]. Gray zone alleles frequently contain a long and uninterrupted (CGG)_n [Zhong et al., 1996]. The longest tract of uninterrupted (CGG)_n appears to determine instability and susceptibility to disease, whereby the presence of a pure CGG-tract of more than 33 triplets appeared to greatly enhance the chance of unstable transmission. Thus, AGG interruptions might stabilize a (CGG)_n [Eichler et al., 1994]. This could be through prevention of formation of stable secondary RNA structures such as hairpins, as has been shown to occur in in vitro studies [Napierala et al., 2005]. Hairpins might decrease the efficiency of FMRP translation initiation [Chen et al., 2003]. AGG interruptions, through influencing RNA structure, predominantly determine the extent of instability of long normal and short permutation (PM) alleles [Napierala et al., 2005].

The remaining repeat range of 55–200 CGGs has been defined as the PM [Maddalena et al., 2001]. Initially, it was thought that the only risk associated with the PM was for the (CGG)_n to expand to an FM allele, as alleles in this size range are quite unstable upon maternal transmission [Nolin et al., 2003]. The risk of expansion from a female PM carrier to an FM in her progeny increases with repeat length [Bat et al., 1997]. In few cases, a contraction of maternal origin is seen. Inheritance of a (CGG)_n of paternal origin shows a less consistent pattern, as expansions, contractions and stable transmissions occur. Expansion of a PM to an FM is restricted to maternal transmission. Also, daughters of a male FM carrier, affected with fragile X, never show clinical or cytogenetical signs of FXS. This was explained by the finding that FM males only carry PM alleles in their sperm [Reyniers et al., 1993]. Thus, both allele size and the parent’s sex determine the risk of expansion from parent to offspring [Nolin et al., 1996].

One in 813 males and one in 259 females of the general population were described to carry the PM [Rousseau et al., 1995; Dombrowski et al., 2002]. A recent study in 40,000 women in Israel revealed a prevalence of 1 in 154. No difference in carrier frequency was seen between women with and without family history of mental retardation and developmental abnormalities [Berkenstadt et al., 2007]. However, it is now known that different ethnic groups show a different prevalence, for example it is less common in Asian populations [Tzeng et al., 2005], while it is more prevalent in Mediterranean groups [Pesso et al., 2000; Toledano-Alhadeef et al., 2001].

Early in situ hybridization studies in mice revealed that *FMRI* mRNA is abundant and widespread in early embryogenesis. Later in embryonic development a specific expression

pattern develops, with high expression mainly in brain, testis and ovary. Adult mice also showed high expression in brain and testis. No *FMR1* expression was seen in mature ovary, despite the high levels in the fetal ovary, when proliferation of oogonia takes place. Thus, the *FMR1* gene might have a function during germ cell proliferation in both sexes [Bächner et al., 1993a,b; Hinds et al., 1993]. *FMR1* mRNA expression in human fetuses was high in the nervous system, but also in some non-neuronal tissues such as cartilage, hepatocytes, the spinal cord and the retina [Abitbol et al., 1993; Agulhon et al., 1999].

In fragile X patients, the (CGG)_n of over 200 trinucleotides (FM) and the upstream promoter region of the *FMR1* gene are usually hypermethylated [Oberlé et al., 1991; Verkerk et al., 1991; Sutcliffe et al., 1992]. As a result, gene transcription is silenced, thus the gene product fragile X mental retardation protein, FMRP, is not produced. The complete lack of FMRP in neurons is the cause of the mental retardation seen in fragile X patients [Verheij et al., 1993]. The functions of FMRP and the consequences of the absence of FMRP in FXS are discussed in more detail below. About 1 in 4,000 males and 1 in 6,000 females have fragile X-linked mental retardation. The clinical spectrum is however much broader than mental retardation, involving learning disabilities and emotional disturbances [Hagerman, 2006].

TIMING OF REPEAT INSTABILITY

When trying to elucidate the mechanism of repeat instability, timing of the event is a major aspect that remains surrounded by question marks. The fact that repeat length changes between generations, tells us that instability occurs at some point between gametogenesis in the parent and early embryogenesis. Attempts have been made to gain insight into what happens around fertilization, although availability of human material is limited. Remarkable observations that expansions to a fragile X FM occur only upon maternal transmission of a PM allele, and that daughters of FM males are clinically and cytogenetically normal, were explained when it was observed that male fragile X patients only have PM alleles in their sperm [Reyniers et al., 1993]. Two models have been proposed to explain this finding (Fig. 1).

First, the prezygotic model predicts that an expansion of PM to FM could occur during maternal meiosis (Fig. 2). This means that the FM must contract to a PM in gametes of male offspring. PM gametes might have some selectional advantage either due to the presence of FMRP, or against the presence of an expanded CGG-repeat. However, FMRP is not required for spermatogenesis [Bakker et al., 1994; Meijer et al., 1994]. FMRP expression might still be beneficial to the spermatogonia as they mature. A selective advantage for cells expressing FMRP was indeed seen in cell culture studies [Rousseau et al., 1991].

In 13-week-old FM fetuses, no PM alleles were detected in testes, while in 17-week-old FM fetuses some primordial germ cells expressing FMRP were observed. This suggests that contraction from FM to PM occurs in the maturation process of the testis, after the initial replication event [Malter et al., 1997]. Paternally derived alleles have been shown to undergo rapid demethylation in murine zygotes, before the first round of replication, while CpG methylation is maintained in maternally derived alleles [Reik et al., 2003]. CpG methylation appears to stabilize the DNA. Hence, paternally derived alleles could be prone

to deletions upon demethylation [Nichol Edamura and Pearson, 2005]. Furthermore, FMs are responsible for a delay in replication of the *FMR1* gene during the cell cycle [Hansen et al., 1993; Samadashwily et al., 1993]. The large number of cell cycles associated with spermatogenesis might enhance a selectional effect [Ashley and Sherman, 1995]. Thus, primordial germ cells with a PM might have a proliferative advantage, thereby overgrowing FM cells. However, if multiple contraction events can take place, it is puzzling why only one distinct PM band is mostly seen in sperm of FM men [Reyniers et al., 1993].

The other model considers a postzygotic expansion, after separation of the germ line. It assumes that the FM allele has never been present in male or female gametes [Devys et al., 1992; Wöhrle et al., 1993]. However, oocytes of a fetal female FM carrier had only FM alleles [Malter et al., 1997]. It is however unknown whether oocytes of a female PM carrier bear PM or FM alleles. A postzygotic expansion mechanism occurring only for a PM allele on the maternal X-chromosome, would require an imprinting mechanism to distinguish it from a paternally derived PM, which would not expand [Reyniers et al., 1993; Trottier et al., 1993].

Based on a simulation study and analysis of tissues of affected fetuses, Moutou et al., 1997 favor a prezygotic model, whereby a selection mechanism accounts for the sole presence of PM alleles in sperm of male offspring. However, they cannot exclude a postzygotic model in which a transition occurs at the early morula stage [Moutou et al., 1997]. Naturally, a final conclusion can only be drawn after analysis of oocytes in ovaries from an embryo carrying a PM allele. Unless the embryo suffers from a different detrimental defect, this material is not available for obvious reasons. Also, oocytes from an adult PM carrier would provide useful information. However, the most likely reason for an ovarium to become available is when a female PM carrier suffers from POF. In this case, naturally, almost no oocytes are present anymore. Expanded (CGG)_n knock-in mouse models have been developed [Bontekoe et al., 2001; Entezamet al., 2007], that show intergenerational repeat instability [Brouwer et al., 2007; Entezamet al., 2007]. These models will be valuable in answering remaining questions.

TRANSCRIPTIONAL SILENCING OF THE *FMR1* GENE

Many studies have addressed the question of how methylation of CpGs in the *FMR1* gene leads to gene silencing. Local chromatin changes as a result of methylation mediate indirect mechanisms, while a direct effect is the inhibition of binding of transcription factors to the promoter [Chiurazzi et al., 1999; Coffee et al., 2002]. Methyl binding proteins (such as MeCP2) recognize methylated CpG residues, upon which a multiprotein complex is recruited. This protein complex includes histone deacetylases (HDACs) [Jones et al., 1998; Nan et al., 1998]. HDACs remove acetyl groups from lysines of histone H3 and H4 amino terminal tails, which result in increased condensation of the chromatin (heterochromatin), which is associated with inactive gene regions [Razin, 1998]. The *FMR1* gene normally shows acetylated histones H3 and H4, while cells of patients with fragile X show reduced acetylation [Coffee et al., 1999]. Experiments in cells of fragile X patients showed that methylation and deacetylation indeed cooperate in silencing chromatin domains, when histone hyperacetylating drugs could synergistically potentiate reactivation of the *FMR1*

gene induced by a demethylating agent [Chiurazzi et al., 1998, 1999]. Further analysis of epigenetic modification in three different regions of the *FMRI* gene revealed that epigenetic codes relevant to the transcription of the gene are concentrated in the 5' UTR region [Tabolacci et al., 2005].

Histones can also be modified in ways other than acetylation, for example, by the addition or removal of phosphate or methyl groups [Turner, 2002]. Methylation of lysines 4 and 9 of histone 3 (H3K4 and H3K9, respectively) has been looked at intensively and it became clear that methylation of H3K4 is associated with a transcriptionally active state, while methylation of H3K9 correlates with transcriptional inactivation [Grewal and Moazed, 2003; Lachner et al., 2003]. Methylated H3K9 has been suggested to recruit histone methyl transferases, associated with HDACs, which then results in histone deacetylation [Cheutin et al., 2003]. However, studies in cell lines suggest that methylation of H3K9 and partial deacetylation, normally associated with heterochromatin, can coexist with H3K4 methylation, which is correlated with active *FMRI* transcription [Tabolacci et al., 2005].

Two brothers with expanded (CGG)_n in the FM range have been described, who were spared from the fragile X phenotype. Although cytogenetical analysis revealed a fragile site, the *FMRI* promoter was unmethylated and they did produce *FMRI* mRNA and FMRP. This indicates that inactivation of *FMRI*, due to methylation and not the expansion itself, is the cause of the symptoms seen in FXS [Smeets et al., 1995]. Characterization of a human embryonic stem cell line derived from a pre-implantation embryo with FM range repeat showed that the *FMRI* gene was unmethylated and expressed, despite the presence of an FM allele. More specifically, it displayed the features of active chromatin [Eiges et al., 2007]. Thus, these results confirm that the (CGG)_n by itself is not sufficient to cause transcriptional silencing of *FMRI* [Sutcliffe et al., 1992]. Upon differentiation (by inducing formation of teratomas in immunodeficient mice) this ES cell line underwent partial transcriptional silencing. Differentiation lead to reduced H3 tail acetylation, and H3K9 became methylated, while *FMRI* mRNA levels decreased. However, the 5' UTR of *FMRI* was still hypomethylated. Thus, the authors concluded that de novo methylation of the upstream regulatory region of *FMRI* follows transcriptional silencing, where-by the chromatin changes take place relatively early during differentiation. DNA methylation appears to contribute to the maintenance, rather than to the induction of transcriptional inactivation in somatic cells in patients with fragile X [Eiges et al., 2007]. This role for DNA methylation has previously been described with regard to X-inactivation in females [Lock et al., 1987].

However, many questions remain to be elucidated about the precise molecular mechanism by which *FMRI* transcription is regulated, of which the answers might prove valuable for developing therapeutical strategies involving reactivation of the *FMRI* gene in FXS. Contrary to in FXS, PM carriers show enhanced transcription of the *FMRI* gene, which is discussed in more detail below.

THE GENE PRODUCT FMRP

FMRP is an RNA-binding protein (RNA-BP), as it bears two KH-domains and an RGG-box; both are sequences with RNA-binding capacities [Ashley et al., 1993; Siomi et al.,

1993; Darnell et al., 2001]. Among many other mRNAs, FMRP can bind its own message with high affinity in vitro [Ashley et al., 1993]. Furthermore, a nuclear localization signal and a nuclear export signal have been identified, suggesting that FMRP shuttles between the nucleus and the cytoplasm [Eberhart et al., 1996; Fridell et al., 1996; Sittler et al., 1996; Feng et al., 1997b]. Eighty-five percent of cellular FMRP is present on actively translating polyribosomes, underscoring the role of FMRP as an RNA-BP [Feng et al., 1997a]. Ribonucleoprotein (RNP) particle domains were identified within FMRP. RNA was shown to bind in stoichiometric ratios, suggesting that each FMRP molecule bears two RNA-binding sites [Ashley et al., 1993]. A minority of FMRP is found in the nucleus, either at the nucleopore, or associated with the nucleolus [Willemsen et al., 1996; Feng et al., 1997a; Bakker et al., 2000].

Immunocytochemical studies in mice revealed that FMRP is ubiquitously expressed up until day 14 of embryonic development. In later embryonic development a more specific pattern arises with tissues of ectodermal origin (brain, ganglia, hair follicles, sensory cells, adrenal medulla) and the gonads (mesodermal origin) showing the highest expression levels [De Diego Otero et al., 2000]. FMRP expression in human embryos (3–7 weeks) was consistent with the murine expression pattern, showing highest expression in brain and testes [Abitbol et al., 1993; Devys et al., 1993; Tamanini et al., 1997; Agulhon et al., 1999]. In adult human brain, FMRP is present in high quantities in neurons, while little or no FMRP is detected in glia and oligodendrocytes [Abitbol et al., 1993; Devys et al., 1993; Hinds et al., 1993; Wang et al., 2004; Pacey and Doering, 2007]. High levels of FMRP expression in human tissue coincide with clinical involvement in FXS [Devys et al., 1993; Malter et al., 1997; Tamanini et al., 1997; Khandjian et al., 1998]. The absence of evident neuropathological abnormalities of fragile X fetuses suggests that the *FMR1* gene product FMRP is not crucial in early stages of development of the nervous system [Agulhon et al., 1999].

After identification of the *FMR1* gene research has focused on the cellular function of FMRP. The RNA-binding capacities, association with ribosomes and the cytoplasmic localization in dendrites pointed towards a function for FMRP in mRNA transport or translation. The RGG-box has been identified as the motif of specific mRNA interaction. The RNA motif consists of a set of four quartets of purines that form an intramolecular stem-loop structure termed a G-quartet [Darnell et al., 2001; Schaeffer et al., 2001]. mRNAs containing a G-quartet have been identified that are potential targets for FMRP, including important neuronal proteins like microtubule-associated protein 1B (MAP1B) and semaphorin [Brown et al., 2001; Darnell et al., 2001; Miyashiro et al., 2003].

mRNA transport and translation in dendrites is important for neuronal function, including modulation of synaptic plasticity. This is essential in memory consolidation and learning [Kiebler and DesGroseillers, 2000; Steward, 2002]. Altered spine morphology (long and thin dendritic spines) has been observed in post-mortem brains of fragile X patients [Rudelli et al., 1985; Hinton et al., 1991; Irwin et al., 2001] and in *FMR1* KO mice [Comery et al., 1997; Nimchinsky et al., 2001; De Vrij et al., 2008]. Target mRNAs of FMRP that are involved in synaptic function or dendritic growth have been identified, which is very interesting in light of the aberrant spine maturation [Darnell et al., 2001]. FMRP was found to strongly inhibit translation of several mRNAs in vitro, through inhibition of the assembly

of the 80S ribosomal complexes on target mRNA. The second KH-domain appears crucial in this process, as the I304N mutant FMRP, with a mutation in this RNA-binding domain, failed to inhibit translation [Laggerbauer et al., 2001]. Thus, a model has been proposed that FMRP binds specific mRNAs and mediates the targeting of these transcripts into the dendrite. During transport they remain translationally inactive until appropriate synaptic input allows translation. Thus, FMRP might have a role in transport and/or translational efficiency of specific mRNAs at the synapse. More specifically, It has been hypothesized that FMRP may link transport and translational control of dendritic mRNAs [Willemsen et al., 2004]. FMRP was postulated to associate directly with the dendritic non-messenger RNA *BCI*, which can form an RNA duplex with a number of mRNAs that are potential targets for FMRP via base pairing to this mRNA. This suggests that FMRP acts through *BCI*, thereby determining the specificity of FMRP function [Zalfa et al., 2003]. However, this function for *BCI* is now under debate [Bagni, 2008; Iacoangeli et al., 2008a,b].

The absence of FMRP could lead to translational dysregulation of a subset of mRNAs at the synapse. Translational dysregulation of mRNAs normally associated with FMRP is proposed to be the underlying cause of mental retardation in fragile X [Brown et al., 2001; Darnell et al., 2001; Willemsen et al., 2004]. fxS patients do not present with gross brain abnormalities. However, both FXS patients and *FMR1* KO mice show an immature phenotype of dendritic protrusions, which partly form the synaptic connections between neurons. Much of what we know about the function of FMRP, stems from studies in *FMR1* KO mice. *FMR1* KO mice suffer from mild cognitive impairment [Bakker et al., 1994; Van Dam et al., 2000] and they show macroorchidism [Bakker et al., 1994]. Concerning the cause of the cognitive impairment, evidence supporting the metabotropic glutamate receptor (mGluR) theory on FXS has accumulated [Bear et al., 2004]. Three groups of mGluR exist and stimulation of group 1 mGluRs (mGluR1 and mGluR5) can lead to long-term depression (LTD). As predicted by the mGluR theory, the internalization of AMPA receptors (one type of glutamate receptor), which is triggered by mGluR5 stimulation [Snyder et al., 2001], is exaggerated in *FMR1* KO mice. Also, enhanced hippocampal LTD was found in knockout mice [Huber et al., 2002; Bear et al., 2004]. It is hypothesized that FMRP normally plays a role in inhibiting the translation of several local mRNAs that are involved in the regulation of AMPA receptor internalization. An S6K1-PP2A signaling pathway has now been described to regulate FMRP function by controlling the phosphorylation status of FMRP, thereby inducing or blocking protein synthesis [Narayanan et al., 2008]. Recently it was shown that FMRP deficient dendrites indeed show aberrant AMPA receptor trafficking resulting in a significantly reduced number of AMPA receptors at the plasma membrane [Nakamoto et al., 2007]. The number of AMPA receptors in the postsynaptic density is correlated with protrusion shape. Thus, this could explain the immature protrusion morphology that has been found in different brain areas of both fragile X patients [Hinton et al., 1991] and *FMR1* KO mice [Comery et al., 1997; Nimchinsky et al., 2001; Koekkoek et al., 2005; Grossman et al., 2006]. Interestingly, a significant rescue of protrusion morphology in primary hippocampal mouse neurons of *FMR1* KO mice was seen after treatment with an mGluR5 antagonist [De Vrij et al., 2008].

CLINICAL SYMPTOMS ASSOCIATED WITH THE PREMUTATION

As mentioned above, it was long thought that carriership of the PM was not associated with clinical problems, other than the risk of expansion to an FM upon transmitting the PM allele to the next generation. However, a small subgroup of PM carriers was reported to have mild learning disabilities, developmental delay, obsessive thinking, ADHD, autism, and in some cases mental retardation and social phobias, or anxiety disorder [Hagerman and Hagerman, 2002, 2004; Farzin et al., 2006].

Primary Ovarian Insufficiency

Furthermore, it became apparent that 20% of female PM carriers manifests premature ovarian failure (POF: cessation of menstruation at or before 40 years of age) [Sherman, 2000]. Women with the PM, even if they are still cycling, have higher levels of follicle stimulation hormone (FSH) than do healthy women [Welt et al., 2004]. It has recently been suggested that anti-Müllerian hormone (AMH) may be a better marker of ovarian decline. AMH is expressed only in growing follicles, thus serves as a marker for the size of the primordial follicle pool in women. Indeed, female PM carriers, with (CGG)_n lengths beyond 70, had lower AMH levels than did female PM carriers with (CGG)_n shorter than 70 nucleotides. Thus, lower AMH levels are suggestive of early ovarian decline in women with (CGG)_{>70} [Rohr et al., 2008]. Penetrance and age of onset of POF, as well as the increase of FSH levels, correlate with (CGG)_n length [Sullivan et al., 2004]. However, a non-linear relationship has been described for age at menopause and PM size, in which PMs in the mid-size range are at greatest risk for POF, while larger repeat tracts are associated with a lower risk [Ennis et al., 2005]. It has been proposed that primary ovarian insufficiency (POI) is a more accurate term for the disorder, to describe the broad range of clinical manifestations associated with what used to be classified as POF [Wittenberger et al., 2007].

Fragile X-Associated Tremor/Ataxia

Over the past few years, it has become apparent that PM carriers are also at risk of developing a progressive neurodegenerative disorder, which is clinically and neuropathologically entirely distinct from FXS [Hagerman et al., 2001; Jacquemont et al., 2003, 2004a]. In 2001, five male PM carriers were described who suffered from progressive action tremor, causing executive function deficits, cerebellar dysfunction, cognitive decline, and Parkinsonism associated with generalized brain atrophy. Also autonomic dysfunction and peripheral neuropathy were reported [Hagerman et al., 2001]. Since it had already been established that PM carriers have elevated *FMRI* mRNA levels in their lymphocytes, but only somewhat reduced FMRP levels [Tassone et al., 2000b], it was then hypothesized that the progressive nervous system degeneration as seen in those five male PM carriers might be the result of the elevated *FMRI* mRNA levels [Hagerman et al., 2001]. The new syndrome was called fragile X-associated tremor/ataxia syndrome (FXTAS) [Jacquemont et al., 2003]. However, not all PM carriers develop FXTAS; of male PM carriers ascertained through family members with FXS, more than a third of men older than 50 years showed both tremor and ataxia [Jacquemont et al., 2004b]. Penetrance increases with age, to at least 50% in men aged 70–90 years. It has now been suggested that penetrance increases with CGG repeat

length. For instance; most people with FXTAS had (CGG)_n with over 70CGGs, while in the general population only 22% of PM alleles are of this size or larger [Jacquemont et al., 2006]. Based on the findings of Jacquemont et al. [2006], Allen et al. [2008] defined alleles with over 70 CGGs as “at risk” alleles, while a repeat shorter than 70 trinucleotides was considered a “low risk” allele. Preliminary analyses of neuromotor tests did not reveal a difference between low-risk allele carriers and non-carriers [Allen et al., 2008].

It is as yet unknown whether specific prodromal signs exist for FXTAS. Although elevated *FMRI* mRNA levels in blood from PM carriers (devoid of FXTAS symptomatology) have been found to be associated with more psychological symptoms [Hessl et al., 2005], naturally at the time it was unknown whether these PM carriers will later develop FXTAS. As awareness about FXTAS grows, monitoring of PM carriers (in known fragile X families) is likely to give insight into whether PM carriers are also at risk of developing problems before FXTAS develops and whether possible prodromal symptoms have predictive value for FXTAS development. This will of course also have importance for genetic counseling of fragile X families.

CLINICAL ASPECTS OF FXTAS

FXTAS patients generally present with cerebellar gait ataxia and intention tremor [Jacquemont et al., 2003], but may develop other neurological symptoms such as Parkinsonism, autonomic dysfunction, and peripheral neuropathy and may suffer from cognitive decline ranging from mild frontal executive and memory deficits to global dementia [Grigsby et al., 2006]. The dementia resembles cognitive performance in the frontal variant of frontotemporal dementia, but not dementia in Alzheimer’s disease [Bozeat et al., 2000; Grigsby et al., 2007]. There is substantial variation in clinical presentation, as illustrated by the broad range of diagnoses given to patients who were later diagnosed with FXTAS, once the syndrome had been identified and described [Hall et al., 2005].

Evidence is accumulating that the severity of some aspects of the disorder is correlated with the length of the (CGG)_n. For instance, age at death negatively correlated with the number of (CGG)_n [Greco et al., 2006]. (CGG)_n length was also reported to negatively correlate with age of onset of the major clinical motor symptoms, action tremor, and gait ataxia [Tassone et al., 2007]. Two earlier studies had not found any association between (CGG)_n length and age of onset of clinical symptoms [Jacquemont et al., 2003; Grigsby et al., 2006]. This difference was explained by cohort size and the width of the repeat range tested. Age of onset of symptoms was neither correlated to *FMRI* mRNA levels, nor to FMRP levels [Tassone et al., 2007]. This was explained by the finding that *FMRI* mRNA levels were measured in peripheral lymphocytes. The increase of *FMRI* mRNA levels in brain tissue from a FXTAS patient relative to a control subject is smaller than when *FMRI* mRNA levels in blood are compared. This is due to the higher abundance of *FMRI* mRNA in brain versus in blood (measured relative to GUS mRNA), also in controls. Differences in transcript levels in brain vary considerably over different regions, although they are driven by the same (CGG)_n length, which is generally consistent throughout the brain [Tassone et al., 2004a]. It is possible that region-specific transcriptional regulation by transcription factors determines the variable *FMRI* expression levels throughout the brain. A lack of correlation between

FMRI transcription and onset of motor problems is not surprising, as FMRP levels are not deviant from normal controls [Tassone et al., 2007]. (CGG)_n length was also described to correlate with increased cognitive and functional impairment in PM carriers [Grigsby et al., 2006]. Total brain volume reductions and increased volume of white matter disease correlated with the number of CGGs [Loesch et al., 2005; Cohen et al., 2006]. Similar, though smaller volumetric changes were seen in PM carriers without symptoms of FXTAS, suggesting that neuropathological changes take place before clinical symptoms become apparent [Cohen et al., 2006].

It has been described that the abnormal elevation of *FMRI* mRNA is associated with increased psychological symptoms such as anxiety, depression, and irritability. Thus, increased psychopathology is not confined to FXTAS patients, but can occur in all adult PM carriers, although especially in males [Jacquemont et al., 2004a; Hessler et al., 2005; Bacalman et al., 2006; Bourgeois et al., 2006]. However, the observed neuropsychiatric phenotypes do not show a very consistent pattern, due to methodological limitations. Therefore, very recently a controlled study into neuropsychiatric functioning was conducted in male PM carriers, who did not show any signs of FXTAS, who were compared to controls who have a similar family environment (care of a family member with fragile X) and other non-PM carrier controls. This study did not find significant differences in psychopathology between PM carriers and non-PM carrier family and other controls, except that PM carriers were impaired in utilizing working memory and accessing recall check. Also, when comparing PM carriers with normal controls, but not family controls, PM carriers showed more alcohol abuse [Kogan et al., 2007]. A recent study investigated the prevalence of mood and anxiety disorders in PM mothers of at least one child with FXS. The most prominent finding was a higher rate of lifetime major depressive disorder (MDD). This did not appear to be fully explained by the stress of raising a child with FXS. Interestingly, a longer (CGG)_n was associated with less likely occurrence of lifetime MDD. Preliminary analysis might suggest a non-linear relationship between (CGG)_n length and MDD, such that women with midrange lengths are at increased risk, while females that carry the longest repeats have a lower risk [Roberts et al., 2008]. Although speculative, this is in line with recent findings for POI prevalence in PM females [Allen et al., 2007]. As yet, no controlled study has been performed for PM carriers with FXTAS. A study which thoroughly investigated cognitive functioning in PM carriers with and without FXTAS, found substantial executive impairment and dysfunction in a wide range of aspects of cognitive functioning in patients with FXTAS, while unaffected PM carriers performed worse than controls on executive cognitive functioning and declarative learning and memory [Grigsby et al., 2008]. All relevant clinical aspects of FXTAS have been nicely summarized in a recent review by Berry-Kravis et al. [2007] and other clinicians and others involved in the field.

In 2004, the first female cases with FXTAS were described. Female PM-carriers are much less likely to develop FXTAS, probably because of a protective effect due to the expression of the normal allele in part of their neurons. Also, estrogen might have an alleviating effect on the disease mechanism [Hagerman et al., 2004]. The diluting effect of the presence of a normal *FMRI* allele is also the likely cause of the less severe clinical outcome seen in female FXTAS patients, as compared to male patients with FXTAS [Hagerman et al., 2004;

Zuhlke et al., 2004]. Positively skewed X-inactivation, wherein the normal allele is preferentially active, could lessen the toxic effects of elevated *FMRI* mRNA levels. In these first known females with FXTAS, no skewed X-inactivation was seen [Hagerman et al., 2004]. However, another study described two sisters who are both PM-carriers, displaying a different degree of clinical involvement, which correlated with the pattern of X-inactivation. The sister who had a skewed X-inactivation pattern, favoring a higher percentage of cells expressing the PM-allele, suffered from more severe symptomatology. Although genetic background may influence severity of FXTAS, this is not likely a major factor in these sisters. Thus, this study provides a clear view on the effect of X-inactivation on the clinical outcome in FXTAS [Berry-Kravis et al., 2004].

DIAGNOSTIC CRITERIA FOR FXTAS

MRI studies of the first patients identified to suffer from FXTAS revealed generalized brain atrophy and enlarged ventricles [Hagerman et al., 2001]. Later, neuropathological post mortem studies of patients with manifestations of FXTAS generally showed global brain atrophy, as well as cerebellar and subcortical cerebral white matter disease. Also substantial Purkinje cell dropout with Bergmann gliosis is seen [Greco et al., 2002]. Another prominent feature is the so-called MCP sign, namely increased signal intensities in T2-weighted MRI recordings of the middle cerebellar peduncles (MCPs), which is caused by spongiosis of the deep cerebellar white matter [Brunberg et al., 2002; Jacquemont et al., 2003]. This MCP sign is observed in about 60% of PM carriers with tremor and/or ataxia and was therefore included in the diagnostic criteria for FXTAS. Diagnosis is based on the following criteria, presented in Table I, on top of the mandatory criterion of a (CGG)_n length between 55 and 200 [Hagerman and Hagerman, 2004]. Testing guidelines for FXTAS, as well as the proposed diagnostic criteria have recently been described and summarized in a review by Berry-Kravis et al. [2007].

MOLECULAR CORRELATES OF FXTAS

The development of FXTAS is restricted to carriers with the PM, who have a transcriptionally fully active *FMRI* gene [Tassone et al., 2004b]. PM carriers, irrespective of showing signs of FXTAS, have been shown to have up to eightfold elevated *FMRI* mRNA levels in peripheral blood leucocytes, despite close to normal FMRP levels [Tassone et al., 2000a,b; Kenneson et al., 2001; Tassone and Hagerman, 2003]. Relative *FMRI* mRNA levels in peripheral blood leucocytes are more elevated in comparison with normal controls than are relative *FMRI* mRNA levels in brain [Tassone et al., 2004a]. The level of *FMRI* mRNA in blood leukocytes is highly and positively correlated to (CGG)_n length in the PM range [Tassone et al., 2000a,b; Kenneson et al., 2001; Allen et al., 2004]. The mechanism underlying the elevated *FMRI* mRNA levels is unknown. The observation that increased transcript levels were also measured when constructs bearing the *FMRI* 5' UTR with PM sized (CGG)_n, fused to a luciferase reporter were transfected into two different cell lines [Chen et al., 2003], suggests that the expanded (CGG)_n itself, rather than the reduced FMRP levels, is responsible for increased transcription [Tassone et al., 2000a; Kenneson et al., 2001; Willemsen et al., 2004]. Multiple *FMRI* transcriptional initiation sites have been identified, with different efficiencies, dependent on the length of the (CGG)_n in the 5' UTR

downstream of the initiation sites. Thus, it appears that the (CGG)_n exerts some effect at the level of transcription initiation [Beilina et al., 2004]. Cell culture studies on the decay of *FMR1* mRNA indicated that increased stability is not the cause of elevated *FMR1* mRNA levels [Tassone et al., 2000b]. Later, it was shown that increased *FMR1* mRNA levels are due to increased transcriptional activity of both spliced and unspliced mRNA. Also, the majority of *FMR1* mRNA was found to be localized in the cytoplasm, thus excluding the possibility that nuclear retention of *FMR1* mRNA contributes to the higher levels (as occurs in myotonic dystrophy, which will be discussed later) [Tassone et al., 2004b].

Although different methods and different repeat ranges tested show somewhat different results, it seems that within the PM range, FMRP levels gradually decrease with increasing (CGG)_n length [Tassone et al., 2000a,b; Kenneson et al., 2001; Tassone and Hagerman, 2003]. The reduced levels have been found to reflect impaired translational efficiency of the (CGG)_n containing *FMR1* mRNA [Primerano et al., 2002]. Since none of the characteristic features of FXTAS have been observed in FM carriers, who express little or no FMRP, it is very unlikely that this syndrome originates from a protein-deficiency.

NEUROPATHOLOGICAL HALLMARK OF FXTAS

As can be seen in Table I, apart from generalized atrophy, another neuropathological feature has been found to be characteristic for FXTAS brain pathology. Neurohistological examination of brains of PM carriers who displayed the neurological phenotype which would later be diagnosed as FXTAS, revealed the presence of eosinophilic, intranuclear inclusions in neurons and astrocytes. These inclusions are discrete, round bodies, 2–5 µm large, and were seen in various regions throughout the cerebrum and brain stem [Greco et al., 2002]. They are most numerous in the hippocampus. A strong correlation was found between repeat length and number of intranuclear inclusions, in neurons as well as astrocytes [Greco et al., 2006] (Fig. 3). The intranuclear inclusions stain positively with antibodies against ubiquitin. No positive staining in inclusions was obtained with antibodies against tau, cytokeratin, desmin, αB-crystallin, vimentin, GFAP, or neurofilament. The antibody against neurofilament, however, showed swollen axons in the granular cell layer of the cerebellum (Purkinje axonal torpedoes). The GFAP-antibody revealed Bergmann gliosis. Furthermore, Purkinje cell drop out was seen in the cerebellum. No inclusions were observed in Purkinje cells. Brains of two female PM carriers unaffected by FXTAS were also examined; none of the neuropathological changes associated with FXTAS were observed [Greco et al., 2002].

Previous neuropathological examinations of brains of elderly carriers of the FM did not show inclusions [Rudelli et al., 1985; Sabaratnam, 2000]. Some neuroanatomical changes do, however, overlap between PM carriers with FXTAS and FM carriers; Purkinje cell loss and Bergmann gliosis have also been described in an older male with the FM [Sabaratnam, 2000]. Also, shrinkage of the temporal lobe [Reiss et al., 1994] and enlarged ventricles [Reiss et al., 1995] have been seen in FM carriers. However, overall brain volume tends to be greater in FM carriers [Reiss et al., 1994; Schapiro et al., 1995]. Thus, the intranuclear inclusions seem to represent a neuropathological hallmark specific for FXTAS and their presence is therefore used as a post-mortem diagnostic criterion (Table I).

Based on the toxic RNA gain-of-function model proposed for FXTAS, which will be described in detail later, it was predicted that *FMRI* mRNA is present within the intranuclear inclusions. Indeed, *FMRI* mRNA was detected in inclusions isolated from brain of a 70-year-old patient. This finding serves as evidence for an association between elevated *FMRI* transcript levels and formation of intranuclear inclusions. Since the presence of inclusions is associated with the development of FXTAS, these results strongly point towards an RNA gain-of-function mechanism [Tassone et al., 2004a]. Another prediction of the model is that RNA-BPs interfere with the abundant (CGG)_n containing mRNA, which then ultimately leads to the clinical phenotype [Iwahashi et al., 2006]. To investigate which proteins might mediate the toxic effects of the expanded-repeat *FMRI* mRNA, a systematic analysis was under-taken into the composition of purified intranuclear inclusions of FXTAS brain. Over 20 proteins were found to co-localize with ubiquitin-positive inclusions, among which α B-crystallin, Hsp27 and Hsp70, muscleblind-like protein 1 (MBNL1), several intermediate filaments, and microtubule components, as well as myelin associated proteins and the RNA-BP heterogeneous nuclear RNP A2/B1 (hnRNP A2/B1) [Iwahashi et al., 2006]. Another group demonstrated co-localization of Pur- α and ubiquitin in intranuclear inclusions in human FXTAS brain [Jin et al., 2007], which had not been identified in the purified inclusions in the study by Iwahashi et al. [2006]. Thus, the exact composition and formation of inclusions is as yet unknown as well as how they are related to the clinical symptoms seen in FXTAS.

PATHOGENESIS OF FXTAS

Most studies on the pathogenesis of FXTAS currently focus on the proposed RNA toxic gain-of-function model (Fig. 4). The evidence for this pathogenesis model, in which the *FMRI* mRNA itself is the cause of the neurological disease, originates from the observations that *FMRI* mRNA levels are elevated in peripheral blood leucocytes of PM carriers, *FMRI* mRNA is present in the intranuclear inclusions in brain and that no symptoms of FXTAS are seen among older carriers with the FM [Hagerman et al., 2001; Greco et al., 2002; Jacquemont et al., 2003], reviewed in Hagerman and Hagerman [2004]. This model parallels the RNA toxic gain-of-function model proposed for another non-coding expanded repeat disorder, myotonic dystrophy. Myotonic dystrophy is either caused by expansion of a (CTG)_n in the 3' UTR of the myotonic dystrophy protein kinase (*DMPK*) gene (DM1: congenital form), or by expansion of the (CCTG)_n in intron 1 of the *ZNF9* gene (DM2: late onset form). In both forms of DM, RNA-BPs are sequestered to the mRNA that bears long C(C)TG-repeat tracts. One of these proteins is MBNL1. Nuclear foci are seen in DM, similar to intranuclear inclusions in FXTAS (reviewed in: Mankodi et al. [2002] and Ranum and Day [2004]). These foci contain the repeat-containing RNA involved in DM, as well as MBNL1. The effect of the sequestration of MBNL1 into the nuclear foci is dysregulation of splicing of several target mRNAs, which in most cases can explain symptoms seen in DM [Miller et al., 2000; Fardaei et al., 2001]. The toxic gain-of-function effect has been confirmed in transgenic mouse models in which either an expanded (CTG)_n was placed within a non-coding region of a heterologous gene [Mankodi et al., 2002] or by introducing a transgene containing the human *DMPK* gene with an expanded (CTG)_n [Seznec et al., 2000]; both transgenic mouse lines developed myotonia. Thus, it has been proposed that the

situation in FXTAS is similar to DM, based on the previously described observations. It has furthermore been considered that POF in female PM carriers might also arise from an RNA-mediated toxicity [Conway et al., 1998]. Cellular and animal models, which will be described in more detail hereafter, provide further evidence that indeed an RNA gain-of-function mechanism underlies the clinical syndromes in FXTAS. However, the protein targets that are sequestered by the (CGG)_n containing mRNA remain to be elucidated, as well as the down-stream effects. It will be difficult to answer these questions when solely investigating human samples, because in the case of the most interesting organ, the brain, one is limited to using patient material only at the end stage of the disease. Naturally, it is important to know about the onset and the course of the disease as well. Therefore, in vivo models as an alternative to conducting research in humans are necessary. Animal models have proven and will continue to prove very helpful in answering questions about different stages of the disease and will be discussed in detail below.

Also cellular in vitro models can give valuable insight into the mechanisms of toxicity. Inducible expression of constructs with non-coding (CGG)_n of different lengths in HEK293 cells showed higher levels of apoptosis with increasing repeat length. Non-induced cells, thus only expressing endogenous levels of *FMRI* mRNA, showed reduced cell death in comparison to the induced cells, suggesting that cell death is related to the amount of (CGG)_n RNA that is transcribed [Handa et al., 2005]. Another cellular model mimics another aspect of FXTAS, namely the formation of inclusions, which occurred in human neuroblastoma cells after introduction of an expanded (CGG)_n. Another result of the expression of the (CGG)_n containing RNA is disruption of lamin A/C architecture [Arocena et al., 2005]. Lamin A/C was one of the intermediate filaments found to be a component of the intranuclear inclusions in FXTAS brain [Iwahashi et al., 2006]. Controlled expression of (CGG)_n tracts in cellular models might provide further insight on the altered cellular function as a result of toxic RNA species.

ANIMAL MODELS FOR FXTAS

Several animal models have been generated in the past, in an attempt to mimic repeat instability as well as neurodegeneration associated with (CGG)_n expansion in the *FMRI* gene. The different existing animal models and their contributions to our understanding of the pathogenesis of FXTAS are discussed.

Drosophila melanogaster Models for FXTAS

A *Drosophila* model generated by Jin et al. [2003] provided useful insight into the effect of ectopic expression of different amounts of different (CGG)_n lengths in the fly. Strong expression of a construct with 90 CGGs in all neurons of the peripheral and central nervous system caused lethality, as did ubiquitous expression in the embryo. Epithelial expression of this construct did not cause any effect, which shows that neuronal cells suffer more from the toxic effect of the long (CGG)_n containing RNA. Moderate or strong expression of constructs containing part of the human *FMRI* 5' UTR with either normal or PM-sized CGG-tracts was also specifically directed to the retina. Moderate expression of a tract of 60 CGGs hardly caused any aberrant phenotype in the eye, whereas 90 CGGs lead to rough

eye, loss of pigmentation and disturbed architecture. Strong expression of either repeat length caused a more severe phenotype, with an even stronger effect for the (CGG)₉₀, including major cell death. These experiments show that both the length of the CGG-tract and the expression level play a role in the severity of the neurodegenerative eye phenotype in *Drosophila*. Furthermore, disruption of the eye morphology increased with age in transgenic flies expressing 90 CGGs, mimicking the progressive nature of FXTAS. It was also shown that the phenotypes represent late onset symptoms rather than developmental defects. Immunohistochemical studies of the retina of flies strongly expressing the long repeat demonstrated the presence of inclusions, positive for ubiquitin, hsp70, and proteasome. These inclusions were found both in nuclei and cytoplasm, which is different from what is seen in humans and mice. Also, they were not so consistent in size and shape as in humans and mice. None of the aspects of the neurodegenerative phenotype could be induced with expression of a control construct containing enhanced green fluorescent protein (EGFP) only. Since hsp70 has been shown to be a universal suppressor of many neurodegenerative models caused by mutant proteins, for example, polyglutamine-containing due to an expanded (CAG)_n in the coding region of a gene, flies were generated that co-express the (CGG)₉₀ and hsp70. Hsp70 was found to be capable of suppressing the CGG-RNA induced degeneration, despite the absence of a mutant protein, like in other neurodegenerative disorders where hsp70 could act as a chaperone in refolding mutant proteins. Thus, these results are promising since they provide a proof-of-principle that the RNA-induced phenotype can be rescued [Jin et al., 2003].

Subsequent studies in this model were aimed at testing the hypothesis that FXTAS is an RNA-mediated neurodegenerative disease caused by the titration of RNA-BPs by the (CGG)_n. A genetic screen of candidate RNA-BPs identified CUG binding protein (CUGBP1) as a modifier of the eye phenotype induced by the repeat tract of 90 CGGs. Overexpression of CUGBP1 was capable of suppressing the (CGG)_n RNA-induced neurodegenerative eye phenotype. CUGBP1 was found to interact with the (CGG)_n via hnRNP A2/B1 [Sofola et al., 2007b], a ribo-CGG binding protein, of which the presence has been demonstrated in inclusions in human FXTAS brain [Iwahashi et al., 2006]. An article published simultaneously demonstrated that hnRNP A2/B1 directly binds to the (CGG)_n [Jin et al., 2007] and overexpression of this protein and its two *Drosophila* homologues also suppresses the repeat RNA-induced eye phenotype [Sofola et al., 2007b]. In addition, Purα, an RNA-BP expressed in neuronal cytoplasm, involved in dendritic mRNA transport, was also found to be associated with (CGG)_n RNA and overexpression of Pur α suppressed the eye neurodegeneration phenotype. Pur α was also present in the inclusions in *Drosophila* [Jin et al., 2007], as well as in human FXTAS brain [Iwahashi et al., 2006]. Thus, these findings strongly support the current pathogenesis model for FXTAS, namely that PM-sized (CGG)_n sequester RNA-BPs, which leads to altered cellular function and ultimately neuronal cell death [Jin et al., 2007].

More evidence for the hypothesis that the levels of repeat-RNA are important in inducing a neurodegenerative phenotype comes from the observation that simultaneous expression of a (CGG)_n and a (CCG)_n, which is present in the 5' UTR of the *FMR2* gene, in *Drosophila* suppress their independent toxicity. The *FMR2* gene is located about 600 kb distal to the

FMRI gene and is involved in non-syndromic X-linked mental retardation. Expression of only a (CCG)_n of 90 CCGs also induced a neurodegenerative phenotype of the eye. The protective effect of co-expression was found to be dependent on the RNAi pathway; toxicity was reversed through a reduction of transcript levels, possibly through formation of double stranded RNA, which is then processed and cleaved. This is hopeful in light of potential therapeutical strategies involving complementary RNA molecules targeting mutant mRNAs carrying expanded trinucleotide repeats [Sofola et al., 2007a].

Mouse Models for FXTAS

Initial attempts to make transgenic mouse models expressing expanded (CGG)_n tracts focused on investigating the mechanisms underlying (CGG)_n instability, since FXTAS had not been discovered at the time. The first transgenic mouse model expressed a (CGG)₈₁, with two interruptions, but with a pure tract of 60 CCGs. Although in humans, this repeat length is sufficient to cause instability upon transmission to the next generation, all transgenic mice showed exactly the same repeat length. Thus, the repeat was stably inherited in all animals tested [Bontekoe et al., 1997]. In another transgenic mouse line, a randomly (autosomally) integrated *FMRI* PM allele ((CGG)₂₂TGG(CGG)₄₃TGG(CGG)₂₁) was also stably transmitted [Lavedan et al., 1997]. (CGG)_n usually have a few AGG interruptions. Expansion of the (CGG)_n appears to be initiated upon loss of the interruptions of the otherwise pure (CGG)_n-tracts. Interruptions are predominantly lost at the 3' end of the repeat tract, causing a long pure (CGG)_n-tract [Richards and Sutherland, 1992; Kunst and Warren, 1994]. Therefore, Lavedan et al. [1998] created new transgenic mouse lines with even longer uninterrupted (CGG)_n. In addition, because it was recognized that chromosomal context might be important for (CGG)_n instability to take place, they situated the (CGG)_n tract within the context of the first exon of the human *FMRI* gene and included the flanking CpG island. However, including nearby *cis*-acting elements in the transgene proved not to be sufficient to reproduce repeat instability as seen in humans, since only minor intergenerational instability was observed in these mice [Lavedan et al., 1998].

Another approach was taken with the use of yeast artificial chromosomes (YACs), which can contain large fragments of exogenous DNA. YACs in transgenic mice have been demonstrated to appropriately express the genes present on the YAC, as all splice isoforms and other regulatory elements can be included [Gaensler et al., 1993; Peterson et al., 1993]. Several transgenic lines were created using YAC transgenes carrying (CGG)_n of different lengths and with varying amounts of flanking sequences. Length-dependent intergenerational instability (small expansions and contractions) was seen. Parental origin of the repeat allele did not influence the magnitude or direction of instability [Peier and Nelson, 2002]. To investigate the effect of potential *cis*- and *trans*-factors promoting expansions, the transgenic mice described by Lavedan et al. [1998] were subjected to a low-folate or folate-free diet, or crossbred with mice deficient in genes involved in DNA replication (Wn helicase) and repair (p53). Also, the potential effect of parental age was taken into consideration. Small changes in repeat length were seen in almost all allele transmissions. Large deletions occurred about 10% of transmissions. No large expansions were seen, neither somatic instability. The pattern of inter-generational instability was not affected by

the absence of the proteins involved in DNA replication and repair, nor by folate levels or the parental age [Fleming et al., 2003].

Since flanking of the (CGG)_n with part of the *FMRI* gene proved not to be sufficient to recapitulate all aspects of repeat instability in humans, another approach was taken by Bontekoe et al. [2001]. They generated a knock-in mouse model in which the endogenous murine (CGG)₈ was exchanged with a human (CGG)_n of 98 trinucleotides long. When cloning the human expanded repeat into the murine *FMRI* promoter, minimal changes were made. Comparison of the promoter sequence in mice and humans showed that all known regulatory elements are conserved. After the initial breedings, infrequent (15 out of 155 transmissions studied) and minor instabilities were observed, two of which were contractions, both of paternal origin. Expansions occurred upon transmission of either one of the parental alleles [Bontekoe et al., 2001].

After FXTAS was first recognized and described, the aging expanded (CGG)_n knock-in mouse (from now on called (CGG)_n mouse) was analyzed with respect to neurohistology, biochemistry, and molecular aspects. The main findings were that *FMRI* mRNA levels in brain were increased throughout life, as compared with wild type animals [Willemsen et al., 2003], which mimics the human situation [Tassone et al., 2000a,b]. Importantly, also intranuclear inclusions were found in many different areas throughout the brains of the (CGG)_n mice. Numbers and size of the inclusions increase with age; the first appearing around 30 weeks, but with percentages up to 55% in certain brain regions at 72 weeks of age, which correlates with the progressive nature of the disorder in humans. In addition, a correlation was recognized between the presence of inclusions in certain brain areas and clinical features in patients with FXTAS. This clearly suggests a role for the formation of intranuclear inclusions in the development of the tremor/ataxia syndrome in elderly PM carriers. Interestingly, inclusions were only seen in neurons [Willemsen et al., 2003], while in humans also astrocytes bear inclusions [Greco et al., 2002]. Other neuropathological features observed in patients with FXTAS like neuronal loss, gliosis, and Purkinje cell dropout have not been seen in the (CGG)_n mice. Molecular chaperone Hsp40 and the 20S proteasome complex were found to be present in the inclusions. Among many other proteins, FMRP, α -synuclein, poly-glutamine, and tau were not present in the inclusions. Minor somatic repeat instability was seen in some organs at 52 weeks of age, as compared to in tail DNA taken 10 days postnatally [Willemsen et al., 2003].

The (CGG)_n mice were subjected to several behavioral tests. No severely aberrant behavioral phenotype was seen; only mild age-dependent learning disturbances and decreased performance in a neuromotor task [Van Dam et al., 2005]. Thus, although the progressive character of the disorder is also seen in mice, the mouse appears to be less affected by the expression of the expanded (CGG)_n than humans are, both with regard to neurodegeneration (neuronal cell loss) and the clinical and behavioral phenotype. The origin of these differences remains to be elucidated.

This first knock-in mouse model later furthermore turned out to be more promising in light of repeat instability than the earlier models with non-targeted autosomal (CGG)_n, when considerable repeat instability was reported. The (CGG)_n in these mice has been expanding

over generations since it was generated and now has reached lengths, which in humans represent the FM range. Despite (CGG)_n lengths over 200 CGGs, no methylation of the promoter region has been detected thus far in these mice [Brouwer et al., 2007]. Also, alterations in hypothalamus–pituitary gland–adrenal gland (HPA) axis physiology, among which increased stress hormone levels, have been observed in this mouse model, which might explain the increased psychopathology seen in PM carriers [Brouwer et al., 2008].

Another, similar knock-in mouse model was generated by Entezam et al. [2007], which had an initial repeat tract of ~118 CGGs. Similarly, these mice show high repeat instability with a bias towards expansions. They show an increase in *FMR1* mRNA levels with (CGG)_n length and an inverse correlation with FMRP levels. Also, they showed area-specific decreases in F expression throughout the brain in mice with expanded (CGG)_n. For the first time, large expansions into the FM range were seen within a single generation in a mouse model, but the gene was not found to be methylated, nor silenced, as FMRP was still present [Entezam et al., 2007].

Thus, animal models have proven useful and will continue to prove useful in elucidating the molecular mechanisms underlying the clinical symptoms in FXTAS and thus might also provide potential therapeutical targets.

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FIG. 1. (CGG)_n length and *FMR1* expression and clinical outcome. This figure shows the consequences of the different repeat length categories on transcription, translation and clinical phenotype.

FIG. 2.

The prezygotic model of expansion of the fragile X mutation. This model assumes that an expansion of a maternal PM to an FM takes place during meiosis. The fertilized oocyte carries an FM allele. After separation from the embryo proper, the primordial germ cells (PGCs) have an FM. Some alleles will contract to PMs. To explain why FMs are only transmitted through females, some selection must exist against FMs in the male germ line during spermatogenesis. In the mature testes, PM alleles predominate. In somatic cells and

the female germ line, this selection did not take place. Cells with a PM are shown in green and cells with an FM are depicted in orange.

FIG. 3. Ubiquitin-positive intranuclear inclusions, the neuropathological hallmark of FXTAS in A: human neurons, B: human astrocytes, C: murine neurons. No inclusions have been seen in murine astrocytes.

FIG. 4.

A schematic representation of the RNA gain-of-function mechanism proposed for the pathogenesis of FXTAS. The *FMR1* gene is transcribed in the nucleus and transported to the ribosomes. The expanded (CGG)_n present in the 5'UTR of the *FMR1* gene hampers translation, leading to lower FMRP levels. Through an as yet unknown mechanism, transcription is upregulated, leading to increased *FMR1* mRNA levels. In an attempt to get rid of the excess of *FMR1* mRNA, the cell might attract chaperones or elements of the ubiquitin/proteasome system. Also (CGG)_n-binding proteins might be recruited. These processes could lead to formation of intranuclear inclusions. Sequestration of proteins into the inclusions might prevent them from exerting their normal function, thereby disturbing cellular function, which in the end might cause neurodegeneration. Also, it cannot be excluded that neuroprotection takes place, such that cells that are capable of capturing the toxic transcripts in the inclusions are the cells that survive.

TABLE I

Diagnostic Criteria for FXTAS

Clinical		Radiological		Neuropathological (post-mortem)
Criteria: diagnosis	Major	Minor	Major	
Definite FXTAS	1: Intention tremor OR gait ataxia AND:		MCP sign OR:	Presence of intra nuclear inclusions
Probable FXTAS	2: Intention tremor AND gait ataxia OR:	Parkinsonism AND:	MCP sign	
Possible FXTAS	1: Intention tremor OR gait ataxia AND:		Cerebral white-matter lesions, generalized atrophy	

Mandatory criterion: 55–200 CGGs in *FMR1* (from Hagerman and Hagerman, 2004).

The table should be read from left to right, for each level of probability of the diagnosis.