

# Ectopic expression of CGG containing mRNA is neurotoxic in mammals

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**Fragile X-associated Tremor/Ataxia Syndrome (FXTAS) is a progressive neurodegenerative disorder that has been diagnosed in a substantial fraction of older male fragile X premutation carriers. Patients affected by FXTAS have elevated levels of ribo-rCGG repeat containing *FMR1* mRNA with normal to slightly reduced levels of FMRP in blood leukocytes. Coupled with the absence of FXTAS in fragile X syndrome patients, this suggests premutation-sized elongated rCGG repeats in the *FMR1* transcript rather than alterations in the levels of FMRP are responsible for the FXTAS pathology. Mice expressing rCGG in the context of *Fmr1* or the enhanced green fluorescent protein specifically in Purkinje neurons were generated to segregate the effects of rCGG from alterations in *Fmr1* and to provide evidence that rCGG is necessary and sufficient to cause pathology similar to human FXTAS. The models exhibit the presence of intranuclear inclusions in Purkinje neurons, Purkinje neuron cell death and behavioral deficits. These results demonstrate that rCGG expressed in Purkinje neurons outside the context of *Fmr1* mRNA can result in neuronal pathology in a mammalian system and demonstrate that expanded CGG repeats in RNA are the likely cause of the neurodegeneration in FXTAS.**

## INTRODUCTION

Within the last decade, a late age of onset neurodegenerative disorder, termed Fragile X-associated Tremor/Ataxia Syndrome (FXTAS), has been recognized in older males of fragile X syndrome (FXS) families and is uncoupled from the neurodevelopmental disorder, FXS. Although both disorders involve repeat expansions in the *FMR1* gene, the clinical presentation and molecular mechanisms underlying each disease are completely distinct. The most common clinical feature of FXTAS is a progressive action tremor with ataxia. More advanced or severe cases may show a progressive cognitive decline that ranges from executive and memory deficits to dementia (1). Magnetic resonance imaging of adult male patients affected with FXTAS demonstrated mild-to-moderate global brain atrophy, most common in the frontal and parietal regions as well as the pons and the cerebellum (2–4). The most significant radiological findings were the increased T2

intensities of the middle cerebellar peduncle and adjacent cerebellar white matter not seen in controls (3). This finding serves as a major diagnostic criterion for FXTAS. Nearly all case studies on autopsy brains of symptomatic premutation carriers demonstrated degeneration of the cerebellum, which includes Purkinje neuronal cell loss, Bergman gliosis, spongiosis of the deep cerebellar white matter and swollen axons (5,6). The major neuropathological hallmark and post-mortem criterion for definitive FXTAS is eosinophilic, ubiquitin-positive intranuclear inclusions located in broad distribution throughout the brain in neurons, astrocytes and the spinal column (5). The inclusions are both tau and  $\alpha$ -synuclein negative, which indicates that FXTAS is not a tauopathy or synucleinopathy.

Interestingly, these patients are carriers of a premutation size CGG repeat (55–200 triplets) in the 5'-UTR of the *FMR1* gene. Symptomatic patients with repeat lengths between 70 and 135 triplets have been described (6).

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The repeat is expressed in the mature *FMRI* mRNA in premutation carriers and in individuals with normal CGG repeat lengths. A study of the penetrance of the tremor and ataxia among premutation carriers, ascertained through families with known probands with FXS, revealed greater than one-third of carriers, aged 50 years and older, show symptoms of FXTAS and that the penetrance of this disorder exceeds 50% for men over 70 years of age (7). The prevalence of the premutation alleles is approximately 1 of 800 for males and 1 of 250 for females in the general population; however, it is estimated that 1 in 3000 men older than 50 years in the general population will show symptoms of FXTAS (8,9). The degree of brain atrophy and severity of the tremor and ataxia are associated with the CGG repeat length (10). Some female carriers also develop clinical features of FXTAS (11–13), but at a much lower frequency than males (7), which is thought to be due to partial protection offered by random X-inactivation of the premutation allele (14).

An RNA gain of function mechanism was suggested for FXTAS (2,6,15,16) based on the observation of increased levels of CGG containing *FMRI* mRNA (17), along with either no detectable change in FMRP (18) or slightly reduced FMRP levels, observed in peripheral blood leukocytes (17,19) and brain regions (20) of premutation carriers. Tassone *et al.* (21) demonstrated the presence of premutation *FMRI* RNA transcripts in the FXTAS inclusions of a 70-year-old male who died with FXTAS. The absence of FXS, which results from the loss of function of the *FMRI* gene product, in FXTAS patients along with absence of FXTAS symptoms in older individuals with FXS, also suggests a role for the expanded ribo-CGG (rCGG) repeat in FXTAS pathology.

A knockin mouse model of FXTAS, generated by the Oostra group, which replaced the endogenous CGG<sub>8</sub> of the mouse *Fmr1* gene with a premutation length CGG<sub>98</sub> repeat of human origin was developed to study instability in the murine *Fmr1* gene (22). Interestingly, these animals demonstrated features of FXTAS such as intranuclear neuronal inclusions positive for ubiquitin, Hsp40 and the 20S catalytic core complex of the proteasome. This model also exhibited cognitive decline, neuromotor and behavioral disturbances assessed by various behavioral tests. Knockin mice with expanded repeat lengths between 100 and 150 have significantly higher levels of *Fmr1* mRNA expression in brain compared with wild-type controls; however, expanded knockin brains with repeat lengths between 151 and 200 repeats have normal levels of *Fmr1* mRNA, suggesting that at least in the case of this expanded knockin mouse, there is no relationship between CGG repeat length and *Fmr1* mRNA levels (23). *Fmrp* expression in brain was normal in expanded knockin animals ranging 100–150 triplets, but when the repeat exceeds 151 triplets the *Fmrp* levels drop slightly below normal levels when compared with wild-type controls. A proportional increase in neuronal inclusion size and number was previously reported in the CGG98–200 expanded knockin (18), however animals with repeats greater than 200 triplets show marked reduction in the number of observable inclusions (23).

A second knockin mouse model, generated by the Usdin group (24), shared key features of human premutation carriers not seen in the previous mouse model such as expansion to a full mutation sized allele in a single generation. This model

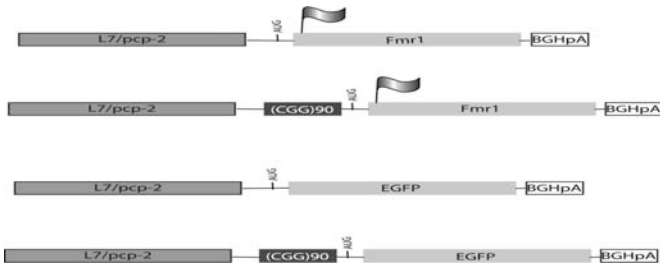
has serial ligated, short stable CGG•GCC-repeat tracts knocked into exon 1 of the endogenous mouse *Fmr1* gene. These mice develop Purkinje neuron pathology such as abnormal calbindin staining, swollen axons and torpedoes, and Purkinje neuron dropout. Additionally, this model exhibits a proportional relationship between the repeat number and *Fmr1* mRNA levels. Unlike the Oostra knockin model, the Usdin knockin model exhibits an inverse relationship between the FMRP levels and repeat number; however, the number of mice examined per repeat length is not described. Although the mouse models suggest a direct rCGG involvement, it still remains unclear which part of the expanded message is necessary or whether FMRP plays any role and to what capacity. The rCGG transgenic fly study reported the first evidence that rCGG outside of the context of *FMRI* could induce neurodegeneration and inclusion formation (16).

In order to demonstrate the direct toxicity of rCGG, Jin *et al.* (16) developed a *Drosophila* model of FXTAS where rCGG was expressed in a heterologous transcript (enhanced green fluorescent protein, EGFP). Expression of 90 CGGs in this transcript was found to cause neurodegeneration along with the formation of inclusions when expressed in the fly eye. This model has been used to identify genetic modifiers (25,26). These studies strongly implicate expanded rCGG as sufficient to induce neurodegeneration; however, it has remained unclear whether premutation length rCGG outside of the context of the *Fmr1* gene was sufficient to recapitulate FXTAS pathology in a mammalian system.

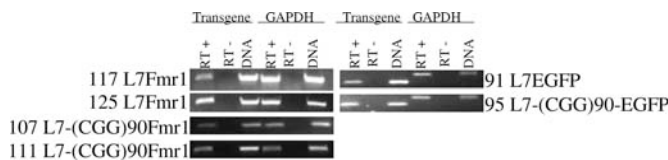
To test this hypothesis, we developed four transgenic mouse models that express *Fmr1*, EGFP CGG<sub>90</sub>*Fmr1* or CGG<sub>90</sub>EGFP driven by a Purkinje neuronal specific promoter, L7/*pcp2*, to explore the molecular requirement for the neurodegeneration observed in FXTAS. In contrast to the Oostra knockin model, which showed inclusion formation in several neuronal cell types but almost never in Purkinje neurons, we observed large and frequent intranuclear inclusions in this cell type. Our model allowed us to evaluate the direct pathogenesis of expanded rCGG and is the first mammalian model to distinguish the effect of rCGG from the effects of the levels of FMRP. We show that rCGG outside of the context of *Fmr1* is sufficient to produce ubiquitin-positive intranuclear inclusion formation. Furthermore, we demonstrate that rCGG overexpression leads to Purkinje neuron axonal swellings and neurotoxicity. Finally, we show that rCGG expressing animals show a progressive, age-dependent decline in neuromotor learning abilities.

## RESULTS

To examine whether the *FMRI* premutation rCGG could cause neurodegeneration in mammals, we expressed a human *FMRI* rCGG repeat in Purkinje neurons of *Mus musculus*. Transgene expression was spatially regulated by the Purkinje neuron-specific L7 promoter. The L7 promoter replaced the CMV promoter in the pcDNA3.1 vector. Cloning of the human premutation length (90) CGG repeat upstream of either *Fmr1* or EGFP was the last step due to instability of the repeat. As controls, transgenic animals with L7*Fmr1* or L7EGFP alone were generated. Transgene constructs are shown in Figure 1.



**Figure 1.** Schematic representation of L7-(CGG)<sub>90</sub> and L7 constructs. The transgenes were driven by and Purkinje cells specific, L7 promoter. A human genomic *FMR1* DNA fragment containing 90 CGG repeats was inserted upstream of the *Fmr1* or EGFP coding region between the transcriptional and translational start sites. The *Fmr1* containing transgenes have a FLAG epitope engineered into the 5' of the gene. A BGH polyadenylation site was inserted 3' of the transgenes.



**Figure 2.** Expression of *Fmr1* and EGFP transgenes. RT-PCR was performed using primers specific for *Fmr1* and EGFP. RNA was isolated from mouse cerebellum of all listed genotypes. RT-PCR of GAPDH was also performed to as a control for RNA quality.

To examine the expression of the transgenes, we used RT-PCR (Fig. 2) using primers specific for the *Fmr1* or EGFP coding sequences. Seven of the lines generated demonstrated expression of their respective transgene.

To determine whether the *FMR1* premutation rCGG repeat could cause neurodegeneration in mice similar to the human FXTAS, we assayed each line for the presence of intranuclear inclusions with antibodies against ubiquitin that could positively detect inclusions in human FXTAS. Sections of cerebella revealed the presence of ubiquitin positive nuclear inclusions present in Purkinje neurons of animals with the expanded rCGG repeat constructs in both the *Fmr1* and EGFP contexts. No inclusions were detected in animals expressing *Fmr1* or EGFP without rCGG. Inclusions were evident in sections from animals beginning at 8 weeks of age. In addition to ubiquitin (Fig. 3A), the inclusions stained with antibodies to the 20S core complex of the proteasome (Fig. 3B), Hsp40 (Fig. 3C) and Rad23B (Fig. 3D). We did not detect staining of Purkinje neuron nuclear inclusions with antibodies to hnRNPA2/B1 and Pur $\alpha$ , which have been observed in the human FXTAS. Additionally, we did not detect Tau or  $\alpha$ -synuclein staining of the inclusions.

These results demonstrate an essential role for rCGG in nuclear inclusion formation. Inclusions were limited to Purkinje neurons in each L7CGG<sub>90</sub>*Fmr1* (Fig. 4B) and L7CGG<sub>90</sub>EGFP (Fig. 4D) line, but were totally absent in L7*Fmr1* (Fig. 4A) or L7EGFP lines (Fig. 4C). The frequent formation of inclusion bodies in Purkinje neurons in the L7CGG<sub>90</sub>EGFP mouse line suggests that inclusion formation is not directly correlated to elevated *Fmr1* RNA levels, but rather to the expression of expanded rCGG repeat as

demonstrated in animals expressing the repeat outside of the context of the *Fmr1* gene.

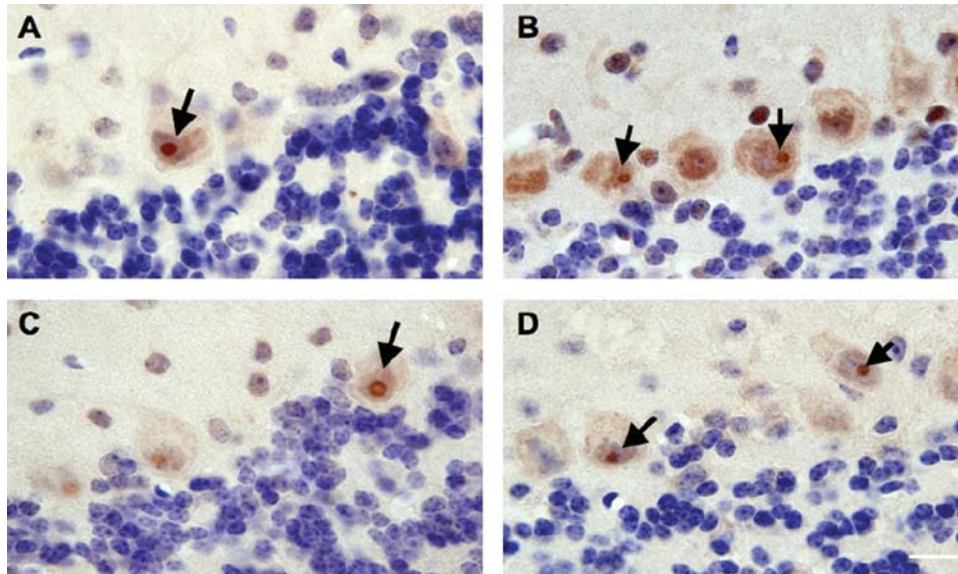
There was significant Purkinje neuron cell loss, comparable to the human FXTAS, for L7CGG<sub>90</sub>*Fmr1* animals compared with wild-type (data not shown) or L7*Fmr1* ( $P < 0.001$ ) (Fig. 5A and B). Comparison of 10-, 25- and 33-week-old L7CGG<sub>90</sub>*Fmr1* mice showed greater cell loss ( $P < 0.001$ ) compared with age-matched L7*Fmr1* mice (data not shown). L7CGG<sub>90</sub>EGFP mice also showed greater cell loss when compared with L7EGFP mice ( $P = 0.001$ ) (Fig. 5C and D). Detailed examination of the cerebellum at higher resolution revealed Purkinje cell axonal swellings (torpedoes) for both L7CGG<sub>90</sub>*Fmr1* and L7CGG<sub>90</sub>EGFP mice (Fig. 6A and B), indicating cell damage in living cells in addition to the increased cell loss.

In order to determine whether Purkinje cell loss resulted in loss of cerebellar neuromotor performance and equilibrium were analyzed using an accelerating Rotarod apparatus for male and female L7*Fmr1* and L7CGG<sub>90</sub>*Fmr1* mice aged 20 and 40 weeks. Initial analysis of variance revealed that there was no genotype X gender interactions; therefore, the Rotarod data were analyzed using three-way (genotype X age X trial) ANOVA with repeated measures. Although the genotype X age interaction was not significant ( $P > 0.05$ ), the present experimental design allowed us to use an a priori planned comparisons to determine if there was an effect of age in the two different genotypes. Overall, the performance of both genotypes improved with training (effect of trial,  $F_{7,385} = 47.37$ ,  $P < 0.0001$ ). In addition, the L7*Fmr1* mice performed significantly better than the L7CGG<sub>90</sub>*Fmr1* mice (effect of genotype,  $F_{1,55} = 29.486$ ,  $P < 0.0001$ ). Finally, a priori planned comparisons revealed that although there was no effect of age in the L7*Fmr1* mice ( $F_{1,12} = 0.298$ ,  $P = 0.595$ ), the effect of age in the L7CGG<sub>90</sub>*Fmr1* mice was significant ( $F_{1,47} = 8.55$ ,  $P = 0.005$ ) (Fig. 7). These results demonstrate that the fragile X premutation repeat causes a progressive age-dependent neurological decline over time, which is characteristic of the human FXTAS.

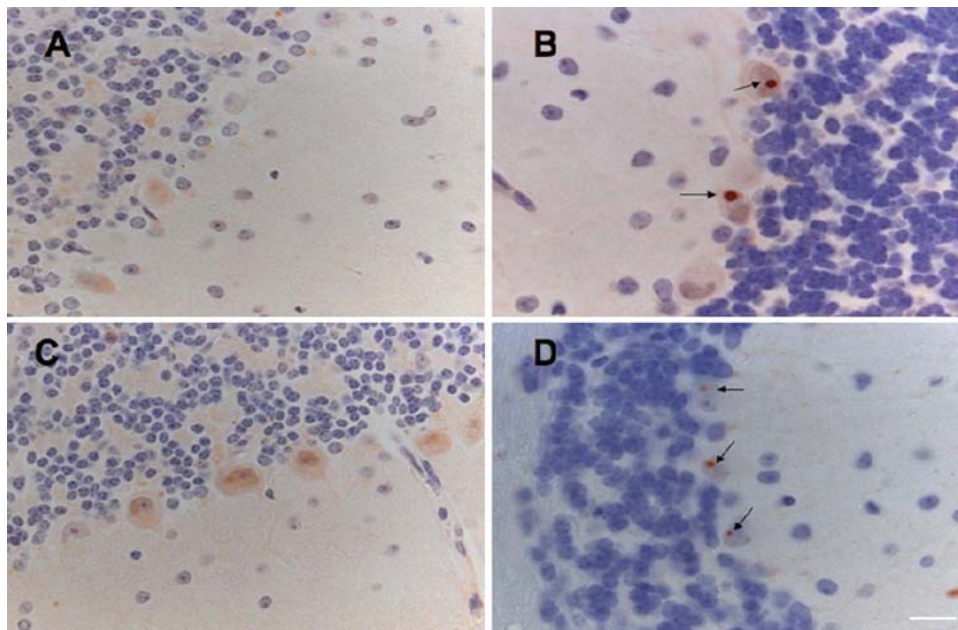
## DISCUSSION

In the present study, Purkinje neuron-specific transgenic FXTAS mouse models expressing a premutation length CGG repeat in the context of *Fmr1* 5'-UTR or EGFP 5'-UTR exhibited neuropathological and molecular features of FXTAS. These features include ubiquitin-positive intranuclear neuronal inclusions, Purkinje neuron dropout and consequent behavioral anomalies. Ubiquitin-positive intranuclear inclusions were observed in transgenic animals expressing CGG repeats in the context of *Fmr1* or EGFP. Our study presents the first evidence in a mammalian system that expanded CGG repeats outside the context of *Fmr1* are sufficient to cause inclusion formation and neurodegeneration.

Our models, similar to a knockin mouse model developed by the Oostr group, which replaces the endogenous mouse CGG<sub>8</sub> with a 98 human premutation length CGG repeats (18,22), develop intranuclear inclusions; however, the presence of large and frequent ubiquitin positive inclusions in Purkinje neurons is unique to our model. Also similar to a knockin



**Figure 3.** Staining for inclusion content. Immunohistochemistry demonstrates cerebellar Purkinje neuron inclusions stain with antibodies against (A) ubiquitin, (B) 20S subunit of the proteasome, (C) Hsp40 and (D) Rad23B in the line expressing the L7CGG<sub>90</sub>EGFP constructs as well as all L7CGG90*Fmr1* (data not shown) lines.

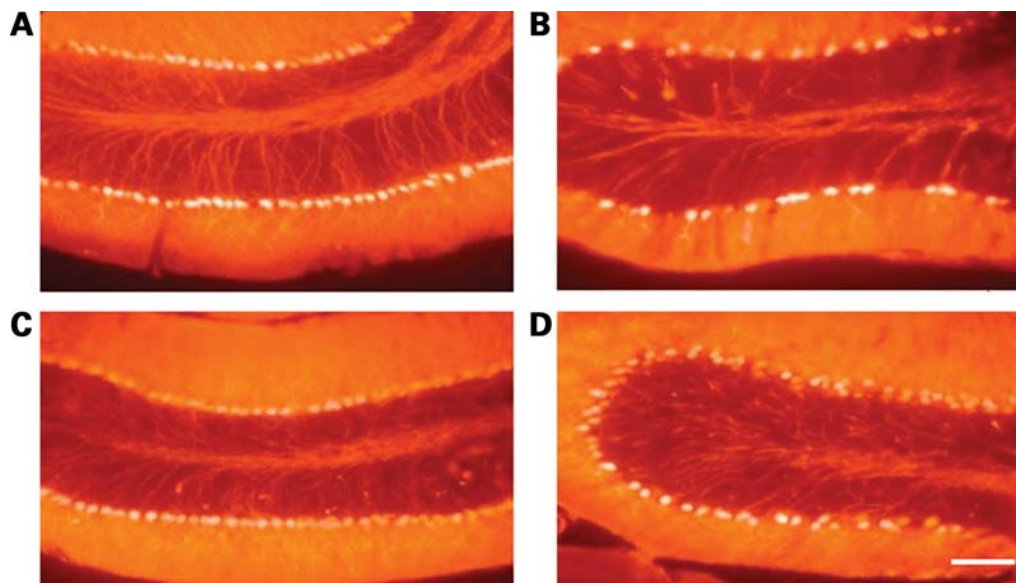


**Figure 4.** Immunohistochemistry demonstrates rCGG repeat is necessary and sufficient to cause the formation of intranuclear inclusions. (A) L7*Fmr1*, (B) L7CGG<sub>90</sub>*Fmr1*, (C) L7EGFP and (D) L7CGG<sub>90</sub>EGFP.

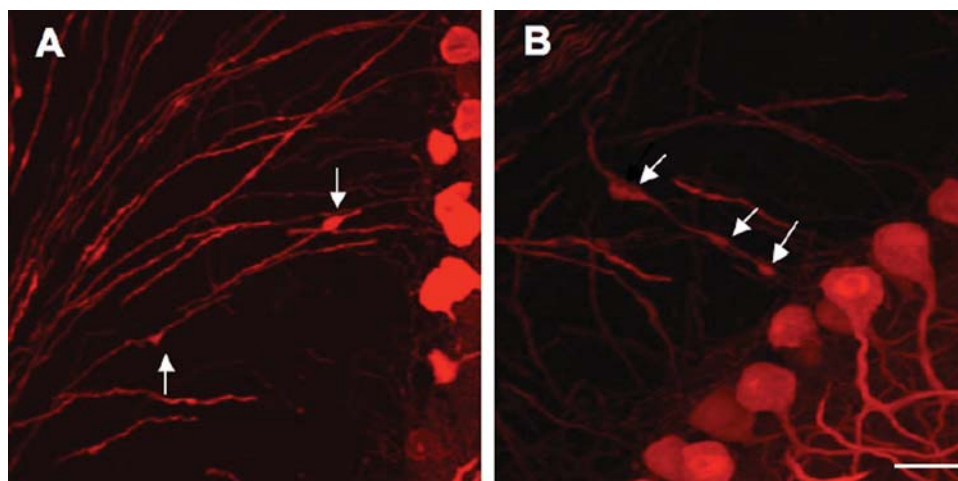
mouse model generated by the Usdin group, which replaced the endogenous mouse CGG<sub>8</sub> with serial ligated CGG•GCC-repeat tracts (24), our model shows neurodegeneration of the Purkinje neurons. However, the Usdin knockin lacks observable Purkinje neuronal inclusions. Interestingly, a murine prion protein promoter SCA7 mouse model, which expresses polyQ expanded Ataxin-7 throughout the cerebellum in both neurons and non-neuronal cells, but not in Purkinje neurons, demonstrated marked degeneration of Purkinje cells (27,28). This model helps us to appreciate the

possibility of a non-cell-autonomous mechanism for the pathogenesis and behavioral defects observed in human FXTAS and knockin mouse models.

To alleviate the potentially confounding effects of pathology stimulated by changes in interacting cells, which could make it difficult to interpret a phenotype, we focused on a single cell type. Specific expression of transgenes in Purkinje neurons provides several advantages. The L7/*Pcp2* promoter more robustly and rapidly produces pathological and behavioral changes compared with the use of the endogenous *Fmr1*



**Figure 5.** Purkinje cells stained with anti-calbindin Ab in sagittal cerebellum sections of 32-week-old mice. (A) *L7Fmr1*, (B) *L7CGG<sub>90</sub>Fmr1*, (C) *L7EGFP* and (D) *L7CGG<sub>90</sub>EGFP*. There was significant cell loss for *L7CGG<sub>90</sub>Fmr1* mice compared with *L7Fmr1* ( $P < 0.001$ ). *L7CGG<sub>90</sub>EGFP* mice also show greater cell loss when compared with *L7EGFP* mice ( $P = 0.001$ ).

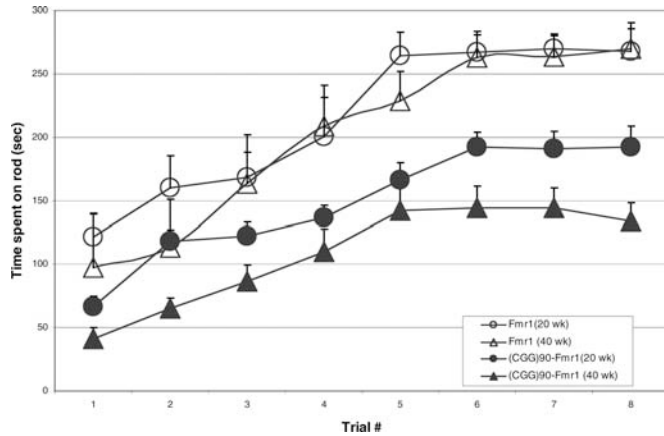


**Figure 6.** Purkinje cell axonal swellings indicate ongoing cell damage in living cells. (A) *L7CGG<sub>90</sub>Fmr1* and (B) *L7CGG<sub>90</sub>EGFP*.

promoter with an expanded repeat. The Oostra knockin model takes 30 weeks to show inclusions, which peak in numbers after 1 year of age, and 72 weeks to exhibit a Rotarod phenotype (29). This is in contrast to our models that show inclusions by 8 weeks and behavioral deficits at 20 weeks for *L7CGG<sub>90</sub>Fmr1* mouse lines.

Our transgenics, like the Oostra knockin, possess ubiquitin, 20S core complex of the proteasome, Hsp40, as well as Rad23B positive intranuclear inclusions. The presence of these proteins in the inclusions suggests a role for the proteasomal degradation pathway in inclusion formation. Bergink *et al.* (30) observed mammalian homologs of Rad23, HR23A and HR23B are constituents of the neuronal inclusions in the Oostra knockin mouse model. Rad23B binds ubiquitin (31,32) and is thought to participate as a shuttle factor for translocating proteins to the proteasome for degradation

(33). Rad23B also plays a role in the regulation of the GG-nucleotide excision repair reaction by possible protection against degradation by the ubiquitin/proteasome system (34). Interestingly, Ataxin-3, the gene product associated with CAG expansion disease, Machado–Joseph disease, was shown to interact with the two mammalian homologs of Rad23 through their ubiquitin-like domain and is found in intranuclear inclusions in 293 cells with mutant Ataxin-3 (35). Coincidentally, human fibroblasts overexpressing GFP-polyQ peptides induce HR23B-positive inclusions without hampering DNA repair (30). The similarities in contents between the inclusions of these two diseases may indicate a common mechanism for inclusion formation. Although there is potential for crossover between the pathways of the disease mechanisms for FXTAS and Machado–Joseph, several studies suggest that polyglutamine toxicity



**Figure 7.** Age-dependent decline in motor learning on the accelerating Rotarod. *L7Fmr1* 20-week-old mice (open circle) and *L7Fmr1* 40-week-old mice (open triangle) do not show an age-related phenotype on the accelerating rod. *L7CGG<sub>90</sub>Fmr1* 20-week-old mice (closed circle) perform worse than *L7CGG<sub>90</sub>Fmr1* 40-week-old mice (closed triangle). The effect of age X genotype for the *L7CGG<sub>90</sub>Fmr1* mice was significant ( $P = 0.005$ ), whereas *L7Fmr1* was not ( $P > 0.05$ ).

plays an important role in the disease. Polyglutamine repeats can change the function of the protein and may therefore lead to downstream events that lead to neurodegeneration and inclusion formation. This is in contrast to FXTAS where the expanded CGG repeat does not encode protein and therefore may not be mechanistically relevant to FXTAS disease progression. The Bonini group recently observed that RNA toxicity is involved in CAG-mediated degeneration in *Drosophila*. Flies expressing an untranslated CAG repeat of pathogenic length in the 3'-UTR of the dsRed reporter gene exhibit degeneration of the eye and nervous system, early death and loss of climbing activity (36). These observations suggest a role for rCAG toxicity in polyglutamine diseases. Interestingly, flies expressing rGCC repeats, which have not been associated with neurodegenerative disease in humans, demonstrate pathology indistinguishable from the rCGG FXTAS fly model (37). These observations provide additional impetus to understand the mechanisms by which simple sequence repeats in RNA can be toxic to cells.

Elevated levels of premutation length CGG repeat containing *FMRI* message are present in peripheral blood leukocytes of premutation carriers (17) with and without FXTAS. This finding offers biochemical evidence of alterations in premutation *FMRI* gene expression. A correlation has also been observed between larger premutation length repeats and a reduction in FMRP (19). It has been proposed that the cells carrying premutations attempt to compensate for the reduction in FMRP by increasing the expression of the *FMRI* transcript (17), however; reduced translation efficiency of premutation transcripts was suggested to result from reduced association with polysomes in blood leukocytes of premutation carrier males (38). This observation raises the question of the localization of the premutation rCGG transcript if it indeed has reduced association with polysomes. Although it has been shown that premutation transcripts localize to inclusions in humans (21), it is uncertain what fraction of the transcript may be bound up in inclusions and what fraction is translated.

It would be interesting to investigate the translation efficiency of the premutation transcripts in other mouse models of FXTAS. A previous study demonstrated that the increased premutation *FMRI* message was not due to increased stability of the message (17), but increased primary transcription (19).

RNA gain-of-function mechanisms have been suggested for simple repeat diseases such as the myotonic dystrophies (DM1 and DM2) whereby increasing numbers of triplet repeats in the transcripts lead to aberrant interaction with RNA binding proteins or other unknown proteins resulting in the formation of intranuclear inclusions. These interactions, in turn, may alter normal functioning of the proteins involved, in a manner similar to that proposed for the CUG repeats expanded in DM1 mRNA. Several pieces of evidence now support this RNA toxicity model for FXTAS. RNA binding proteins hnRNP A2/B1 (26) and Pur $\alpha$  (25) can interact directly with rCGG *in vitro*, although the consequences of this physical interaction are unclear. Overexpression of the RNA binding proteins hnRNP A2/B1 (26) or Pur $\alpha$  (25) can suppress the rCGG-mediated neurodegenerative phenotype observed in the *Drosophila* eye (16). Interestingly, hnRNP A2/B1 was identified as a component of the intranuclear inclusions in a previous study, which set out to isolate and identify the composition of human intranuclear inclusions (39). *Drosophila* modifier Pur $\alpha$  was also demonstrated as a component of the intranuclear inclusions in the FXTAS fly model and FXTAS post-mortem patient brain samples (25); however, Pur $\alpha$  and hnRNPA2/B1 could not be detected in the inclusions present in our transgenic mouse models or the Oostra knockin model (personal communication). Interestingly, Pur $\alpha$  knockout mice develop tremors and neurodegeneration of Purkinje neurons (40). It would be interesting to determine if a Pur $\alpha$  mutation (loss of function or overexpression) can modify the neurodegenerative and behavioral phenotypes observed in our Purkinje neuron-specific mouse model of FXTAS. Our Purkinje neuron-specific mouse models allow us to perform genetic screens for modifiers of behavior and neurodegeneration and may provide a more rapid phenotypic read out compared with either of the current knockin mouse models, which develop inclusions or neurodegeneration much later than our models.

The fly model may also help uncover additional modifiers of the neurodegenerative phenotype. Some important advantages of the fly model are the ability to use it to perform rapid genetic screens for modifiers and to assess behavioral anomalies relatively quickly. This model may also afford us the ability to investigate drug and gene therapies for FXTAS. Although the fly model may help us uncover additional modifiers of the neurodegenerative phenotype, it is important to consider that the *Drosophila* inclusions were almost always cytoplasmic (16), which is unlike those observed in patients or in mouse models; therefore, the composition of the inclusions and pathways involved in neurodegeneration could differ significantly.

It is unknown whether inclusion formation is a protective mechanism for the cell. Additional mouse models are necessary to help us understand the role of the inclusion in FXTAS pathology. One way to answer this question is to develop an inducible transgenic mouse model much like the conditional transgenic mouse model of spinocerebellar ataxia type 1 (SCA1).

The *SCA1* transgenics expressed Ataxin-1 with 82 polyglutamine repeats driven by a tetracycline responsive element and were crossed to animals expressing a tetracycline responsive activator under the control of a Purkinje neuron-specific promoter. Doxycycline administration inhibited the expression of the mutant Ataxin-1. When mutant Ataxin-1 was turned off Purkinje neurons were able to recover from most of the pathological changes observed, including Purkinje neuronal inclusions, but not Purkinje neuron loss (41). For *SCA1*, at least, it has been demonstrated that the inclusions are not protective because upon clearance of the inclusion, an improvement of the Rotarod phenotype was observed. It is still unclear as to why the cell responds to the premutation repeat by elevating the CGG containing *FMRI* transcript levels in FXTAS or whether the inclusions are protective; however, we have confirmed the primary role of rCGG portion of the transcript is to cause neurodegeneration in mammals.

## MATERIALS AND METHODS

### Generation of L7/pcp2 mouse lines

Four plasmids were developed with either a FLAG-tagged *Fmr1* cDNA or an EGFP cDNA with or without a CGG<sub>90</sub> repeat. For the *Fmr1* containing constructs, an *EcoRI/MscI* fragment of the mouse *Fmr1* cDNA containing a 5'-FLAG tag was cloned into the *EcoRI/NotI* site of the pCDNA3.1 vector (Invitrogen, Carlsbad, CA, USA). For the EGFP constructs, a *NheI/XhoI* fragment from a pEGFP vector (Clontech) was cloned into the *NheI/XhoI* site of the pCDNA 3.1 vector. To generate the transgene constructs, the *SpeI* fragment of the CMV promoter in the pCDNA3.1 vector was replaced by a *HindIII/BamHI* fragment of L7/pcp2 promoter. Owing to instability of the CGG repeat, the last step was cloning of an *EcoRI* fragment containing the human CGG<sub>90</sub> repeat previously described (22) into the *EcoRI* site of both the L7/pcp2-*Fmr1* (L7*Fmr1*) and the L7/pcp2-EGFP (L7EGFP) constructs now known as L7CGG<sub>90</sub>*Fmr1* or L7CGG<sub>90</sub>EGFP, respectively (Fig. 1). The inserts were excised from their respective cloning vectors using a *SalI* restriction enzyme and microinjected into oocyte donors of C57B/6 mice. Tail DNA was extracted from each of the offspring and PCR analysis was performed to verify transgene presence and CGG length where applicable. Founder transgenic lines were mated to C57B6 wild-type mice to obtain offspring.

### Determination of repeat size

Length of the CGG repeat was verified by digestion with *EcoRI* and visualization by electrophoresis on 4% acrylamide gels. CGG repeat number and localization of AGG interruptions were determined using a previously described bisulfite sequencing method (42) with some modifications (43). Briefly, 5 µg of DNA was treated with sodium bisulfite. Following the bisulfite treatment, the CGG region was amplified by PCR using primers FCDF and FCDR (42). The PCR fragments were cloned into a vector and miniprep DNA from individual clones was sequenced in both directions to determine length and composition of the CGG repeat.

### RNA isolation and RT-PCR analysis

Total RNA from mouse cerebellum was isolated with Trizol (Invitrogen) according to manufacture's instructions. Total RNA was treated with DNase I (Invitrogen) according to manufacture's instructions. The cDNA was synthesized using SuperScript II reverse transcriptase (Invitrogen) with a mixture of oligo(dT)<sub>12-18</sub> and random hexamer primers. PCR amplification was performed with primers that recognize the *Fmr1* transgene or the EGFP transgene. The forward and reverse primer sequences for the detection of the *Fmr1* transgene are as follows: *Fmr1*ITG forward, 5'-CGACGATCATTC CCGAACAG-3'; *Fmr1*ITG reverse, 5'-GGCACGGGGGAGG GGCAAACAAC-3'. The forward and reverse primers for the detection of the EGFP transgene are as follows: EGFP forward, 5'-GTGGCTGTTGTAGTTGTAATCC-3'; EGFP reverse, 5'-AGCTGACCCTGAAGTTCATCTG-3'. PCR amplification was performed in a final volume of 25 µl containing 0.2 µM of each primer, 2.5 mM MgCl<sub>2</sub> and 1 U of *Taq* polymerase (Roche, Branchburg, NJ, USA), with the following conditions: 5 min at 94°C, followed by 25 cycles of 30 s denaturation at 94°C, annealing for 40 s at 55°C and a 1 min 20 s extension at 72°C, followed by a 7 min final extension at 72°C and an indefinite hold at 4°C. Amplification of GAPDH mRNA was used as an internal control using the forward primer, 5'-ACCACAGTCCATGCCATCAC-3' and the reverse primer, 5'-TCCACCACCCTGTTGCTGTA-3'. A water control was included as a control for DNA contamination. PCR products were loaded in either 3% agarose gel or 5% polyacrylamide gels.

### Immunohistochemistry

For inclusion staining, mice ranging in age from 8 to 56 weeks old were sacrificed. Brain tissue was isolated, cut in sagittal sections and immediately fixed in 10% neutral buffered formalin followed by paraffin embedding. Brain sections were immunostained with rabbit anti-ubiquitin (1:500, DAKO), mouse anti-Hsp72 (1:100, Amersham), rabbit anti-Hsp70 (1:100, San-vertch), rabbit anti-Hsp40 (1:100, Stressgen), goat anti-Hsp27 (1:100, Sanvertch), rabbit anti-core complex of 20S proteasome and RAD23B (30), rabbit anti-FMRP (1:100, Abcam), rabbit anti- $\alpha$ -synuclein (1:100, Chemicon), mouse anti-TAU (BROI, 1:500, Innogenetics), CUGBP1 (clone 3B1) and mouse anti-Pur $\alpha$  antibody (clone 10B12).

To determine cell loss, 53-week-old mice were transcardially perfused with 4% paraformaldehyde. The brains were post-fixed overnight in the same solution and dehydrated in 20% sucrose/0.1 M PBS. Serial sagittal sections were cut on a freezing microtome at 40 µm thickness. After rinsing in 0.1 M PBS, free-floating sections were incubated in a blocking solution containing 2% normal goat serum (Jackson ImmunoResearch) and 0.3% Triton X-100 in 0.1 M PBS for 1 h at 4°C. The sections were then incubated for 48 h at 4°C with anti-calbindin (D28K, 1:1000, Sigma) in the blocking solution. After rinsing, sections were incubated for 48 h at 4°C with a Cy3 goat anti-mouse IgG H+L secondary antibody (1:600, Jackson ImmunoResearch) in blocking solution. Sections were washed and visualized under a confocal microscope. Cerebella immunostained with calbindin were quantitatively

analyzed for Purkinje cell number. The number of Purkinje cells was determined as 10 times for every fourth 40 mm thick section starting at the midline for 10 sections. The *t*-test was used to determine the significance between the two sets of data. A *P*-value of < 0.05 was considered significant.

### Accelerating Rotarod

Equilibrium and motor coordination were examined on an accelerating Rotarod apparatus (Jones and Roberts 7650, Ugo Basile, Italy). Each mouse was placed on the rotating rod for four test trials on two consecutive days during which the rotation speed gradually increased from 4 to 40 r.p.m. Mice were allowed to rest for 30 min before and 30 min after each trial. The time any animal could stay on the rod was timed up to 5 min.

### Statistical analysis

Calbindin-stained Purkinje neurons were counted using 10 midline, 40 mm thick sagittal cerebellar sections for each genotype and a *t*-test was used to determine the significance between two data sets. A *P* < 0.05 was considered significant. Initial analysis of variance revealed that there were no genotype X gender interactions, therefore, the Rotarod data were analyzed using a three-way (genotype X age X trial) ANOVA with repeated measures. Although the genotype X age interaction was not significant (*P* > 0.05), the present experimental design allowed us to a priori planned comparisons to determine if there was an affect of age in the L7*Fmr1* and L7CGG90*Fmr1* genotypes.

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