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RNA-mediated neurodegeneration in fragile X-associated tremor/ataxia syndrome

Yujing Li and Peng Jin

Department of Human Genetics, Emory University School of Medicine, Atlanta, Georgia, USA

Abstract

Carriers of fragile X syndrome (FXS) have FMR1 alleles, called premutations, with a number of 5'-untranslated CGG repeats somewhere between patients, who have over 200 repeats, and normal individuals, with fewer than 60 repeats. Fragile X-associated tremor/ataxia syndrome (FXTAS), a late-onset neurodegenerative disorder, has been recognized in older male fragile X premutation carriers, and FXTAS is uncoupled from the neurodevelopmental disorder, FXS. Several lines of evidence have led to the proposal of an RNA (fragile X premutation rCGG repeat)-mediated gain-of-function toxicity model for FXTAS, in which rCGG repeat-binding proteins (RBPs) could become functionally limited by their sequestration to lengthy rCGG repeats. In this review, we will discuss the recent progress towards understanding the molecular basis of RNA-mediated neurodegeneration in FXTAS.

Introduction

Fragile X syndrome (FXS) usually results from expansion of the CGG trinucleotide repeat in the 5' untranslated region (5' UTR) of the fragile X mental retardation 1 (FMR1) gene (Warren, 2001). Normal individuals generally possess between 5 and 54 repeats, but fully affected individuals have more than 200 CGG repeats on what are referred to as full mutation alleles (Sherman, 2002); premutation alleles (55–200 CGG repeats) of the FMR1 gene, on the other hand, are known to contribute to the fragile X phenotype via genetic instability and could expand into the full mutation during germline transmission (Hagerman and Hagerman, 2002) (Figure 1). Fragile X-associated tremor/ataxia syndrome (FXTAS), a late-onset neurodegenerative disorder, affects many male premutation carriers in or beyond their fifth decade of life (Hagerman and Hagerman, 2004). Given the high prevalence of fragile X premutation carriers among the general population (~1 in every 800 males and 250 females) and the high risk of developing FXTAS among the male carriers, the molecular basis of FXTAS has been a subject of intense investigation for the past several years.

Clinical features of fragile X-associated tremor/ataxia syndrome

Fragile X-associated tremor/ataxia syndrome (FXTAS) is seen in older males of FXS families and is uncoupled from the neurodevelopmental disorder, FXS. Although both disorders involve repeat expansions in the FMR1 gene, the clinical presentation and

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*Corresponding author: Peng Jin, Ph.D., Department of Human Genetics, Emory University School of Medicine, 615 Michael Street, Suite 301, Atlanta, Georgia 30322, USA, TEL: (404) 727-3729, FAX: (404) 727-5408, peng.jin@emory.edu.

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molecular mechanisms behind 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 (Grigsby et al., 2006). Patients may also present with common psychiatric symptoms, such as increased anxiety, mood liability, and depression (Bacalman et al., 2006; Hessler et al., 2005). Patients also complain of fluctuating muscle weakness and numbness and/or pain in the lower extremities, suggesting the disease may not be purely neurological (Jacquemont et al., 2004). Magnetic resonance imaging (MRI) of adult male patients affected with FXTAS revealed mild to moderate global brain atrophy, most common in the frontal and parietal regions, as well as the pons and the cerebellum (Jacquemont et al., 2004). The most significant radiological findings were increased T2 intensities of the middle cerebellar peduncle (MCP) and adjacent cerebellar white matter, not seen in controls (Brunberg et al., 2002). This finding serves as a major diagnostic criterion for FXTAS.

Nearly all case studies on the brains of symptomatic premutation carriers at autopsy demonstrated degeneration of the cerebellum, including Purkinje neuronal cell loss, Bergman gliosis, spongiosis of the deep cerebellar white matter, and swollen axons (Greco et al., 2002; Greco et al., 2006). The major neuropathological hallmark and postmortem criterion for definitive FXTAS is eosinophilic, ubiquitin-positive intranuclear inclusions located in broad distribution throughout the brain in neurons, astrocytes, and in the spinal column (Greco et al., 2006). The inclusions are both tau and α -synuclein negative, which indicates that FXTAS is not a tauopathy or synucleinopathy. The FXTAS inclusions share the ubiquitin-positive hallmark of several other inclusion disorders, such as the polyglutamine disorders, although FXTAS inclusions do not stain with antibodies that recognize polyglutamine, pointing to a defect in the proteasomal degradation pathway (Greco et al., 2002; Hagerman and Hagerman, 2004; Tassone et al., 2004). It is important to note that, unlike the polyglutamine disorders, there is no known structurally abnormal protein associated with FXTAS.

One logical explanation for the variability in the FXTAS clinical phenotype would be variability in the size of the premutation alleles of individual patients. Interestingly, these patients are carriers of a premutation-size CGG repeat (55–200 triplets) in the 5' UTR of the FMR1 gene. The repeat is expressed in the mature FMR1 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 FXS probands, revealed that over a third of carriers aged 50 years and older showed symptoms of FXTAS and that the penetrance of this disorder exceeds 50% for men over 70 years of age (Jacquemont et al., 2004). The prevalence of premutation alleles is approximately 1/800 for males and 1/250 for females in the general population; however, estimates are that 1 in 3000 men over the age of 50 in the general population will show symptoms of FXTAS (Dombrowski et al., 2002). These estimates do not take size bias, which considers the correlation of the age of onset of symptoms to the size of the repeat, into account. Recent studies have correlated age of onset of clinical FXTAS symptoms with the length of expanded repeats, with larger CGG repeats representing an increased risk for the development of FXTAS (Jacquemont et al., 2006; Leehey et al., 2008). The degree of brain atrophy and severity of the tremor and ataxia are associated with the CGG repeat length (Leehey et al., 2008). Furthermore, some female carriers also develop clinical features of FXTAS, but at a much lower frequency than males, which is believed to be the result of partial protection offered by random X-inactivation of the premutation allele (Hagerman et al., 2004; Jacquemont et al., 2004; Zuhlke et al., 2004).

Molecular signature of fragile X premutation allele

At the molecular level, the premutation exhibits differences that distinguish this allelic class from both normal and full mutation alleles. Besides the obvious difference in the CGG repeat length itself, there are a number of other recognized distinctions.

In cells from premutation carriers with a wide range of repeat lengths, the level of FMR1 mRNA was elevated some 2–8 times normal levels, while the stability of FMR1 mRNA was not changed. Indeed, even in the high-end normal range (~55 repeats), the FMR1 message level was near double that found in normal alleles (~30 repeats) (Kenneson et al., 2001; Tassone et al., 2000). In addition, some hypomethylated full mutation alleles produce substantial amounts of FMR1 message (~4.5-fold of normal) (Tassone et al., 2000) (Figure 1). Although the precise mechanism for this overexpression remains a mystery, it is likely that the increasing length of the CGG repeat near the FMR1 promoter proportionally opens the chromatin, allowing more ready access to transcription factors.

Despite this elevation in FMR1 levels, FMR1 protein is modestly reduced in the premutation range (~80% of normal), and may be almost entirely absent in cells expressing FMR1 message with very long repeats (>300 repeats) (Feng et al., 1995; Kenneson et al., 2001) (Figure 1). This paradoxical reduction in translation despite elevated mRNA levels can be explained by the observation that long CGG repeats in the FMR1 mRNA impede 40S ribosomal subunit migration from the 5' cap to the initiating codon. Overexpression of FMR1 mRNA is unlikely to be a response to the reduced FMRP levels, as I304N mutant cells with 30 CGG repeats, which produce nonfunctional protein, show no elevated FMR1 mRNA (Feng et al., 1997; Kenneson et al., 2001).

RNA-mediated neurodegeneration in FXTAS

An RNA gain-of-function mechanism has been suggested for FXTAS, based on the observation of increased levels of CGG-containing FMR1 mRNA, along with either no detectable change in FMRP or slightly reduced FMRP levels, in premutation carriers (Greco et al., 2002; Jacquemont et al., 2004; Jin et al., 2003; Tassone et al., 2000; Willemsen et al., 2003) (Figure 2). Because FXS results from the loss of function of the FMR1 gene product, the absence of FXS in FXTAS patients, along with the lack of FXTAS symptoms in older individuals with FXS, also point to a role for the expanded ribo-CGG (rCGG) repeat in FXTAS pathology. This type of RNA gain-of-function mechanism has been suggested as a mechanism for triplet repeat-related ataxias, such as SCA8, SCA10, and SCA12, and for myotonic dystrophy (DM) (Ranum and Cooper, 2006). The untranslated repeat expansion in DM has yielded major insight into the underlying molecular mechanisms of FXTAS. DM1 is caused by a CTG repeat expansion that is in a region of transcribed RNA, but is not translated into protein, the 3' UTR of the *DMPK* gene. The mutant transcripts sequester MBNL and other proteins, which form ribonuclear foci or inclusions. Indeed, using in situ hybridization, Tassone et al. detected the presence of expanded FMR1 RNA transcripts in the FXTAS inclusions of a 70-year-old male who died with FXTAS (Tassone, 2004).

Beyond the observation of increased levels of CGG-containing FMR1 mRNA in fragile X premutation carriers, several lines of evidence lend further support to an RNA-mediated gain-of-function toxicity model for FXTAS. First, in “knock-in” mouse models, in which the endogenous CGG repeats (5 CGG repeats in the wild-type mouse *Fmr1* gene) were replaced with a ~100 or longer CGG repeat fragment, intranuclear inclusions were found throughout the brain, with the exception of cerebellar Purkinje cells (Entezam et al., 2007; Willemsen et al., 2003). Increases in both the number and size of the inclusions were seen over the life course, which correlates with the progressive character of the phenotype observed in humans (Greco et al., 2006). Second, neuropathological studies in humans have revealed a highly

significant association between length of the CGG tract and frequency of intranuclear inclusions in both neurons and astrocytes, indicating that the CGG repeat length is a powerful predictor of neurological involvement both clinically (age of death), as well as neuropathologically (number of inclusions) (Greco et al., 2006). Third, intranuclear inclusions can be formed in both primary neural progenitor cells and established neural cell lines, as was revealed using a reporter construct with an FMR1 5' UTR harboring expanded (premutation) CGG repeats (Arocena et al., 2005; Hoem et al., 2011). Fourth, we have described a model of FXTAS using *Drosophila* expressing the FMR1 untranslated-CGG repeats 5' to the EGFP coding sequence; we showed that premutation-length riboCGG (rCGG) repeats are both toxic and sufficient to cause neurodegeneration (Jin et al., 2003). Finally, 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. rCGG was found to be necessary and sufficient to cause pathology reminiscent of human FXTAS. The models exhibit the presence of intranuclear inclusions in Purkinje neurons, Purkinje neuron cell death, and behavioral deficits (Hashem et al., 2009). These results demonstrate that rCGG expressed in Purkinje neurons outside the context of Fmr1 mRNA can result in neuronal pathology and that expanded CGG repeats in RNA are the likely culprit behind the neurodegeneration in FXTAS.

rCGG repeat-binding proteins in FXTAS

Because rCGG repeats are sufficient to cause neuronal cell death, it has been suggested that over-produced fragile X premutation rCGG repeats may sequester the rCGG-binding proteins from their normal cellular functions, thereby contributing to the pathogenesis of FXTAS. During the past several years, research has uncovered several such RNA-binding proteins. In our own work, we found that two RNA-binding proteins, Pur α and hnRNP A2/B1, bind rCGG repeats specifically in both mammalian and *Drosophila* brains (Jin et al., 2007; Sofola et al., 2007). Both Pur α and hnRNP A2/B1 are found to be present in the inclusions of FXTAS brain tissues. Furthermore, overexpression of either Pur α or hnRNP A2/B1 can alleviate neurodegeneration in the fly model of FXTAS (Jin et al., 2007; Sofola et al., 2007). In particular, Pur α knock-out mice appear normal at birth, but develop severe tremor and spontaneous seizures at two weeks of age, suggesting that the depletion of Pur α alone could lead to ataxia (Hokkanen et al., 2012; Khalili et al., 2003). Detailed morphological analyses of Pur α knock-out mice suggest a role for Pur α in the regulation of the expression and cellular distribution of dendritic and axonal proteins (Hokkanen et al., 2012) (Qurashi et al., 2011). More recently, to investigate the role of Pur α in rCGG-mediated neurodegeneration further, we took a proteomic approach to identify the proteins that interact with Pur α . We found over 100 proteins, including several known interactors, such as Fmrp, that interact with Pur α in vitro (Qurashi et al., 2011). To evaluate the physiological role(s) of Pur α -interacting proteins in rCGG-mediated neuronal toxicity, we further tested their genetic interactions with rCGG repeats using our FXTAS fly model and identified several interactors of Pur α that could genetically modulate the toxicity caused by rCGG repeats. Among these, Rm62, the *Drosophila* ortholog of the p68 RNA helicase, was of particular interest. Rm62, physically interacting with Pur α , could modulate rCGG-mediated neurodegeneration, and biochemically, fragile X rCGG repeats could decrease the expression of Rm62 posttranscriptionally, leading to the accumulation of *Hsp70* transcript, a previously identified target of Rm62, in the nucleus. Further microarray analyses revealed the nuclear accumulation of additional mRNAs involved in stress and immune responses in fragile X premutation flies. These findings suggest an unexpected nuclear accumulation of specific mRNAs caused by fragile X premutation rCGG repeats and point to likely deficits in the nuclear export of specific mRNA as a possible cause of the compromised stress response in neurons expressing rCGG repeats (Qurashi et al., 2011).

Aside from Pur α , hnRNP A2/B1 is the other RNA-binding protein that interacts directly with fragile X rCGG repeats (Jin et al., 2007; Sofola et al., 2007). From a genetic screen using a collection of candidate RNA-binding proteins, CUGBP1, an RNA-binding protein discovered for its ability to bind CUG repeats and implicated in myotonic dystrophy type 1 (DM1), was identified to modulate the neuronal toxicity caused by rCGG repeats (Timchenko et al., 1996). Interestingly, CUGBP1 protein was found to be capable of interacting with the CGG repeats via hnRNPA2/B1, and this interaction between hnRNP A2/B1 and rCGG repeats could interfere with the biological function(s) of hnRNP A2/B1 and hnRNPA2/B1-interacting protein, CUGBP1. Given the role of both hnRNP A2/B1 and CUGBP1 in RNA splicing, identifying common targets of these two proteins in neurons is important, because such common targets could contribute to FXTAS pathogenesis. Besides its role in RNA splicing, hnRNP A2/B1 is also known to serve as a trans-acting factor required for dendritic delivery in neurons. More recently, Tiedge and colleagues showed that binding to hnRNP A2/B1 and ensuing dendritic delivery could be effectively competed for by rCGG repeats, which suggests that the cellular dysregulation seen in the presence of rCGG repeats may result from molecular competition in neuronal RNA transport pathways (Muslimov et al., 2011). Furthermore, our recent data also suggest that hnRNP A2/B1 could interact with heterochromatin protein 1 (HP1) to modulate the activation of specific retrotransposons, which could contribute to the neurodegeneration caused by rCGG repeats, as well (Tan et al., 2012). These findings together suggest that the depletion of hnRNP A2/B1 protein may alter multiple biological pathways that would lead to neuronal cell death.

In addition, another RNA-binding protein, Sam68, was found to be involved in FXTAS pathogenesis (Sellier et al., 2010). mRNAs containing expanded CGG repeats were discovered to form large and dynamic intranuclear RNA aggregates that recruit several RNA-binding proteins sequentially: first Sam68, then hnRNP-G and MBNL1. The splicing of several genes, including Bcl-x, SMN2, and ATP11B, were found to be altered similarly by rCGG repeats and loss of Sam68 (Sellier et al., 2010). It was suggested that Sam68 could be sequestered by expanded rCGG repeats, and thereby lose its splicing-regulatory function, which would contribute to FXTAS pathogenesis; however, it is unclear whether Sam68 can directly bind to rCGG repeats.

Development of therapeutic intervention for FXTAS

Currently, there is no effective treatment available for FXTAS. In recent years, *Drosophila* has emerged as a premiere model system for the study of human neurodegenerative diseases, due to the fact that flies and humans share many structurally and functionally related gene families (Bonini and Fortini, 2003; Hirth, 2010; Lessing and Bonini, 2009; Todd and Paulson, 2010; Zoghbi and Botas, 2002). The development of fly disease models allows us to address specific hypotheses concerning disease progression, test candidate modifier genes or therapeutic drug compounds, and potentially identify novel small molecules via unbiased chemical screens. Recently, we used our own *Drosophila* model to conduct an unbiased chemical screen and identified the small molecules that can ameliorate the toxic effects of fragile X premutation rCGG repeats. Specifically, we found that inhibition of PLA2 activity could suppress the neuronal toxicity caused by fragile X premutation rCGG repeats (Qurashi et al., 2012). Indeed, altered PLA2 activity has been linked to several neurodegenerative diseases and brain trauma (Balsinde et al., 1999; Farooqui and Horrocks, 2006; Farooqui et al., 2006; Sun et al., 2004). Further testing of PLA2 inhibitors using the existing FXTAS mouse models would be in order at this point.

Summary

Recent work provides strong support for an RNA gain-of-function mechanism to explain FXTAS neuropathology. Many RNA-binding proteins are known to be involved in FXTAS pathogenesis. There is emerging evidence that multiple biological pathways, including RNA splicing, nuclear export, and mRNA transport, could be altered by fragile X premutation rCGG repeats. These findings point to possible pathways to target in the future development of therapeutic interventions for FXTAS.

Very recently a large hexanucleotide repeat expansion (GGGGCC) within C9ORF72 on chromosome 9p21 accounts for approximately 40% of cases of familial amyotrophic lateral sclerosis (ALS) and 30% of cases of familial frontotemporal dementia (FTD) (DeJesus-Hernandez et al., 2011; Orr, 2011; Renton et al., 2011). It was hypothesized that this expanded hexanucleotide repeat RNA could be pathogenic. Intriguingly hnRNP A2/B1, the rCGG repeat-binding protein, was predicted to also bind to this hexanucleotide repeat (DeJesus-Hernandez et al., 2011). Whether hnRNP A2/B1 is involved in ALS/FTD would require further investigation. What we learned from FXTAS might also provide the insight into ALS/FTD pathogenesis as well.

Highlights

We review the most recent progress towards understanding the molecular basis of FXTAS.

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Figure 1. Genomic structure of the fragile X mental retardation 1 (FMR1) gene

The 5' untranslated region (5'-UTR) of the fragile X mental retardation 1 (FMR1) gene is highly polymorphic. While normal individuals generally possess between 5 and 54 repeats, fully affected individuals have more than 200 CGG repeats on what are referred to as full mutation alleles. Premutation alleles (55–200 CGG repeats) of the FMR1 gene are known to contribute to the fragile X phenotype via genetic instability and could expand into the full mutation during germline transmission.

Figure 2. Genotype-phenotype correlation at the FMR1 locus

The expression of the FMR1 gene, both mRNA and protein, is indicated. The length of the yellow stretch represents the size of CGG repeats. In the full mutation, the hypermethylation of CGG repeats leads to transcriptional silencing of the FMR1 gene.