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Fragile X-associated Tremor/Ataxia Syndrome (FXTAS) Motor Dysfunction Modeled in Mice

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Abstract

Fragile X-associated tremor/ataxia syndrome (FXTAS) is a late onset neurodegenerative disorder that affects some carriers of the Fragile X premutation (PM). In PM carriers there is a moderate expansion of a CGG trinucleotide sequence (55-200 repeats) in the fragile X gene (*FMR1*) leading to increased *FMR1* mRNA and small to moderate decreases in the Fragile X Mental Retardation Protein (FMRP) expression. The key symptoms of FXTAS include cerebellar gait ataxia, kinetic tremor, sensorimotor deficits, neuropsychiatric changes, and dementia. While the specific trigger(s) that cause PM carriers to progress to FXTAS pathogenesis remains elusive, the use of animal models has shed light on the underlying neurobiology of the altered pathways involved in disease development. In this review, we examine the current use of mouse models to study PM and FXTAS, focusing on recent advances in the field. Specifically we will discuss the construct, face and predictive validities of these PM mouse models, the insights into the underlying disease mechanisms and potential treatments.

Keywords

Fragile X Premutation; Fragile X-associated Tremor/Ataxia Syndrome (FXTAS); Fragile X mental retardation (*FMR1*) gene; mouse models; CGG trinucleotide repeat; neurodegenerative disorder

Introduction

The Fragile X gene (*FMR1*) is located on the X chromosome and codes for the Fragile X Mental Retardation Protein (FMRP), which is critical for proper neuronal development and synaptic plasticity (1, 2). The 5′ untranslated region (UTR) of the *FMR1* gene contains a sequence of DNA, CGG trinucleotide, which is typically repeated 5-44 times (3, 4). However, the length of the CGG repeat sequence can expand over generations in some families. Individuals in which the CGG sequence has expanded to 55-200 repeats are referred to Fragile X premutation (PM) carriers, and are at risk for developing a late-onset neurodegenerative disease called Fragile X-associated Tremor/Ataxia Syndrome (FXTAS)

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(5). This neurodegenerative disorder is characterized by a wide-range of symptoms of varying severity including kinetic tremor, cerebellar gait ataxia, brain atrophy, heightened immune response, neuropsychiatric features and cognitive impairments or dementia (5, 6). Furthermore, expansion of the CGG trinucleotide segment to over 200 repeats leads to epigenetic inactivation of the *FMR1* gene, no production FMRP, resulting in Fragile X syndrome (FXS) with cognitive impairment.

According the Center for Disease Control, the PM occurs fairly frequently in the general population affecting 1:151 females and 1:468 males in the United States (7). Interestingly, not all carriers develop FXTAS. FXTAS pathogenesis occurs in about 40% male carriers and only 11-18% of female carriers over the age of 50, which is estimated to affect 1:4000 and 1:7800 of the general population, respectively (8-11). The factors responsible for a PM carrier to progress to FXTAS are unknown, but it is hypothesized that certain PM carriers may be more susceptible to unknown external factors or stressors which, in turn, may result in the pathogenesis of FXTAS (12). Interestingly, compared to other neurodegenerative disorders, FXTAS is much less common than essential tremor or Parkinson's disease (1:100) but it does have a similar prevalence to that of other disorders such as spinocerebellar ataxias (1-4:100,00) and amyotrophic lateral sclerosis (ALS, 4:100,000) (13-15). While not the focus of this review, it is important to note that female PM carriers can develop a reproductive disorder called Fragile X-associated Primary Ovarian Insufficiency (FXPOI) (16, 17). To date, there is no cure or treatment for PM and FXTAS, which is why the development and validation of mouse models that could be used for drug development is important for understanding these complex neurological disorders.

For decades, the laboratory mouse (*Mus musculus*) has been utilized for studying human genetic disorders, ranging from simple Mendelian-inherited diseases such as FXS to complex polygenic disorders such as autism. While challenging, modeling human disease with mice has resulted in the discovery of important information about a variety of neurological disorders including FXS, Parkinson's and Huntington's diseases and FXTAS. In this context, longitudinal/prospective human genetic studies may eventually help clarify the contribution of the Fragile X premutation to the different neurological and psychiatric phenotypes, as well as to the time-course of the disease(s). However, such longitudinal studies in human are difficult to conduct and necessarily take many years to complete. In the absence of such human studies, rodent animal models can significantly advance our knowledge about these disorders and help with the generation of testable hypotheses concerning the mechanism(s) of pathogenesis of the PM and FXTAS. Verification of findings in mouse models can then accelerate development of new rationale treatments for these disorders.

Here, we consider the literature on both well-established and newer mouse models of PM and FXTAS. Specifically, we will compare the validities of the different PM mouse models as well as the insights they have given us into the altered molecular pathways underlying FXTAS pathogenesis. Additionally, we will focus on the use of these models to examine the motor system dysfunction in PM and FXTAS.

Modeling human PM and FXTAS in mice

For an animal model to be considered clinically relevant, it must meet several criteria to determine if it is a suitable and appropriate model which includes construct, face and predictive validities (18). In this section we will review the validities of different mouse models currently used to model PM and FXTAS.

Genetic-based models are typically created by either direct genetic manipulation (via insertion or deletion of a DNA segment) or through the use of mutagenic drugs to alter an organism's DNA. Unfortunately, mouse models rarely completely exhibit all of the aspects of human disease, and this is true in the case of PM and FXTAS. However, these mouse models have allowed researchers to characterize disease pathogenesis and progression, identify underlying neurobiological changes as well as develop and test potential therapeutics. The identification of the *FMR1* premutation as the underlying genetic cause of FXTAS and FXPOI gave not only a genetic explanation for these two new clinical entities (16, 19, 20), but also widened the clinical spectrum ready to be explored using mouse models.

Current Models

Dutch Mouse—The “Dutch mouse” model of PM was generated by the Willemsen laboratory at the Erasmus Medical Center in Rotterdam. In this model the endogenous mouse *Fmr1* 8CGG repeat was replaced with a human 98CGG repeat tract by homologous recombination in embryonic stem cells (21, 22). Importantly, the region flanking the repeat in the human *FMR1* was included, with only minimal changes to the endogenous murine *Fmr1* promoter (Figure 1A). This knock-in (KI) mouse model recapitulates the genetic abnormality associated with the PM and FXTAS (i.e., expanded CGG repeat tract within the 5′-UTR of *FMR1*), as well as much of the histopathology (e.g., ubiquitin staining intranuclear inclusions), molecular changes (i.e., elevated *Fmr1* mRNA) and neurobehavioral symptomatology (e.g., anxiety, visuomotor deficits) (23).

NIH Mouse—The “NIH mouse” model was developed using a *Fmr1* mouse transcript obtained from a bacterial artificial chromosome (BAC) clone and small CGG tracts generated *in vitro* and added up to obtain a final 120CGG tract (24). An advantage of this NIH KI premutation model is that it contains minimal differences from the WT murine *Fmr1* gene, promoter and the regions flanking the repeat (Figure 1A).

Inducible Mouse—Recently, a doxycycline (dox)-inducible (i.e., using a Tet-On system) mouse has been developed that allows experimenter control over the activation of a 90CGG trinucleotide repeat by exposing the mice to dox in their drinking water (Figure 1A) (25, 26). Bigenic mice are obtained by crossing TRE-90CGG-eGFP transgenic “target” mice with “driver” mice carrying the tetracycline reverse transactivator (i.e., rtTA) under control of various promotors, including the heterogenous nuclear ribonucleoprotein (hnRNP), prion protein (PrP) and CaMKII-α promoters (25, 26). The use of these dox-inducible mouse models allows for studies to determine when during development the disease process begins in FXTAS, as well as whether it is possible to halt progression or possibly reverse the disease process if expression of the CGG trinucleotide repeat is halted at the appropriate

developmental period (26). Additionally, the expression of this 90CGG transgene should not alter *FMR1* transcription or translation because it is not expressed within the context of the FMR1 gene. Trinucleotide repeat expansions have been implicated in the pathology of other neurological diseases including spinocerebellar ataxias (27). This inducible model will be an important tool for determining the direct contribution of CGG repeat expansion in FXTAS pathology, including the role of repeat-associated non-AUG translation (discussed later).

Construct validity

In order to accurately study the underlying biology, disease progression and therapeutic effectiveness of uniquely human disorders, animal models should reflect as closely as possible how the disease manifests in patients. This is referred to as construct validity for the model system (18). Along these lines, the pathophysiological "insult" of the model should be the same as that occurring in the human condition, and in turn should also lead to the characteristic molecular and cellular pathologies of that disorder. As PM and FXTAS result from an expansion of a CGG trinucleotide sequence upstream of the *FMR1* gene, various mouse models have been designed using different genetic manipulations to model these gene mutations. In terms of construct validity, there are advantages and disadvantages for each of the PM and FXTAS mouse models as considered in the sections below.

Molecular Pathology—An important aspect of construct validity is that the model should reasonably mimic the molecular and cellular pathology of the disease they were developed to model. In FXTAS, the molecular hallmarks are elevated *Fmr1* mRNA transcript levels, normal to slight reductions of FMRP expression and the presence of intranuclear inclusions in both neuronal and glial cells in the brain (28). Both the Dutch and NIH mouse models show these molecular features of FXTAS, and therefore appear to have reasonable construct validity (Figure 1B) (23, 29).

In carriers of the PM the length of the CGG sequence upstream of the *FMR1* gene can be unstable, and the trinucleotide repeat can expand substantially, or contract, upon maternal transmission (30). The Dutch mouse model shows only modest repeat instability that can occur from both maternal and paternal transmissions, with a rate of expansion and contraction between generations of around 10% (21, 31). On the other hand, the NIH mouse model with 120 CGG repeat segments has a much higher repeat instability than the Dutch mouse, with a few expansions to over 200 repeats in one generation (24), a finding more similar to that described in humans. A possible explanation for the lack of congruence observed in repeat instability between the Dutch and the NIH mouse models may reside in the different origins of the transgene, human versus mouse, respectively. The use of the human version of the repeat in a mouse model may have an additional effect on the regulatory machinery. At this time, the repeat instability associated with PM and FXTAS cannot be assessed using the inducible mouse model of FXTAS because the CGG repeat expansion is ectopically expressed outside of the context of the *FMR1* gene.

At the mRNA level, 2-3.5 fold elevations in *Fmr1* transcripts were reported in the Dutch mouse brain which is consistent with that found in postmortem FXTAS brain tissue (22, 32, 33). However, the mRNA increase did not result in large reductions of FMRP expression

levels in the Dutch KI mouse model (22, 34). The magnitude of reduction in FMRP was significantly correlated with the length of the CGG repeat expansion (34). In contrast, the NIH mouse model shows increased *Fmr1* transcript levels associated with CGG expansion size, and a strong negative correlation between CGG size and FMRP levels, i.e. the larger the CGG size the lower the FMRP levels. These findings indicate that disease severity and FXTAS-susceptibility may be strongly related to CGG repeat length.

Cellular pathology—The presence of intranuclear inclusions in neurons and glia that stain for ubiquitin is a key histopathological hallmark of FXTAS (28). Similar appearing ubiquitin-positive inclusions were observed in neurons of both the Dutch and NIH mouse models (Figure 1C) (22, 24, 35). Wenzel and colleagues (35) also identified the presence of these intranuclear inclusions in astrocytes of the neocortex as well as in Bergmann glia and astrocytes of the cerebellum in the Dutch CGG KI mouse model. Conversely, there are no reports of inclusions in glial cells in the NIH mouse model (24, 36). Rodent and human postmortem studies have also reported the presence of the inclusions in a variety of other tissue types including the heart, pancreas, gastrointestinal, adrenal gland, etc. (37), which may explain the wide variety of symptoms and complications associated with FXTAS. While brain atrophy, white matter disease and loss of cerebellar Purkinje cells have been observed in FXTAS patients, it remains unknown whether these intranuclear inclusions are cytotoxic or cytoprotective or simply mark the presence of a disease state but do not contribute directly to CNS injury (38).

To date, no Purkinje cell degeneration has been found in the Dutch mouse model (22). On the other hand, the NIH mouse model show evidence of Purkinje pathology, including abnormal calbindin staining, swollen axons and torpedoes and Purkinje cell drop-out similar that the seen in tissue from FXTAS patients (24). In FXTAS, the Purkinje cell loss observed in patients ranges from mild to severe, and was also accompanied by corresponding Bergmann gliosis (28).

Ubiquitin-positive intranuclear inclusions are also observed in the dox-inducible PrP-TRE-90CGG mouse model as early as 8 weeks after activation of the CGG repeat-containing transgene (Figure 1C) (26). This finding suggests that expression of the CGG expansion mRNA, outside the context of the *Fmr1* gene, is sufficient to produce ubiquitin-positive intranuclear inclusions similar to those found in FXTAS patients. The expression of an expanded CGG repeat alone (outside the context of the FMR1 gene) has been previously shown using Drosophila (Jin et al., 2003) and L7-promoter in mice (Hashem et al., 2009). In both cases, expression of the 90CGG RNA led to neurodegeneration and FXTAS-hallmark pathology (intranuclear inclusions). Another important feature of the human disorder modeled in the inducible model is the production of poly-glycine and poly-alanine peptides by repeat-associated non-AUG mechanism (i.e., RAN translation) (26), discussed later, which can impair the ubiquitin proteasome control system, leading to inclusion formation (39). Importantly, these recently discovered translation products are found associated with the intranuclear inclusions in neurons in postmortem human FXTAS brains, brains from the Dutch CGG KI mouse model, but not the NIH mouse model. While these RAN translation products are present in brain tissue from control brains, they only aggregate into inclusions in FXTAS patients or animal models (40).

Face Validity

In addition to having construct validity that produces the molecular and cellular pathologies of a disease, a complete model should also display the key phenotype changes relevant to the symptoms associated with that human disorder (18). With this theory in mind, an ideal FXTAS mouse model should exhibit signs of tremor and ataxia as well as cognitive deficits. However, modeling face validity for FXTAS is more challenging, because not all PM carriers develop FXTAS and those that do can present with varying degrees of symptom severity. The current PM mouse models display alterations in several phenotypes including motor system dysfunction (summarized in Table 1). However, it is unclear as to what extent the motor alterations described in the models are relevant for the human pathology.

Hyperactivity—The open field is a widely used laboratory test that measures general locomotor activity by quantifying horizontal and/or vertical movements in a novel test environment. Both the Dutch and the NIH mouse models show an increased total distance travelled compared to their respective wildtype control animals (36, 41). This result may reflect the hyperactivity, which is often reported in premutation individuals presenting with a variety of neurodevelopmental conditions, such as attention-deficit hyperactivity disorder (ADHD) (42-44). A study by Farzin and colleagues (43) shows that 93% of PM boys, recruited as probands, had symptoms of ADHD, a number that goes down to 38% in the premutation non-proband group, but still very significant. Also in this respect, many men with FXTAS have a history of attention problems in childhood that may have led to a diagnosis of ADHD (43).

Motor System Dysfunctions—Cerebellar gait ataxia is a major clinical criterion proposed for definite FXTAS (45). O'Keefe and colleagues (46) reported recently on the first attempt to quantify gait and mobility in PM individuals with and without FXTAS. Only PM carriers with FXTAS showed deficits in almost all gait parameters analyzed including stride velocity, gait cycle time and turn duration (46). Therefore, testing PM mouse models in behavioral tests that reflect the cerebellar function is of critical relevance. Performance in the Rotarod provides a measure of motor coordination and learning assessing cerebellum function. In an accelerated Rotarod paradigm, van Dam and colleagues (41) report an age-dependent (from 20 to 52 to 72 weeks of age) deterioration of motor performance by the expanded CGG KI Dutch model that is not observed in WT animals. Still, at each time point no differences were observed between genotypes in the latency to fall off the rod, indicating that the deficits were only modest (41). Also, no differences in Rotarod performance were observed between the NIH mice and WT animals (36) although these animals were tested at a fairly young age (13 weeks), and possibly later time points should be examined in the future. Footprint pattern is another test useful to assess gait ataxia in rodents by analyzing several relevant variables of the stride such as stance width and step variability (47). No differences were found between expanded Dutch and control mice in any of the stride parameters analyzed.

Although a complete behavioral battery in the inducible PrP-TRE-90CGG mice is not available yet, our preliminary evidence from a pilot study showed that ataxia could be modeled in the inducible mice when testing mice in the Rotarod and analyzing footprint

patterns (data not shown). Except for the promising evidence from the pilot study with inducible PrP-TRE-90CGG mice (data not shown), there is no convincing modeling of gait ataxia symptoms in the Dutch and NIH mouse models.

Skilled limb movement—Skilled motor performance tests can be more sensitive for subtle sensorimotor deficits using behavioral assays such as the ladder-rung or forelimb-reaching task (47). Performance in certain motor tests revealed motor deficits in Dutch CGG KI mouse model when tested between 2-6 months of age (37). Specifically, when tested in the ladder rung test that requires mice to traverse a horizontal ladder with narrow rungs, both male and female expanded mice show an increased number of foot slips as compared to WT animals. Although seen in younger mice, the effect was not age dependent and was also found in CGG KI mice older than 6 months of age (37). In a skilled forelimb-reaching task, female Dutch CGG KI mice are slow in learning to use their forelimb to reach and grasp a small food pellet, and do not reach the same level of performance as wildtype mice (48). These impairments in skilled walking and reaching abilities appear to resemble early motor symptoms that have been detected in men and women PM carriers (49). Subtle motor coordination deficits have also been described in female PM carriers without FXTAS, as measured by impaired finger tapping and slower reaction times as compared to controls (50). Male PM carriers aged >50 years have an increased risk for ataxia, tremor and decreased manual coordination (51). Continued research on motor impairments in skilled tasks in both mice and humans may be helpful to understand motor systems impacted by the PM, as well as to evaluate efficacy of therapeutic interventions.

Sensorimotor behavior—Evidence from human and mouse studies suggest that sensorimotor gating is impaired in PM carriers and it is sensitive to early dysfunction, before the appearance of more obvious and severe FXTAS symptoms. Acoustic startle response (ASR) measures the magnitude of the reflexive response (muscle contraction) in response to a loud auditory stimulus or startle tone (52). Alterations in the ASR were detected in both young and old expanded CGG NIH mice as compared to WT controls (53). The startle reflex can be inhibited by pairing the loud startle tone with a softer prepulse tone and is referred to as prepulse inhibition (PPI), which is a measurement of sensorimotor gating. Young (2 to 5 months old) NIH CGG KI mice showed a deficit in ASR with preserved PPI (53). Deficits in PPI were evident in aged mice (7 to 8 months old) which is consistent with the sensorimotor gating alterations in FXTAS patients also measured using the ASR and PPI (54).

Eye movements have been used to assess inhibitory control, a component of executive function, in PM carriers asymptomatic for FXTAS (55). Impairments were detected in the oculomotor domain of these individuals; specifically, PM carriers show increased reaction times in the anti-saccade task and increased inhibitory cost from early life, as compared to healthy controls. The optokinetic reflex (OKR) is a motor reflex driven by visual stimulation. The vestibulo-ocular reflex (VOR) is a reflex based on vestibular input evoked by head rotation to generate the visually-enhanced VOR (VVOR) (56). Both OKR and VOR reflexes work together to ensure clear vision. Hukema and colleagues (26) reported that in the dox-inducible mouse model activation of the 90CGG repeat transgene expression for 20 weeks impair performance in the optokinetic test. Specifically, these mice showed a lower

gain of OKR, VOR and VVOR than that of appropriate control mice. These reflexes rely on proper function of the vestibulo-cerebellum, a brain region that shows high concentration of the hallmark intranuclear inclusions in this mouse model (26).

Dysfunction of the vestibulo-cerebellum system in human and mouse premutation carriers is reflected in both behavioral and neuropathology studies. In this respect, neurological (motor) reflexes are sensitive to the function of the vestibular system (57). Since deviations in the achievement of motor milestones are early indicators of future neurological disease, assessing these milestones by monitoring neurological reflexes may constitute a tool for early assessment of PM effects/pathology and therapeutic interventions.

Insights from the PM Mouse Models

Disease Reversibility

Predictive Validity—Predictive validity means that the model will respond to drugs and treatments in a similar manner as patients with that particular disorder. Animal models are used in the development and testing of treatments, thus, having a model that shows predictive validity will bring power for drug efficacy in the human condition. In the case of the mouse models of PM and FXTAS discussed here, predictive value of these models is still lacking as pharmacological validity has not been fully assessed.

No targeted treatment exists to prevent or halt disease progression in FXTAS. However, treatments focusing on other aspects of PM and FXTAS haven proven to be beneficial in alleviating some symptoms in a small sub-group of patients (reviewed in Hagerman *et al*, 2008) (58). Psychiatric problems such as depression, anxiety and ADHD were reported in a subgroup of PM carriers often preceding the onset of FXTAS (6, 59). The use of antidepressants such as selective serotonin re-uptake inhibitors (SSRI; ex. sertraline, citalopram and escitalopram) is recommended to treat these psychological symptoms in FXTAS. PM animal models show heightened anxiety phenotype (face validity), which could be treated by administration of anxiolytics such as the ones used in clinics to check for predictive validity.

In FXTAS, difficulties in gait can be, in part, attributed to cerebellar ataxia and/or Parkinsonism (60). A treatment for cerebellar ataxia, such as amantadine or buspirone drugs, may prove helpful in FXTAS patients (8, 61). It would be interesting to assess the effectiveness of these drugs in the PM mouse models, for example, to rescue motor coordination deficits.

More recently, some alterations in the ASR and PPI were reported in FXTAS patients and in the NIH CGG KI mouse model, as described above (53, 54). The mechanism for altered PPI in FXTAS patients and CGG KI mice is not known. FXS patients and *Fmr1*-knockout (KO) mice show alterations in PPI that are reversible with mGluR 1/5 antagonists (62). Interestingly, mGluR-dependent synaptic weakening was shown in CGG KI mice (63), and thus it is tempting to speculate that the same mechanism could underlie both disorders. This hypothesis can be easily tested in the expanded CGG KI mice using mGluR antagonists while performing PPI of the acoustic startle, and will bring important mechanistic

knowledge. Nevertheless, in a controlled trial administration of memantine - an NMDA receptor uncompetitive antagonist - did not return benefic results for FXTAS patients (64). An explanation for these results could be the proxies used to assess recovery, such as intention tremor (using CATSYS) and executive function (using behavioral dyscontrol scale), when mGluR could be more related to sensorimotor gating.

Interestingly, inclusion formation that occurs in the cerebellum lobule X of dox-inducible mice after 8 weeks on dox can be reversed by removing dox from the water for a period of 12 weeks (26). However, longer exposure to dox for up to 18 weeks results in more intranuclear inclusions of larger size that could not be reversed by washout. These data indicate that in FXTAS early intervention might be necessary in order to prevent pathology.

RAN Translation as a Toxic Pathological Mechanism in FXTAS

More recently, our understanding of the altered molecular pathways and neurobiology of PM and FXTAS has been shifted based on the findings from animal model studies. In PM carriers, there is an increase in transcription of the *Fmr1* mRNA with the CGG expanded repeats. While this results in some reduction in FMRP expression levels, the CGG trinucleotide expansion mRNA was originally thought not to be translated. However, Todd *et al.* (40) identified that toxic polyglycine (FMRpolyG) and polyalanine (FMRpolyA) peptides were actually being produced from the CGG repeats via Repeat Associated Non-AUG initiated (RAN) translation. Toxic RAN translation products have been implicated in other nucleotide repeat expansion disorders including spinocerebellar ataxia type 8 (SCA8) and C9-orf72-associated amyotrophic lateral sclerosis (27, 65). The mechanism of RAN translation is not fully understood, however, evidence suggests that the scanning ribosome may stall along the CGG repeat expansion which, consequentially, leads to the use of an alternative non-AUG translation start site and a shift in the reading frame (40).

Immunodetection showed that the FMRpolyG actually accumulates in the ubiquitin-positive intranuclear inclusions in the brain associated with FXTAS. Moreover, this finding was confirmed in *Drosophila*, cell culture, mouse models and FXTAS patient brains (40). Interestingly, FMRpolyG ubiquitin inclusions were only present in the Dutch and Inducible mouse models, not the NIH mouse (26, 40). While the functional consequences of the FMRpolyG have yet to be determined, it is important to note that its presence in ubiquitin-positive inclusions from other non-CNS organs has also been identified, suggesting this toxic FMRpolyG may play a role in other symptoms associated with PM and FXTAS. For example, FMRpolyG-positive inclusions were found in ovarian tissue from both the Dutch mouse model and female PM patients with FXPOI (26). The use of current and new mouse models of FXTAS will be an important resource for designing and testing novel therapies for targeting this RNA toxic gain-of-function mechanism in FXTAS.

Developmental changes and early behavioral deficits in the Dutch CGG KI mouse

FXTAS was initially described as a late onset neurodegenerative syndrome based on a study of five elderly men carrying the Fragile X PM. These individuals developed progressive action tremor, executive function deficits and generalized brain atrophy, accompanied by elevated *FMR1* mRNA and slightly reduced levels of FMRP (5). Later research found that

younger male PM carriers without FXTAS, previously thought to be asymptomatic, showed deficits in executive cognitive function, short-term working memory and visuospatial processing (66-68), with young female PM carriers without FXTAS showing social anxiety deficits and impaired attention and postural control (69-71). These studies raised questions about when during development symptoms occur in carriers of the PM. In order to address these questions we carried out a series of studies in the Dutch CGG KI mice to determine how early during development *Fmr1* mRNA and reduced FMRP levels are evident, if embryonic development of the neocortex is affected, and whether neurobehavioral deficits in motor or cognitive function also occur earlier in development than previously thought. As described below, we found the hallmark molecular and cellular features associated with the PM, including elevated *Fmr1* mRNA, reduced FMRP and the presence of intranuclear inclusions occur early in development, and appear to presage the later appearance of neuropsychological problems and neurodegeneration in those carriers who go on to develop FXTAS.

Brain Fmr1 mRNA expression in the Dutch CGG KI mice is elevated as early as embryonic day E11.5 (E11.5)—Levels of *Fmr1* mRNA were determined by quantitative qPCR from 10 WT, 13 low CGG repeat mice (i.e., 82-102) and 9 high repeat mice (i.e., 142-183). As shown in Figure 2, *Fmr1* mRNA expression was significantly elevated as early as E11.5, and at all ages examined through postnatal day P18 compared to levels found in WT mice. The magnitude of increase was related to the length of the CGG repeat segment, with the largest increase (i.e., fold increase versus WT) found in the CGG KI mice with the highest repeat lengths (note – embryonic tissue from high CGG repeat animals was not available for analysis in this study). These results demonstrate that abnormal *Fmr1* mRNA expression occurs in the embryonic brain of CGG KI mice and suggest that pathological processes that become apparent later in development may actually begin prenatally and continue through the neonatal period.

Levels of FMRP in blood and brain are lowered in premutation carriers—FMRP levels are slightly decreased in postmortem brain tissue from premutation carriers and in the brains young postnatal CGG KI mice (5, 36, 72). As shown in Figure 3, using Western blot analyses we find similar reductions in FMRP levels in mice with high CGG repeat lengths at P0, P7 and P18. In addition, levels of FMRP in the low CGG repeat group were significantly decreased on E14.5, but not on E11.5. The reduction in FMRP levels has been attributed primarily to inefficient translation at the ribosome due to the presence of the CGG repeat tract. FMRP plays an important role in brain development and synaptic plasticity, and reduced levels early in development raise the possibility of early abnormalities in brain development as well as possible neurobehavioral effects early in development. In support of this possibility, Cunningham, *et al.* reported abnormal migration and differentiation of neuronal precursors during development of the embryonic cortical plate examined on E17 (73). Specifically, CGG KI mice had more Pax6+ cells in the ventricular zone and fewer TBr2+ neuronal precursor cells in the subventricular zone than WT mice, suggesting that delayed maturation and migration of the Pax6+ cells was occurring in CGG KI mice during cortical development.

Whalsten neurobehavioral test battery for development of sensory and motor functions—The effects of the early increase in *Fmr1* mRNA and decreased levels of FMRP on sensory and motor system development were examined using the Wahlsten developmental test battery (74). The test battery examines the development of basic sensory (e.g., eye opening, ear opening, auditory startle, visual cliff) and motor (e.g., righting reflex, vertical screen climbing, fore and hind-limb grasping) functions from approximately P8 through P18. A composite score is then calculated and used as a measure of development. Using this battery in a large cohort of WT and CGG KI mice (low, high and greater than 200) we find very little evidence of early neurodevelopmental delay or abnormalities (Figure 4). Only in the group of CGG KI mice with CGG repeat tracts greater than 200 in length was there any evidence for developmental delay, and this was a small but statistically significant delay in motor development at P18. However, by 12 weeks of age CGG KI mice are showing the presence of ubiquitin-positive intranuclear protein inclusions in the hippocampus and parietal cortex, as well as deficits in processing spatial relationships dependent on hippocampal function (75).

Considered together these findings show that pathology in PM carriers and those who go on to develop FXTAS may begin as early the gestational period, even though gross motor or sensory symptoms may not become apparent until after the neonatal period. This information is important because it points to the need to discover and begin treatments that may prevent or delay the onset of symptoms early in development. The results are also consistent with recent studies using a dox-inducible mouse model of the PM and FXTAS that allows for the activation or inactivation of expression of a CGG repeat segment at different ages under experimenter control. These studies also indicate that disease progression can be halted and possibly reversed provided that expression of the abnormal CGG repeat segment is stopped at an early age (i.e., during the first 3-4 months of age in the mouse model).

Summary

Our understanding of the underlying mechanisms, natural history and behavioral sequela of FXTAS have benefitted greatly from research using mouse models of the PM and FXTAS. Their contributions should continue to be an important role in the research as new models become available, including inducible model that allow the experimenter to control the onset and termination of expression of the CGG repeat segment expansion as described in this chapter. Mouse models have been particularly useful in the study of developmental aspects of the PM and FXTAS because they have allowed for research to be carried out from the embryo to the aging organism. The results from these studies have been particularly enlightening by showing that the disease processes, including elevation of *Fmr1* mRNA expression and intranuclear inclusions formation in brain begins much earlier than previously thought. More recent studies in dox-inducible mouse models have demonstrated that it is possible to halt and maybe even reverse the pathology seen in carriers of the PM and in FXTAS by reducing expression of the mutant CGG trinucleotide repeat segment at the appropriate time during development. These are exciting findings that provide encouragement in the development of rationale therapies to improve neurological function in patients with FXTAS. Important questions remain to be answered, including the role of the

recently discovered FMRpolyG peptide in disease, what molecular mechanisms lead to elevated FMR1 mRNA expression, and what determine whether or not a PM carrier will eventually go on to develop FXTAS with its associated tremor/ataxia, cognitive decline and brain atrophy. It is expected that the search for answers to the critical questions will be pursued with the help of mouse models.

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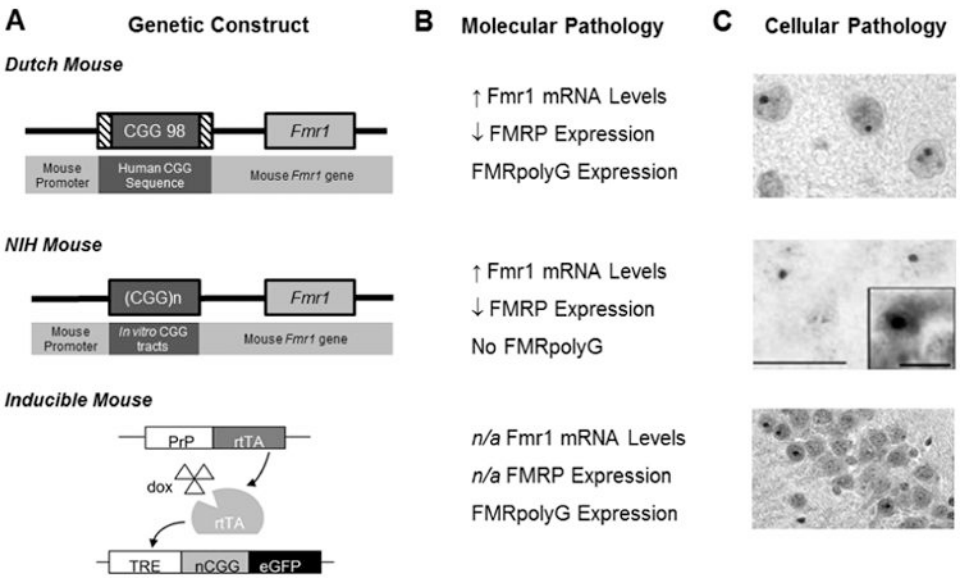

Figure 1.

Mouse models of the fragile X premutation. A) Schematic drawings representing the genetic constructs designed for each of the PM mouse models: Dutch (21), NIH CGG KI (24) and the Inducible PrP-CGG90 mouse model (26). Both the Dutch mouse model has an intact mouse promoter followed by a human genetic sequences flanking the inserted CGG repeat expansion upstream of the mouse *Fmr1* gene. The NIH CGG KI mouse has an *in vitro*-generated CGG repeat tract inserted to replace mouse CGG8 also keeping the mouse *Fmr1* gene and promotor intact. The Inducible mouse transgenically expresses the CGG repeat expansion outside the context of the *Fmr1* gene in the mice which is activated by doxycycline (dox). **B**) Summary of the molecular pathological changes reported in each mouse model. The Dutch and NIH CGG KI mice show similar mRNA and FMRP expression changes, however only the Dutch and Inducible mouse models also produce the toxic FMRpolyG peptide. **C**) Representative images showing immunodetection for ubiquitin-positive intranuclear inclusions, the hallmark histopathology of PM and FXTAS, in brain tissue from each of the mouse models. Images were reprinted and/or modified with copyright permissions (21, 22, 24, 26)

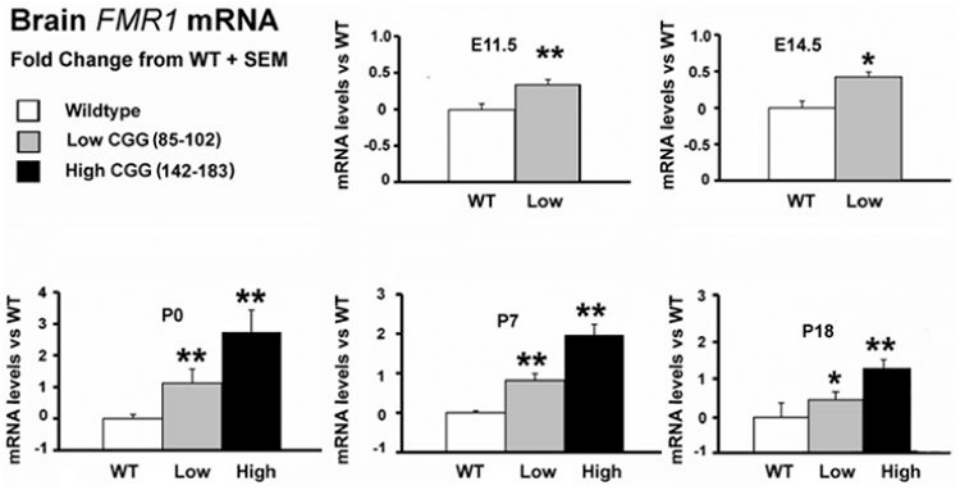

Figure 2.

Brain FMR1 mRNA levels in the Dutch CGG KI mice with Low (85-102) and High CGG (142-183) trinucleotide repeat lengths at embryonic ages 11.5 and 14.5 days and postnatal days P0, P7 and P18 compared to wildtype mice (WT). The ordinate represents mean fold-changes from WT (+ SE). *p<0.05, **p<0.01)

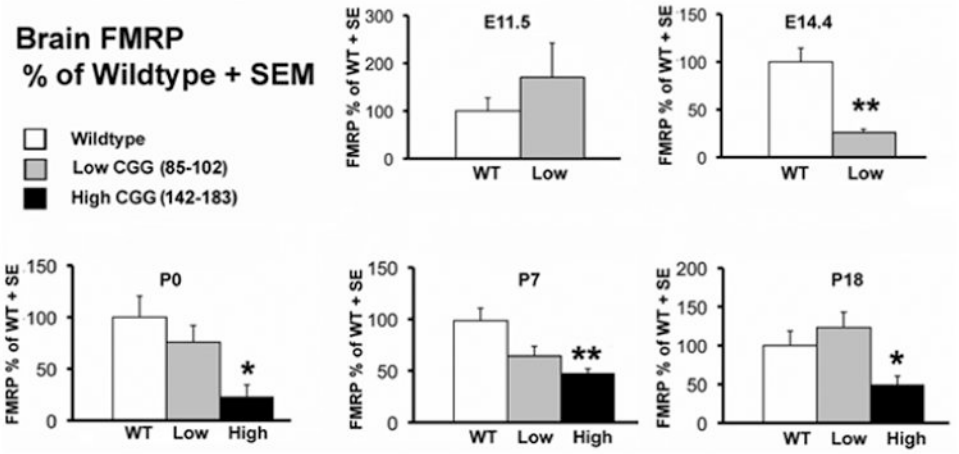

Figure 3.

Brain FMRP levels in the Dutch CGG KI mice with Low (85-102) and High (142-183) CGG trinucleotide repeat lengths at embryonic ages 11.5 and 14.5 days and postnatal days P0, P7 and P18 compared to wildtype mice (WT). The ordinate represents percent change (% +SE) from wildtype (WT). *p<0.05, **p<0.01)

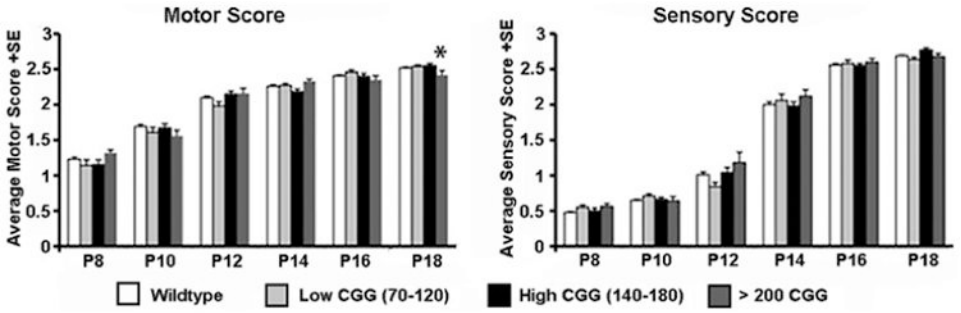

Figure 4.

Wahlsten neurodevelopmental neonatal test battery for motor and sensory functions from postnatal day 8 (P8) through postnatal day 18 (P18) on Dutch CGG KI mice. Average scores for motor (e.g., forelimb and hind limb grasp, righting reflex, etc) and sensory (eye opening, auditory startle, etc) functions were calculated as described previously (Wahlsten, 1974). *P<0.05 versus WT.

Table 1

Motor Dysfunction in Mouse Models of FXTAS. Unless otherwise stated, all behavioral tests were performed in male animals. WT, wild type; KI, knock in; ASR, acoustic startle response; PPI, pre-pulse inhibition; n/a, not assessed.

Motor test	The Dutch: 98CGG	The NIH: 120CGG	Inducible transgenic PrP-90CGG
Open field	- *20 weeks old: trend to hyperactivity exhibited by expanded CGG mice as compared to WT animals* - 52 and 72 weeks old: no differences observed between genotypes in exploratory activity	- 7 to 9 weeks old: CGG KI animals show increased total distance travelled as compared to WT animals	*n/a*
Home cage activity	- 72 weeks old: no differences observed between genotypes in the 23 hour circadian activity	*n/a*	*n/a*
Rotarod -Accelerated	- Significant decrease in performance with age (from 20 to 52 to 72 weeks of age) eminent in 98CGG, but not WT mice	- 11 to 13 weeks old: no differences were found between CGG KI and WT animals in their motor performance	*n/a*
Wire suspension test	- 20, 52 and 72 weeks old animals: no differences were observed between genotypes in the latency to let go the wire and fall	*n/a*	*n/a*
Stationary beam task	- 72 weeks old: expanded CGG mice show a reduced number of segments crossed as compared to WT	*n/a*	*n/a*
Gait test	- 72 weeks old: no differences between genotypes in different stride parameters analyzed	*n/a*	*n/a*
Ladder rung task	Males and females were tested - Young (<6 months) and old (>7 months) animals: number of foot slips in premutation CGG mice was significantly higher than that of WT animals.	*n/a*	*n/a*
Skilled forelimb reaching task	Test performed in female animals - CGG KI (low and high) animals showed a lower % of successful reaches than WT animals (6 months of age)	*n/a*	*n/a*

Motor test	The Dutch: 98CGG	The NIH: 120CGG	Inducible transgenic PrP-90CGG
Acoustic startle response (Muscle contraction to a loud auditory stimulus)	*n/a*	- 2 to 5 months old: reduced startle amplitude in young CGG KI mice as compared to WT animals - 7 to 8 months old: increased startle response in old CGG KI mice as compared to controls	*n/a*
Pre-pulse inhibition	*n/a*	- 2 to 5 months old: no differences in % PPI were found between genotypes in young animals - 7 to 8 months old: decrease in % PPI in old CGG KI as compared to WT controls	*n/a*
Vestibulo-ocular reflex	*n/a*	*n/a*	- 20 weeks of doxycycline: gain of VOR and VVOR was lower in 90CGG than in 11CGG controls
Optokinetic reflex	*n/a*	*n/a*	- 8 weeks of doxycycline: no differences observed between genotypes - 20 weeks of doxycycline: gain of OKR was lower in 90CGG than in 11CGG controls
References	(37, 41, 48)	(36, 53)	(26)