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CGG-repeat length and neuropathological and molecular correlates in a mouse model for fragile X-associated tremor/ataxia syndrome

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Abstract

The 5'untranslated region (UTR) of the *FMR1* gene contains a CGG-repeat, which may become unstable upon transmission to the next generation. When repeat length exceeds 200, the *FMR1* gene generally undergoes methylation-mediated transcriptional silencing. The subsequent absence of the gene product Fragile X Mental Retardation Protein (FMRP) causes the mental retardation seen in fragile X patients. A CGG-repeat length between 55 and 200 trinucleotides has been termed the premutation (PM). Predominantly elderly male PM carriers are at risk of developing a progressive neurodegenerative disorder: fragile X-associated tremor/ataxia syndrome (FXTAS). All PM carriers have elevated *FMR1* mRNA levels, in spite of slightly decreased FMRP levels. The presence of intranuclear ubiquitin-positive inclusions in many brain regions is a neuropathological hallmark of FXTAS. Studies in humans attempting to correlate neuropathological outcomes with molecular measures are difficult because of the limited availability of tissue. Therefore, we have used the expanded CGG-repeat knock-in mouse model of FXTAS to examine the relationship between the molecular and neuropathological parameters in brain. We present *Fmr1* mRNA and Fmrp levels and the presence of intranuclear inclusions at different repeat lengths. Contrary to existing hypotheses, our results suggest that inclusion formation may not depend on the elevation *per se* of *Fmr1* transcript levels in aged CGG mice.

Keywords

CGG repeat; Fmr1; FMRP; fragile X-associated tremor/ataxia syndrome; inclusions; RNA gain-of-function

The (CGG)_n-repeat in the 5'UTR of the fragile X mental retardation 1 (*FMR1*) gene is polymorphic. Normal individuals have alleles that are within the range of 5-44 CGGs (Fu *et al.* 1991). Repeat lengths between 45 and 54 are considered intermediate alleles, which can show minor instability when transmitted to the next generation (Nolin *et al.* 1996; Zhong *et al.* 1996). Over 200 CGGs has been named the full mutation (FM), as this usually leads to C (cytosine) followed by G (guanine) methylation of the *FMR1* promoter and the CGG-repeat, which results in transcriptional silencing of the gene (Oberlé *et al.* 1991; Verkerk *et al.* 1991; Sutcliffe *et al.* 1992). The consequent absence of the gene product FMRP is the cause of mental

retardation in fragile X patients (Pieretti *et al.* 1991; Verheij *et al.* 1993). Unmethylated expansions of 55-200 CGGs are considered pre-mutation (PM) alleles. Initially, it was thought that the only risk associated with the PM was expansion to an FM when a mother transmits the allele to her child. However, it was later recognised that carriers of the PM are at risk of developing a progressive neurodegenerative disorder called fragile X-associated tremor/ataxia syndrome (FXTAS) (Hagerman *et al.* 2001; Hagerman and Hagerman 2002; Jacquemont *et al.* 2003). About 30% of male PM carriers over 50 years of age will develop FXTAS, although penetrance increases with age (Jacquemont *et al.* 2004). Few cases of female PM carriers presenting with FXTAS have been described (Hagerman *et al.* 2004; Zuhlke *et al.* 2004). Females are less severely affected, which can be explained by the presence of a second, normal allele. The process of X-inactivation determines how many PM alleles are on the active X chromosome, thereby influencing the clinical outcome (Berry-Kravis *et al.* 2004; Hagerman *et al.* 2004). About 20% of female carriers are at further risk of developing premature ovarian failure (Sherman 2000; Allen *et al.* 2004).

In FXTAS, patients usually present with progressive intention tremor and/or ataxia. As the disease progresses, symptoms worsen and a wider range of clinical involvement develops, including cognitive decline with impaired memory and executive function, autonomic dysfunction and peripheral neuropathy (Jacquemont *et al.* 2003; Berry-Kravis *et al.* 2007b).

Premutation carriers have elevated *FMRI* mRNA levels in spite of normal or slightly reduced FMRP levels. The increase of *FMRI* mRNA levels seems to be correlated to the length of the (CGG)_n (Tassone *et al.* 2000b,c; Kenneson *et al.* 2001). Expanded (CGG)_n tracts negatively influence translation of the *FMRI* mRNA, such that FMRP gradually decreases with increasing repeat length, despite increased levels of *FMRI* mRNA (Tassone *et al.* 2000b; Kenneson *et al.* 2001; Primerano *et al.* 2002).

As FXTAS is restricted to the PM range, as no aged FM carriers have been observed with signs of FXTAS, FMRP deficiency is unlikely the cause of the disease. This, combined with the fact that *FMRI* mRNA levels are increased in PM carriers, led to the proposal that an RNA toxic gain-of-function mechanism underlies the symptoms seen in FXTAS patients (Hagerman *et al.* 2001; Hagerman and Hagerman 2004). In analogy to the well-described pathogenic RNA gain-of-function model for myotonic dystrophy (DM) [reviewed in (Ranum and Day 2004)], this hypothesis predicts that certain proteins are sequestered to the expanded (CGG)_n-containing RNA, away from their normal function. Loss of the normal function of these (CGG)_n-binding proteins (BPs) could then lead to cellular toxicity or ultimately cell death (Hagerman *et al.* 2001; Hagerman and Hagerman 2004).

Neurohistological studies on postmortem brains of PM carriers with FXTAS have revealed the presence of ubiquitin-positive intranuclear inclusions in neuronal and astrocytic cell types throughout the brain. Furthermore, Purkinje cell dropout and Bergmann gliosis were observed. Purkinje cells did not show intranuclear inclusions (Greco *et al.* 2002). *FMRI* mRNA has also been detected in the intranuclear inclusions, further strengthening its involvement in the pathogenesis of FXTAS (Tassone *et al.* 2004a).

A mouse model originally developed in our group (Bontekoe *et al.* 2001) to study instability of the *FMRI* (CGG)_n is also an adequate model for FXTAS (Willemsen *et al.* 2003; Brouwer *et al.* 2007, 2008). In this animal model, the endogenous mouse (CGG)₈ was exchanged with a human (CGG)₉₈, which is in the PM range in humans. These expanded (CGG)_n 'knock-in' [(CGG)_n] mice show moderate repeat instability upon both maternal and paternal transmission (Bontekoe *et al.* 2001; Brouwer *et al.* 2007). Neurohistological studies performed on (CGG)_n mouse brains revealed ubiquitin-positive intranuclear inclusions in neurons. Number and size of inclusions increase with time, paralleling the progressive nature of the

disease in human patients. Furthermore, the presence of inclusions in distinct brain regions and organs associated with the hypothalamopituitary-adrenal axis in (CGG)_n mice can be linked to clinical features of FXTAS patients (Willemsen *et al.* 2003; Brouwer *et al.* 2008). The mouse model also mimics human FXTAS in that *Fmr1* transcript levels are elevated (Willemsen *et al.* 2003; Brouwer *et al.* 2007).

Efforts have been made to correlate clinical outcomes to molecular measures, although this has mostly been limited to (CGG)_n length (Greco *et al.* 2006; Grigsby *et al.* 2006; Tassone *et al.* 2007a). Human studies using brain tissue are limited because of the availability of material. Therefore, this study is designed to use (CGG)_n mice to establish the relationship between the molecular and neuropathological parameters in brain, the central organ involved in the pathogenesis of FXTAS. In the present study, *Fmr1* mRNA and Fmrp levels and the presence of intranuclear inclusions, the neuropathological hallmark of FXTAS, are described for different repeat length categories. Our results in aged (CGG)_n mice suggest that (CGG)_n length and the sole presence of expanded (CGG)_n *Fmr1* mRNA, rather than elevation of *Fmr1* transcript levels, are important for the neuropathology in FXTAS.

Methods and materials

Mice

The expanded (CGG)_n mice used in this study have been described before (Bontekoe *et al.* 2001; Willemsen *et al.* 2003; Brouwer *et al.* 2007, 2008). Although this mouse model was created by exchanging the murine endogenous (CGG)₈ with a human (CGG)₉₈ (Bontekoe *et al.* 2001), we now also report data on mice carrying (CGG)₇₀. These alleles arose in our colony through a contraction of the longer (CGG)_n. Both the expanded (CGG)_n knock-in (CGG)_n mice and the wild type (wt) mice with an endogenous (CGG)₈ were housed under standard conditions. All experiments were carried out with permission of the local ethics committee. (CGG)_n lengths were determined for the whole colony, but only male mice were used for experiments. All mice had a mixed C57BL/6 and FVB/N genetic background. All mice used in this study were between 55 and 58 weeks old, unless specified otherwise.

Genotyping of the mice

DNA was extracted from mouse tail snips as previously described (Brouwer *et al.* 2007). Determination of the (CGG)_n length was performed by means of PCR using the Expand High Fidelity Plus PCR System (Roche Diagnostics, Indianapolis, IN, USA), with forward primer 5'-CGGAGGCGCCGCTGCCAGG-3' and reverse primer 5'-TGCGGGCGCTCGAGGCCAG-3'. PCR products were visualised on a 6% polyacrylamide gel. As these primers are specific to the knock-in allele, a separate PCR is performed for the wt allele. The wt allele was detected using TaKaRa LA Taq polymerase (using GC buffer II), according to manufacturer's instructions (Takara Bio Inc., Otsu, Shiga, Japan), with 5'-GCTCAGCTCCGTTTCGGTTT-CACTTCCGGT-3' as forward primer and 5'-AGCCCCGCACTTCCACCACCAGCTCCTCCA-3' as reverse primer [detailed description in Brouwer *et al.* (2007)]. Based on their repeat length, mice were grouped into one of five repeat length categories, namely wt, 70 CGG, 100-150 CGG, 151-200 CGG, and > 200 CGG.

Immunohistochemistry

Half (sagittal) brains were fixed overnight in 3% paraformaldehyde. Tissues were embedded in paraffin according to standard protocols. Sections (7 μM) were deparaffinised followed by antigen retrieval using microwave treatment in 0.01 M sodium citrate solution. Endogenous peroxidase activity blocking and immunoincubation were performed as described before (Bakker *et al.* 2000), using a polyclonal rabbit antibody against ubiquitin (Dako, ZO458, Carpinteria, CA, USA) or a monoclonal 2F5-1 antibody specific for Fmrp (Gabel *et al.*

2004). Ubiquitin-positive intranuclear inclusion counts in colliculus inferior and dentate gyrus were quantified by counting the number of inclusion-bearing cells in a field of ~200 (colliculus inferior) or ~400 (dentate gyrus) neurons. At least four such fields were counted for each field of interest. Average inclusion counts and SDs were calculated for mice in each of the five repeat length categories.

Preparation of protein, RNA, and DNA extracts

Half brains (sagittal) were homogenised in 500 μ L HEPES-buffer (10 mM HEPES, 300 mM KCl, 3 mM $MgCl_2$, 100 μ M $CaCl_2$, 0.45% Triton X-100, and 0.05% Tween 20, pH 7.6), with Complete protease inhibitor cocktail (Roche Diagnostics), 3 mM dithiothreitol (Invitrogen, Carlsbad, CA, USA) and 20U RNAsin (Promega, Madison, WI, USA). After incubating the homogenates on ice for 30 min, 100 μ L were taken for RNA isolation, using 1 mL of RNeasy (Tel-Test). 200 μ L chloroform were then added and the mixture was centrifuged for 15 min at 17 000 g at 4°C for phase separation. One volume of isopropanol was added to the aqueous phase to precipitate the RNA. The pellet was washed with 80% ethanol and dissolved in diethylpyrocarbonate-treated MilliQ-H₂O. RNA concentration and purity was determined using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Thermo Scientific, Wilmington, DE, USA).

The remainder of the brain homogenates was centrifuged for 15 min at 17 000 g at 4°C. Protein concentration of the supernatant was determined before use in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The pellet was used for DNA extraction, following the same protocol as described for the mouse tail snips.

Quantitative RT-PCR

Reverse transcriptase was performed on 1 μ g RNA using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) according to manufacturer's instructions. Q-PCR was performed on 0.1 μ L of RT product. Primers used for Q-PCR were as follows: *Fmr1* transition exon 16/17 (5'-CCGAACAGATAATCGTCCACG-3' as forward primer and 5'-ACGCTGTCTGGCTTTTCCTTC-3' as reverse), *Fmr1* transition exon 7/8 (forward: 5'-TCTGCGCACCAAGTTGTCTC-3', reverse: 5'-CAGAGAAGGCACCAACTGCC-3'), *Gapdh* (internal reference) (forward: 5'-AAATCTTGAGGCAAGCTGCC-3', reverse: 5'-GGATAGGGCCTCTCTTGCTCA-3'). Efficiencies of the different primer sets were checked and found to be comparable. C_t values for *Gapdh* mRNA were subtracted from the C_t value of *Fmr1* mRNA for each sample, which gives the ΔC_t . Average ΔC_t of wt mice was then subtracted from ΔC_t of CGG mice, which is designated as the $\Delta\Delta C_t$. The value $2^{-\Delta\Delta C_t}$ then gives the fold change. Average *Fmr1* mRNA levels for each of the repeat length categories were calculated. Differences between the repeat length categories were investigated with one-way ANOVA with fold change *Fmr1* mRNA as dependent variable and repeat length category as the grouping factor. Dunnett's *post hoc* test was performed to compare all expanded repeat categories against the wt animals and to test for significantly elevated *Fmr1* mRNA levels.

In addition, the existence of a correlation between (CGG)_n length and *Fmr1* mRNA levels was investigated by calculation of the Spearman's rho, both for all repeat lengths taken together, and for each repeat category separately.

Western blotting

We loaded 150 μ g of protein onto an 8% SDS-PAGE gel, which was then electroblotted onto a nitrocellulose membrane. The membrane was incubated overnight at 4°C with the monoclonal 2F5-1 antibody specific for Fmrp (Gabel *et al.* 2004) and a monoclonal antibody against Gapdh (Chemicon, Temecula, CA, USA), which served as a loading control. The next day the membrane was incubated with a goat-anti-mouse secondary antibody with 800 nm

conjugate. After washing, the blot was scanned using the Odyssey™ Infrared Imager (Li-Cor Biosciences, Lincoln, NE, USA). Quantification of integrated intensities of the fluorescent signals was performed using the Odyssey™ 2.1 software. All three Fmrp isoforms were included in the calculations. The relative Fmrp levels in the (CGG)_n animals were calculated using the average of the Fmrp levels measured in wt animals as a reference. One-way ANOVA was performed with relative Fmrp levels as the dependent variable and repeat length category as the grouping factor. Dunnett's *post hoc* test was performed to compare all expanded repeat categories against the wt animals and to test for significantly decreased Fmrp levels. Spearman's rho was calculated to reveal the existence of a correlation between (CGG)_n length and Fmrp levels.

Methylation status

Shortly after birth, tail DNA was checked for the presence of methylated C (cytosine) followed by G (guanine) in the *Fmr1* promoter region. We subjected 1 µg of DNA to bisulphite conversion, using the Qiagen Epitect kit, according to manufacturer's instructions. 1.5 µL of converted DNA was then used for two PCR reactions, where one reaction detects methylated alleles and the other PCR detects unmethylated alleles. For the methylated allele, we used the forward primer 5'-GTTTAAATAGGTTTTACGTTAGTGTC-3', and the reverse primer 5'-CGTCCGTTTACTTCACTACCCG-3', followed by a semi-nested PCR on 3 µL of PCR product using forward primer 5'-GAAGAGGTTTTAGTTTTTCGCGGC-3' and reverse primer 5'-CTCAAACGCGACCCCTCACCG-3'. The unmethylated primer was amplified with forward primer 5'-GTTTAAATAGGTTTTATGTTAGTGTT-3' and reverse primer 5'-CATCCATTTACTTCACTACCCA-3', followed by a semi-nested PCR on 3 µL of PCR product using forward primer 5'-GAAGAGGTTTTAGTTTTTGTGGT-3' and reverse primer 5'-CTCAAACACAACCCCTCACCA-3'. The PCR mixtures contained 10× PCR buffer, W-1, 4% dimethylsulfoxide, 0.5 µM of both the forward and reverse primer, one unit Taq Polymerase (all Invitrogen) and 2 mM MgCl₂ for the methylated allele and 1.5 mM MgCl₂ for the unmethylated allele. The PCR program was as follows: 5 min denaturation at 95°C, 35 cycles of 10 s at 93°C, 20 s annealing at 55°C and 30 s at 72°C, followed by a final elongation step of 10 min at 72°C. PCR products were visualised on a 2% agarose gel. The same methylation analysis was performed on DNA isolated from brain.

Results

A threshold for (CGG)_n length exist for inclusion formation

When some mice in our colony showed a contraction to about (CGG)₇₀, we examined how this shorter repeat length affected inclusion formation. Immunohistochemistry for ubiquitin in 72-week-old animals with (CGG)₇₀ and (CGG)_{~100} shows that inclusions were present neither in the colliculus inferior (Fig. 1), nor in other brain areas (data not shown) of (CGG)₇₀ animals. Eight animals have shown contractions to about (CGG)₇₀ over time, none of which showed any inclusions. In contrast, the colliculus inferior of all animals with (CGG)_{~100} showed many inclusions (~15 animals investigated over time, Fig. 1). Thus, a threshold (CGG)_n length exists for the development of inclusions. Fmrp levels were comparable between (CGG)₇₀ and wt animals (Fig. 1).

Elevated *Fmr1* mRNA levels are only seen in lower range PM alleles

With the availability of a range of repeat lengths we extended our findings about the *Fmr1* mRNA levels to a broader range of repeat lengths using more mice (Willemsen *et al.* 2003; Brouwer *et al.* 2007). Figure 2 shows average fold changes of *Fmr1* mRNA from brain in the different repeat categories. Only (CGG)₁₀₀₋₁₅₀ animals had statistically significantly elevated levels (mean difference = 1.60, SE = 0.36, *p* < 0.001), when compared with wt.

No statistically significant correlation was found between (CGG)_n length and *Fmr1* mRNA levels when all categories were taken together (Spearman's $\rho = 0.22$, $p = 0.11$), neither when repeat categories were analysed separately (data not shown).

Not all (CGG)_n animals have inclusions in the dentate gyrus

In our previous studies in which we looked for the presence of inclusions in our expanded (CGG)_n animals, we did not see inclusions in the dentate gyrus at 55 and 72 weeks of age (106 CGGs) (Willemsen *et al.* 2003). When examining mice with a wider range of (CGG)_n lengths, we now see that some mice have many inclusions, while others do not have inclusions in this brain region at 55-58 weeks of age (Fig. 3). Within the repeat length range that shows inclusions in other brain regions, the presence or absence of inclusions in the dentate gyrus does not appear to be dependent on (CGG)_n length (data not shown). This indicates that the dentate gyrus may be less consistent in forming inclusions than other brain regions such as the colliculus inferior.

A threshold for (CGG)_n length exists above which few inclusions are present

Fragile X-associated tremor/ataxia syndrome is proposed to be the result of an RNA gain-of-function mechanism (Hagerman *et al.* 2001), and inclusions are associated with the development of the disease (Greco *et al.* 2002). Therefore, we were curious to see the proportion of inclusion-bearing cells in our mice, which do [(CGG)₁₀₀₋₁₅₀] or do not [(CGG)_{>150}] have significantly elevated *Fmr1* mRNA levels. Percentages of neurons with inclusions in the colliculus inferior and the dentate gyrus are shown in Fig. 3. Mice with repeat lengths exceeding 200 CGGs showed very little inclusion formation (Figs 3 and 4). Not surprisingly, Spearman's ρ revealed a statistically significant correlation for (CGG)_n repeat length and the proportion of inclusion-bearing neurons in the colliculus inferior ($\rho = -0.90$, $p < 0.001$) and the dentate gyrus ($\rho = -0.78$, $p = 0.001$).

Although percentages vary in different regions, an abundance of inclusions is seen in (CGG)_n mice with 100-200 CGGs repeats in most brain regions. The observation that only few inclusions can be seen in the brain areas described here (colliculus inferior and the dentate gyrus) in mice with > 200 CGGs is representative for all other brain areas examined (data not shown).

Fmrp levels are reduced in the upper PM range

Measurement of Fmrp levels by SDS-PAGE followed by Western blotting revealed decreased Fmrp levels in expanded (CGG)_n mice. As only one brain of an animal with (CGG)₇₀ was available for protein measurement, this sample was not included in the statistical analyses. Only mice with expanded alleles of (CGG)_{>150} had statistically significantly lower Fmrp levels than wt mice (see Fig. 5). A significant correlation between CGG repeat length and Fmrp level was found (Spearman's $\rho = -0.58$, $p < 0.01$).

As shown before (Brouwer *et al.* 2007) and confirmed by immunohistochemistry in the present study (data not shown) this decrease in Fmrp is not found in all cells of the brain. For example, while most brain areas showed greatly reduced levels, levels of Fmrp in other regions such as the hippocampal CA2 and CA3 region were relatively unchanged from wt.

Correlations of other parameters

All data (without indication of the variability of the data) are summarised in Fig. 6. Spearman's two-tailed bivariate correlations between all possible combinations of *Fmr1* mRNA, Fmrp and inclusion counts in both regions quantified (i.e. colliculus inferior and dentate gyrus) only revealed a statistically significant correlation coefficient ($\rho = 0.61$, $p = 0.03$) for the relation

between Fmrp levels and the proportion of inclusion-bearing cells in the colliculus inferior (other correlation analyses not shown).

No methylation is seen in the *Fmr1* promoter region

No methylation was detected in any of the DNA samples isolated from tail DNA shortly after birth (data not shown). Nor was methylation seen in DNA isolated from brain at the time of sacrifice at approximately 55-58 weeks of age (data not shown).

Somatic stability in brain

Repeat length analysis in DNA extracted from total brain lysate only showed a single PCR product. Thus, no heterogeneity for repeat length was apparent in the brains of (CGG)_n mice, although we cannot exclude the possibility that with more sensitive methods of visualisation some heterogeneity might be detectable. In addition, comparison with repeat length in tail DNA obtained at 10 days postnatal revealed very minor repeat length differences (data not shown). Thus, we did not find evidence that somatic instability occurs over the course of life in our mouse model.

Discussion

This study was designed to establish the relationships between molecular and neuropathological parameters in an expanded (CGG)_n knock-in mouse model. The results show a high percentage of neurons with intranuclear inclusions in mice with 100-200 CGGs, while mice with > 200 CGGs show few such inclusions. In addition, only mice with (CGG)₁₀₀₋₁₅₀ have significantly elevated *Fmr1* mRNA levels, and Fmrp levels are significantly reduced when (CGG)_n length exceeds 150. Our studies furthermore indicate that a lower (CGG)_n length threshold exists, below which no inclusions are formed, as well as a higher threshold, above which few inclusions are seen.

It is striking that mice with 151-200 CGGs have many inclusions, in spite of relatively normal *Fmr1* mRNA levels. This might imply that it is the presence of mutant (CGG)_n RNA that determines the occurrence of cellular toxicity (i.e. inclusions), rather than the precise level of (CGG)_n mRNA. Because of the limited availability of mice with 151-200 CGGs, it can as yet not be excluded that a significant elevation of *Fmr1* mRNA levels would be seen if a larger group of animals was used. However, as can be seen in the (CGG)₁₀₀₋₁₅₀ and (CGG)_{> 200} categories, variation is large despite the use of large groups.

As there is slightly more Fmrp expressed in mice with 151-200 CGGs when compared with > 200 CGGs, it could also be that a minimal amount of Fmrp is necessary for inclusion formation, which is still present in mice with (CGG)₁₅₁₋₂₀₀. In mice with over 200 CGGs, Fmrp levels might have dropped just below this level, thereby preventing inclusion formation. However, the possibility cannot yet be excluded that repeat tracts with over 200 CGGs adopt different tertiary structures than do shorter repeats, thereby influencing the binding of CGG-BPs and possibly preventing the formation of inclusions. No statistically significant decrease in Fmrp levels was observed in mice with (CGG)₁₀₀₋₁₅₀ in this study. It should be noted that the lack of detecting such a decrease might be a consequence of the small number of wt samples used.

While Fmrp may be important for inclusion formation, it is noteworthy that Fmrp has not yet been detected in inclusions in mice (Willemsen *et al.* 2003) and in humans (Iwahashi *et al.* 2006). Furthermore, inclusions could be induced in a human neuronal cell model expressing a (CGG)_n fused to a *Gfp* reporter (Arocena *et al.* 2005), showing that inclusions can be formed, without the need for the (CGG)_n to be in the context of *Fmr1*. These cells did express endogenous Fmrp levels (Arocena *et al.* 2005). In order to investigate the role of Fmrp in the

formation of inclusions, breedings of female mice homozygous for (CGG)_{>200} with male *Fmr1* knockout mice heterozygous for a yeast artificial chromosome (YAC) containing the entire human *FMR1* gene and some flanking sequences (Peier *et al.* 2000; Musumeci *et al.* 2007) will be set up. This will allow comparison of inclusion formation in the (CGG)_{>200} offspring in the presence (YAC +/-) or absence (YAC -/-) of FMRP expression.

It is possible that a minimal abundance of CGG triplets is needed, either because of elevated transcript levels or because of the length of the repeat tract, or a combination thereof, to cause cellular pathology. Such an effect of 'CGG molarity' in the development of cellular toxicity in FXTAS has been hypothesised in the toxic RNA gain-of-function model (Hagerman and Hagerman 2004). In light of the toxic RNA gain-of-function model, it could be that a longer repeat tract provides more binding sites for (CGG)_n-BPs, such that it exerts toxicity similar to higher levels of a shorter repeat. With regard to the protein sequestration model for DM, it has been found that Muscleblind-like protein 1 binding is proportional to (CUG)_n length (Timchenko *et al.* 1996). Sequestration of (CUG)_n-BPs away from their normal cellular functions, including splice regulation, has been proposed to be the mechanism underlying DM (Timchenko *et al.* 1996; Miller *et al.* 2000; Fardaei *et al.* 2001). Interestingly, Muscleblind-like protein 1 has also been found in intranuclear inclusions in human FXTAS brain (Iwahashi *et al.* 2006), suggesting that a similar pathogenic mechanism may occur in FXTAS. However, no downstream splicing defects have thus far been revealed in FXTAS. In addition, we have not detected Mbnl1 in the inclusions present in our (CGG)_n mouse model (unpublished results).

Our studies in animals carrying a (CGG)₇₀ allele may provide insight on the observation that shorter PM alleles (< 70 CGGs) may pose a lower risk of developing FXTAS for the carrier than do longer repeats (Jacquemont *et al.* 2006). Although some cases with repeat lengths below 70 CGGs have been described (Macpherson *et al.* 2003; Kamm *et al.* 2005), clinical presentation was atypical and thus these findings may be coincidental (Jacquemont *et al.* 2006). It is therefore interesting that mice with (CGG)₇₀ neither show inclusions, nor elevated *Fmr1* mRNA, suggesting that there is a minimum repeat length necessary for the development of disease in mice.

Some correlations between molecular and clinical measures in FXTAS patients have been described. For instance, (CGG)_n length strongly correlates with age of death (Greco *et al.* 2006). (CGG)_n length has also been reported to correlate negatively with age of onset of action tremor and ataxia. Age of onset of these motor symptoms was not correlated to *FMR1* mRNA or FMRP levels in blood. For FMRP levels, this is not surprising, as they are close to normal in FXTAS patients. The lack of correlation with *FMR1* transcript levels might be explained by the fact that mRNA levels are measured in blood, whereas clinical symptoms derive from brain pathology (Tassone *et al.* 2007a). Furthermore, (CGG)_n has been correlated with increased cognitive and functional impairment (Grigsby *et al.* 2006). In addition, (CGG)_n length in humans has been found to be highly correlated to the number of intranuclear inclusions in both neurons and astrocytes, suggesting that it might serve as a powerful predictor of neuropathological involvement (Greco *et al.* 2006). Likewise, a longer (CGG)_n length has been found to correlate with increased motor dysfunction (Leehey *et al.* 2008), neuropathy and reflex impairments (Berry-Kravis *et al.* 2007a) and brain atrophy (Loesch *et al.* 2005).

The presence of inclusions is associated with disease (Greco *et al.* 2006). The size and number of inclusions in murine brain increase with time, reflective of the progressive nature of the disease. In addition, functions of some inclusion-bearing brain regions in expanded (CGG)_n mice can be linked to clinical symptoms in patients with FXTAS. Both findings are suggestive of a role for inclusions in the disease process (Willemsen *et al.* 2003). It is, however, as yet unknown how inclusions cause cellular dysfunction, and eventually neurodegeneration and clinical pathophysiology. Their immunoreactivity to antibodies against ubiquitin and

components of the proteasome, Hsp70 and α B-crystallin suggests involvement of the proteasomal protein degradation pathway (Greco *et al.* 2002; Willemsen *et al.* 2003). In mouse brain, an antibody directed against only poly-ubiquitinated proteins, as well as against mono- and polyubiquitinated proteins, detected inclusions (Willemsen *et al.* 2003). However, in isolated inclusions from human FXTAS brain, there was little evidence that the proteins present in the inclusions are poly-ubiquitinated, which is normally the signal that causes a protein to be degraded by the proteasome. Thus, impaired proteasomal degradation does not seem to play a central role in the formation of the inclusions (Iwahashi *et al.* 2006). It appears more likely that expanded (CGG)_n *FMR1* mRNA acts as a nucleation centre for other proteins, similar to the nuclear RNA foci seen in DM (Hagerman and Hagerman 2004).

In a previous study, we reported elevated *Fmr1* mRNA levels in mice with over 150 CGGs (Brouwer *et al.* 2007), which is in contrast with our current findings. At the time of that study, availability of mice with such long repeat tracts was limited, so few animals could be investigated. Therefore, along with considering the large variability seen for all repeat categories as well as for the wt animals, we believe that our previous findings likely represented a coincidental, biased selection of animals for study. Our conclusion still stands that in mouse brain there is no linear relationship between (CGG)_n length and *Fmr1* mRNA levels, unlike what has been reported in the blood of human PM carriers. In humans, there is no evidence for a drop in *FMR1* mRNA levels above a certain unmethylated (CGG)_n length (Tassone *et al.* 2000b; Kenneson *et al.* 2001; Allen *et al.* 2004). In another expanded (CGG)_n mouse model developed by Entezam *et al.* (2007), a linear increase in *Fmr1* mRNA with increasing (CGG)_n length was reported. However, the number of animals that was used per repeat length was not reported. It is therefore unclear whether those findings were also influenced by high inter-animal variation. Also, *t*-tests were performed to compare the different repeat lengths, without the mention of correction for multiple comparisons.

The *Fmrp* levels measured in this study are consistent with those reported in our previous study (Brouwer *et al.* 2007), such that decreased levels were only seen when (CGG)_n exceeded 150. Again, variability was large and it cannot be excluded that a significant decrease in *Fmrp* levels would have been found in animals with 100-150 CGGs, if more wt animals could have been used. One important realisation of this study is, however, that also wt animals vary greatly in their transcript and protein levels. In the other mouse model for FXTAS *Fmrp* levels had already decreased at (CGG)₁₃₀, but again the number of mice used in that study was not reported (Entezam *et al.* 2007).

The high variability found in *Fmr1* transcript levels in (CGG)_n mouse brain, might explain the fact that not all PM carriers develop FXTAS. Most studies on *FMR1* expression have been done in blood of PM carriers with or without FXTAS, and both show elevated levels on average. However, increases of differing magnitude in *FMR1* mRNA have been described (Tassone *et al.* 2000b; Kenneson *et al.* 2001; Primerano *et al.* 2002). The generally higher fold change of *FMR1* mRNA in human PM carriers versus normal controls, in comparison with lower two- to threefold changes measured in our mice can be explained by the fact that we isolate RNA from whole brain lysate, whereas RNA in human studies is extracted from blood. Thus far, brain *FMR1* mRNA levels have been quantified in one FXTAS patient. Absolute *FMR1* mRNA levels were found to be higher in brain than in peripheral blood leukocytes, although fold increases when compared with normal controls were more pronounced in RNA isolated from blood. Furthermore, differential *FMR1* expression was seen in different brain regions, despite the fact that neurons in each region showed the same repeat length (Tassone *et al.* 2004b). Thus our results in (CGG)_n mice might represent an average of higher and lower regional expression levels throughout the brain. It would be interesting to measure *Fmr1* mRNA and *Fmrp* levels in different mouse brain areas and relate this to the proportion of inclusion-bearing neurons. However, it would be very challenging to reliably dissect the small mouse brain.

In post-mitotic neurons in a mouse model for Huntington's disease, higher (CAG)_n instability was observed in brain regions that show high expression of the mutant allele (Gonitel *et al.* 2008). Thus, if *Fmr1* mRNA expression varies among different brain regions in our (CGG)_n mice, it would be interesting to investigate somatic instability in those specific regions. However, to date there is no evidence that (CGG)_n length heterogeneity exists in human FXTAS brain (Tassone *et al.* 2004b). In the present study, very minor (CGG)_n instability was seen when comparing brain DNA obtained at death with DNA obtained from tail shortly after birth. Naturally, this comparison cannot be made in patients with FXTAS. No evidence for a broader pattern of (CGG)_n lengths was seen in brain DNA when compared with tail DNA, thus repeat length heterogeneity does not seem to be a major occurrence.

The elevated *FMRI* mRNA levels have been attributed to increased transcription, rather than to increased stability of the *FMRI* transcripts (Tassone *et al.* 2000a, 2007b). The cause of increased transcription is unknown, although it could be that the expanded (CGG)_n causes the chromatin to adopt a more open conformation, which could facilitate transcription. In addition, the (CGG)_n length has been shown to influence which transcription start site is used, with the use of more upstream start sites corresponding to increased *FMRI* transcription (Beilina *et al.* 2004). It has been proposed that a feedback system might exist such that lower FMRP levels cause increased *FMRI* transcription (Tassone *et al.* 2000b; Oostra and Willemsen 2003). However, cellular studies show increased expression of reporter constructs containing the *FMRI* 5'UTR containing various (CGG)_n lengths, in the absence of FMRP. This suggests that an intrinsic quality of the *FMRI* 5'UTR and/or the (CGG)_n might be the cause of the elevated transcription. In addition, as the *FMRI* 5'UTR was driven by the cytomegalovirus immediate early promoter in these experiments, the increased transcription must be independent of the *FMRI* promoter. Thus, although it should be noted that this study was performed under conditions of over-expression of the construct, at least part of the elevated transcript levels seen in PM carriers may be a direct *cis*-acting effect of the (CGG)_n, rather than a compensatory response to reduced FMRP levels (Chen *et al.* 2003). This possibility might be reflected by the lack of correlation between *Fmr1* transcript levels and Fmrp levels in this study.

Current evidence suggests that *FMRI* transcripts are not retained in the nucleus, making this an unlikely reason for reduced translation (Tassone *et al.* 2007b). The (CGG)_n has been found to impede the 40s ribosomal unit, leading to suppression of translation (Feng *et al.* 1995). Indeed, FMRP levels are mostly reduced in the upper PM range in humans (Kenneson *et al.* 2001; Tassone *et al.* 2007b), as well as in mice.

In humans, CGG expansions above 200 generally lead to methylation of the repeat and silencing of the gene (Oberle *et al.* 1991; Verkerk *et al.* 1991; Sutcliffe *et al.* 1992). This has not been observed in expanded (CGG)_n mouse models (Brouwer *et al.* 2007; Entezam *et al.* 2007). In general, it has been proposed that mouse models for trinucleotide repeat disorders need longer repeats in order to see major instability than do human repeats (Gourdon *et al.* 1997). The same might be true for methylation of the gene and/or phenotypes associated with the repeats.

The finding that some animals show inclusions in the dentate gyrus, while others do not, irrespective of (CGG)_n length, implies that modifying factors play a role in the formation of inclusions. We cannot exclude that the same effect occurs in other brain regions, as we have not investigated all brain areas extensively. As our breedings are mostly set up to obtain certain repeat lengths, animals have a mixed genetic background. As it is known that genetic background has an influence on behavioral phenotype in different strains of *Fmr1* knockout mice (Spencer *et al.* 2006), a similar influence of the genetic background on inclusion formation in specific regions could have taken place in our mice.

In summary, this study indicates that there is both a lower repeat length threshold, below which no inclusions form, and an upper threshold above which hardly any inclusions develop. There does not appear to be a linear correlation between (CGG)_n and *Fmr1* mRNA levels in brain or the presence of inclusions. Fmrp levels do decrease with increasing repeat length, specifically after the (CGG)_n exceeds 150. Thus, if inclusions are responsible for the clinical outcome, the current results suggest that merely the presence of expanded (CGG)_n *Fmr1* mRNA, rather than the precise level of expression, possibly in combination with a minimal level of remaining Fmrp, determines the occurrence of inclusion neuropathology. Behavioral studies in mice of differing (CGG)_n repeat lengths will be necessary to establish whether an aberrant phenotype is primarily related to the length of the (CGG)_n expansion or to *Fmr1* expression levels.

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Abbreviations used

BPs, binding proteins; DM, myotonic dystrophy; FM, full mutation; FMRP, Fragile X Mental Retardation Protein; FXTAS, fragile X-associated tremor/ataxia syndrome; PM, pre-mutation; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; UTR, untranslated region; wt, wild type; YAC, yeast artificial chromosome..

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Fig. 1.

No ubiquitin-positive inclusions are seen in the colliculus inferior of 72-week-old mice with (CGG)₇₀ (a), while mice with (CGG)₁₀₆ do show ubiquitin-positive inclusions (b) brown, round intranuclear staining). Immunohistochemistry for Fmrp in cortex of (CGG)₇₀ (c) and wt (d) animals gives a similar staining pattern.

Fig. 2.

Average fold change of *Fmr1* mRNA levels (error bars indicate SDs) in the different (CGG)_n length categories. In these experiments, 13 wt, four (CGG)₇₀, 15 (CGG)₁₀₀₋₁₅₀, eight (CGG)₁₅₁₋₂₀₀, and 14 (CGG)_{> 200} animals were used. One-way ANOVA revealed a statistically significant overall difference between the categories ($F = 6.47$, $df = 4$, $p < 0.001$). To determine which individual categories differed, Dunnett's *post hoc* test was performed. This revealed that only animals with 100-150 CGGs differ significantly from wt animals (fold change mean difference = 1.60, SE = 0.36, $p < 0.001$, statistical parameters of other categories not shown).

Fig. 3.

(a) Average percentages of inclusion-bearing cells in the colliculus inferior and dentate gyrus in the different (CGG)_n length categories. Error bars show SDs. Seven (CGG)₁₀₀₋₁₅₀, four (CGG)₁₅₁₋₂₀₀, and six (CGG)_{> 200} animals were used. One-way ANOVA revealed a statistically significant difference between the three inclusion-bearing repeat length categories in both the colliculus inferior ($F = 8.16$, $df = 2$, $p < 0.01$) and the dentate gyrus ($F = 6.13$, $df = 2$, $p = 0.02$). Bonferroni-corrected *post hoc* pair-wise comparisons revealed that inclusion counts did not differ statistically significantly between (CGG)₁₀₀₋₁₅₀ and (CGG)₁₅₁₋₂₀₀ in the colliculus inferior (mean difference = 0.10, SE = 0.15, $p = 1$) and the dentate gyrus (mean difference = 0.08, SE = 0.12, $p = 0.12$). However, inclusion formation in the (CGG)_{> 200} mice differed statistically significantly from (CGG)₁₀₀₋₁₅₀ in the colliculus inferior (mean difference = -0.42, SE = 0.11, $p < 0.01$) and the dentate gyrus (mean difference = -0.30, SE = 0.09, $p = 0.02$), but not from (CGG)₁₅₁₋₂₀₀ (colliculus inferior: mean difference = -0.33, SE = 0.15, $p = 0.15$, dentate gyrus: mean difference = -0.22, SE = 0.12, $p = 0.31$). (b) Dentate gyrus of an animal with (CGG)₁₀₆ that does not show intranuclear inclusions, while a mouse with (CGG)₁₃₀ (c) does.

Fig. 4. Ubiquitin-positive inclusions are present in colliculus inferior (a-c) and dentate gyrus (d-f) of mice with (CGG)₁₃₀ and (CGG)₁₈₀, but not in mice with (CGG)_{>200}.

Fig. 5.

Fmrp levels in mouse brain of the different (CGG)_n length categories. (a) Shows an example of a western blot, visualised with the 2F5 antibody against Fmrp, Gapdh was used as a loading control. Approximate (CGG)_n lengths are indicated. (b) Shows average Fmrp levels relative to the average wt level. Error bars represent SDs. A total of three wt, one (CGG)₇₀, 10 (CGG)₁₀₀₋₁₅₀, five (CGG)₁₅₁₋₂₀₀, and nine (CGG)_{> 200} animals were used for these studies. Note that (CGG)₇₀ has no error bar, as this bar represents one sample. One-way ANOVA revealed that there was a statistically significant difference between (CGG)_n categories ($F = 7.02$, $df = 3$, $p < 0.01$). A Dunnett's *post hoc* test was performed to compare Fmrp levels between mice in each repeat category with wt animals. Animals with (CGG)₁₀₀₋₁₅₀ did not show significantly altered Fmrp levels compared to wt mice (%Fmrp mean difference = -0.19, SE = 0.11, $p = 0.11$). Mice with expanded alleles of (CGG)₁₅₁₋₂₀₀ (%Fmrp mean difference = -0.40, SE = 0.13, $p < 0.01$) or (CGG)_{> 200} (%Fmrp mean difference = -0.44, SE = 0.12, $p < 0.01$) did have statistically significantly lower Fmrp levels than wt mice. Animals with (CGG)_{> 150} have statistically significantly lowered Fmrp levels (see text for details on statistical analyses).

Fig. 6.

Summary of the molecular correlates. Fold change *Fmr1* mRNA (left y-axis), % Fmrp and % of inclusion-bearing neurons in colliculus inferior and dentate gyrus (right y-axis) are given. Please refer to the individual graphs for an indication of the variability of the observed data.