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FMR1 premutation and full mutation molecular mechanisms related to autism

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Abstract Fragile X syndrome (FXS) is caused by an expanded CGG repeat (>200 repeats) in the 5′ un-translated portion of the fragile X mental retardation 1 gene (FMR1) leading to a deficiency or absence of the FMR1 protein (FMRP). FMRP is an RNA-binding protein that regulates the translation of a number of other genes that are important for synaptic development and plasticity. Furthermore, many of these genes, when mutated, have been linked to autism in the general population, which may explain the high comorbidity that exists between FXS and autism spectrum disorders (ASD). Additionally, premutation repeat expansions (55 to 200 CGG repeats) may also give rise to ASD through a different molecular mechanism that involves a direct toxic effect of FMR1 mRNA. It is believed that RNA toxicity underlies much of the premutation-related involvement, including developmental concerns like autism, as well as neurodegenerative issues with aging such as the fragile X-associated tremor ataxia syndrome (FXTAS). RNA toxicity can also lead to mitochondrial dysfunction, which is common in older premutation carriers both with and without FXTAS. Many of the problems with cellular dysregulation in both premutation and full mutation neurons also parallel the cellular abnormalities that have been documented in idiopathic autism. Research regarding dysregulation of neurotransmitter systems caused by the lack of FMRP in FXS, including metabotropic glutamate receptor 1/5 (mGluR1/5) pathway and GABA pathways, has led to new targeted treatments for FXS. Preliminary evidence suggests that these new targeted treatments will also be beneficial in non-fragile X forms of autism.

Keywords Fragile X · Autism · ASD · Premutation · mGluR5 antagonist · Molecular background of fragile X · Molecular background of autism · Targeted treatments · MicroRNA · miRNA · Mitochondrial abnormalities

Introduction

The fragile X mental retardation 1 (*FMR1*) gene gives rise to a family of disorders when its non-coding CGG-repeat element is expanded to either the premutation range (55–200 CGG repeats) or the full mutation range (>200 CGG repeats). Although most individuals with the premutation have normal intellectual abilities, some children with the premutation have developmental problems including attention deficit hyperactivity disorder (ADHD), shyness, social anxiety, and autism spectrum disorders (ASD; Farzin et al. 2006; Aziz et al. 2003; Clifford et al. 2007), although the incidence of each of these problems is awaiting further studies of unbiased populations of carriers. The premutation is also associated with adult-onset disorders, including the fragile X-associated tremor/ataxia syndrome (FXTAS) in both males and females (males 40%; females 16%) over age 50 and fragile X-associated primary ovarian insuffi-

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ciency (FXPOI) in approximately 20% of female carriers under 40 years. The premutation is relatively common in the general population, present in approximately 1 in 130 to 250 women and 1 in 250 to 810 males (Fernandez-Carvajal et al. 2009; Hagerman 2008; Song et al. 2003; Dombrowski et al. 2002). The full mutation is less common at approximately 1 per 2,500 to 1 per 4,000 (Fernandez-Carvajal et al. 2009; Hagerman 2008; Coffee et al. 2009). Those with a full mutation, which gives rise to fragile X syndrome (FXS), are more likely to develop ASD than those with the premutation and approximately 30% of boys with FXS have autism and an additional 30% have pervasive developmental disorder—not otherwise specified (PDD-NOS) (Harris et al. 2008; Hatton et al. 2006; Hernandez et al. 2009).

There are two different molecular mechanisms of involvement leading to pathology in *FMR1* mutations. The full mutation leads to methylation of the gene, causing a lack of transcription and translation and resulting in an absence or deficiency of *FMR1* protein (FMRP). FMRP is an RNA-binding and transport protein that also regulates the translation of many messages that are important for synaptic plasticity, neuronal migration, and adult neurogenesis (Bassell and Warren 2008; Zalfa et al. 2007; Darnell et al. 2005, 2010; Luo et al. 2010; Miyashiro et al. 2003). In stark contrast, the premutation allele produces elevated levels of *FMR1* mRNA (Tassone et al. 2000a; Kenneson et al. 2001; Peprah et al. 2010), leading to an RNA toxic gain of function that is associated with disruption of the nuclear lamin A/C architecture, a significant stress response in both neuronal and non-neural cells, and dysregulation of a variety of proteins (Iwahashi et al. 2006; Sellier et al. 2010a, b; Garcia-Arocena and Hagerman 2010). In addition, recent reports have documented deficits of FMRP in some brain regions of premutation mice (Qin et al. 2011) and also in the blood of some carriers particularly in the upper premutation range (Peprah et al. 2010). Therefore, some of the premutation involvement may also be due to lowered FMRP levels.

Autism is a behaviorally defined disorder (DSM IV TR criteria) that arises through one or more of a large number of genetic and/or environmental factors. Mutations in numerous recognized genes are known to be highly associated with autism, operating through mechanisms that alter/disrupt critical functions, including synaptic plasticity, the balance of inhibitory and excitatory pathways, regulation of mammalian target of rapamycin (mTOR) pathways, mitochondrial function, immune function, and neuronal migration (Nishimura et al. 2007; Belmonte and Bourgeron 2006; de Vries 2010; Wegiel et al. 2010; Ashwood et al. 2009; Giulivi et al. 2010; Betancur et al. 2009; Rubenstein and Merzenich 2003; Gogolla et al. 2009; Gatto and Broadie 2010). Furthermore, premutation and full mutation alleles have overlapping involvement for many of these molecular mechanisms that can lead to autism or ASD (Gatto and Broadie 2010; Hagerman et al. 2010; D'Hulst et al. 2009; D'Hulst and Kooy 2007; Gibson et al. 2008). The mechanistic linkage between autism and FXS underscores the seminal role of FMRP as an mRNA transporter and as a regulator of translation predominantly through inhibition of the many messages that are critical for synaptic maturation and synaptic plasticity (De Rubeis and Bagni 2010; Napoli et al. 2008; Muddashetty et al. 2007). In this regard, the lack of FMRP leads to dramatic upregulation of protein production in the CNS (Qin et al. 2005) and upregulation of the metabotropic glutamate receptor 5 (mGluR5) pathway (Huber et al. 2002; Krueger and Bear 2011) and down-regulation of the $GABA_A$ pathways (D'Hulst et al. 2009; D'Hulst and Kooy 2007). This imbalance between the glutamate and GABA systems can also be seen in autism (Rubenstein and Merzenich 2003; Belmonte et al. 2004) in the context of other mutations associated with autism not associated with FXS (Betancur et al. 2009; Gogolla et al. 2009; Abrahams and Geschwind 2008). This coupling between FXS and autism suggests that the development of new targeted treatments for FXS, including the use of mGluR5 antagonists (Wang et al. 2010), or GABA agonists may turn out to be helpful for autism and other disorders that do not have an *FMR1* mutation, but which do have similar GABA and glutamate imbalances (Wang et al. 2010).

Clinical features of fragile X syndrome

FXS usually presents in the first 2 years of life, with developmental delays in language and with hypotonia. Some children have difficulty coordinating a suck in the newborn period and frequent emesis is common. Physical features in childhood include prominent ears and hyper-extensible finger joints. Macroorchidism (large testicles) typically does not develop until early puberty (Hagerman 2002). Tactile defensiveness, avoidance of eye contact, and hyperactivity usually develop by age 2 and are associated with hyperarousal to sensory stimuli. This hyperarousal can lead to tantrums and irritability, or mood lability in addition to anxiety (Hagerman 2002; Cordeiro et al. 2011; Sullivan et al. 2006; Budimirovic et al. 2006). The presence of autistic-like features, such as hand flapping, perseveration in behavior and language, avoidance of eye contact, and avoidance of light touch, spurred further research on the relationship between FXS and autism. However, detailed studies have demonstrated that it is the severity of the social and language deficits which leads to the diagnosis of autism in about 30% of boys with FXS (Kaufmann et al. 2004;

Lewis et al. 2006), with another 30% meeting criteria for PDD-NOS (Harris et al. 2008). Roberts et al. (2007) have demonstrated that most patients with FXS are initially avoidant of social interactions, but that those with autism continue to be physically and socially avoidant over time. In addition, those investigators found that children with both FXS and autism had higher basal and follow-up cortisol levels compared with those with FXS without autism. Lower cognitive and language abilities are also seen in FXS with autism compared with FXS alone, though the presence of autism is not driven by FMRP levels (Loesch et al. 2007; McDuffie et al. 2010).

The occurrence of autism in those with FXS is likely related to a number of factors that can worsen CNS function in addition to FXS alone. Garcia-Nonell et al. (2008) found that those with FXS and autism had a higher rate of medical problems that affect the CNS, including seizures and additional genetic disorders, such as Down syndrome (Stevens et al. 2010) or the Prader–Willi phenotype (PWP) of FXS (Nowicki et al. 2007). Higher rates of seizures were also found by Berry-Kravis et al. (2010a) in those with FXS and autism compared with FXS alone. The PWP in FXS, first reported by Fryns et al. (1987), is defined by the presence of hyperphagia, obesity, lack of satiation, and hypogenitalia. Several subsequent reports have described this subtype of FXS with severe obesity; it is suggested that the hypogenitalia and the lack of satiation after meals represent more severe hypothalamic dysfunction than what is typically seen in FXS (Fryns et al. 1987; Schrander-Stumpel et al. 1994; de Vries et al. 1993; de Vries and Niermeijer 1994). Individuals with the PWP do not have a deletion at 15q11–13, nor do they have uniparental disomy, but typically have a higher rate of autism compared with those with FXS alone (Nowicki et al. 2007). In addition, individuals with the PWP have down-regulation of *CYFIP1* (located between breakpoints 1 and 2 in the 15q deletion region associated with Prader–Willi syndrome) in peripheral blood compared with those with FXS without the PWP or controls (Nowicki et al. 2007). The downregulation of *CYFIP* in the PWP of FXS is likely to be an epigenetic effect, but the basis for its reduced expression is unknown. Further study of the role of background genetic effects and the role of environment in the development of autism in FXS is needed.

Most boys with FXS are diagnosed with ADHD by the time they are 5 years old and typically do well with stimulant medication (Berry-Kravis and Potanos 2003; Hagerman et al. 1988). Self-injurious behavior is seen in 58% of boys and 17% of girls with FXS, whereas compulsive behavior is seen in 72% of boys and 55% of girls (Hall et al. 2008a). The average IQ of an adult male with FXS is in the 40s, although 15% can present with an IQ>70% because of mosaicism or a lack of methylation in the full mutation (Merenstein et al. 1996; Hall et al. 2008b; Hagerman et al. 1994a). The level of cognitive abilities is positively correlated to the FMRP level, as are many of the physical features of FXS, including prominent ears and hyperextensible finger joints (Loesch et al. 2004). Because of the close association of a low IQ with autism in FXS, one would expect that autism would correlate with FMRP levels, but this is not the case once IQ is controlled for (Loesch et al. 2007; McDuffie et al. 2010). Autism in FXS also improves with age, particularly the reciprocal social communication abilities (Hernandez et al. 2009; McDuffie et al. 2010) as is seen in idiopathic autism (Seltzer et al. 2003; Shattuck et al. 2007).

Girls with FXS are typically less affected cognitively than boys due to the additional normal X chromosome that produces FMRP. Approximately 25% to 33% of girls with the full mutation have an IQ less than 70, and the majority have borderline or low normal cognitive abilities (Cordeiro et al. 2011; Bennetto and Pennington 2002). Executive function deficits are common even in those with a normal IQ (Bennetto et al. 2001). Higher functioning women have a favorable activation ratio (the proportion of cells with the normal X as the active X), and this correlates with a higher FMRP level (Tassone et al. 1999). Approximately 15% to 25% of girls with the full mutation have autism or ASD (Clifford et al. 2007; Cordeiro et al. 2011; Leigh et al., unpublished data), and they also tend to improve their autism over time (McDuffie et al. 2010). Cordeiro et al. (2011) assessed anxiety disorders in 39 girls with the full mutation (mean age 12.4 years; range 5.5–33.3 years) using a standardized psychiatric interview, Anxiety Disorders Interview Scale for DSM IV, and found that 39% met criteria for social phobia, 51.4% for specific phobia, 21% for selective mutism, and 18% for generalized anxiety disorder. They also found that 25% had ASD, whereas only 2% met criteria for full autism. Although fewer girls with the full mutation meet autism criteria compared with males, anxiety disorders are very common and usually respond well to a selective serotonin reuptake inhibitor (SSRI) (Hagerman et al. 2009; Berry-Kravis and Potanos 2004; Amaria et al. 2001; Hagerman et al. 1994b). However, the data regarding response to an SSRI in patients with FXS is from surveys in fragile X clinics, and a controlled trial is needed to demonstrate efficacy.

Molecular aspects of fragile X syndrome

FMRP is an RNA-binding and transport protein that is associated with the transport and stabilization of mRNAs (De Rubeis and Bagni 2010). At the synapse, FMRP regulates the translation of numerous messages (Bassell and Warren 2008; Levenga et al. 2009, 2010). FMRP binds to a

number of proteins, including FXR1P, FXR2P, NUFIP, CYFIP, and kinesin light chain, among others (Dictenberg et al. 2008; Bardoni et al. 1999; Schenck et al. 2001; Davidovic et al. 2007), forming an RNP particle. FMRP suppresses translation of mRNAs at the level of translation initiation (Napoli et al. 2008). It is thought that the microRNA (miRNA) pathway and the ribonucleoprotein RNA-induced silencing complex (RISC) are also part of the RNPs that contain FMRP. Unphosphorylated FMRP associates with Dicer, and this complex processes pre-miRNAs into mature miRNAs (Cheever and Ceman 2009a, b). miRNAs are small single-stranded RNAs that are important for processing mRNA for degradation or translation. In the nucleus, pri-miRNAs are processed by Drosha, an RNAse III endonuclease, into pre-miRNA, which are then processed into mature miRNAs that also work with RISC. Edbauer et al. (2010) have shown that FMRP interacts with miRNA132 and miRNA125b, and this regulates the translation of NMDA receptor subunit 2A. There is an enhancement of protein production in the hippocampus by approximately 20% in the knock out (KO) FXS mouse (Qin et al. 2005; Dolen et al. 2007).

A number of proteins have been shown to bind to FMRP and presumably are regulated by FMRP through translation or stabilization of their messages, including neuroligins 3 and 4, neurorexins, PSD-95, SHANK3, Arc, PTEN, MAPK, JKMIP, HERC, CYFIP1, and others (Darnell et al. 2005, 2010; Miyashiro et al. 2003; Nishimura et al. 2007; Levenga et al. 2010; Dahlhaus and El-Husseini 2010). Many of these proteins, when mutated, can cause an increased risk for autism. Therefore, there is significant overlap in the dysregulation of protein systems between various forms of autism and FXS (Betancur et al. 2009; Hagerman et al. 2010). In addition, Sharma et al. (2010) have demonstrated the there is upregulation of mTOR in the KO mouse, which has also been shown to occur in humans with FXS (Tassone 2010). mTOR is upregulated in other forms of autism, including tuberous sclerosis and PTEN mutations; rapamycin is being tested as a targeted treatment for these disorders (de Vries 2010; Zhou et al. 2009).

Co-immunoprecipitation studies and functional assays have demonstrated that numerous presynaptic and postsynaptic proteins are regulated by FMRP so that, in the absence of FMRP, there is significant dysregulation of synaptic plasticity, which is another reason that there is a close association between autism and FXS (Darnell et al. 2010; Betancur et al. 2009).

One of the important systems upregulated by the absence of FMRP is the mGluR5 pathway that leads to long-term depression (LTD), or weakening of synaptic connections (Huber et al. 2002; Bear et al. 2004). FMRP normally inhibits the protein translation that occurs with mGluR5 stimulation, and this protein translation leads to internalization of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, which weakens synaptic connections, leading to LTD. In the absence of FMRP, LTD is enhanced and morphologically, there are long, thin, and immature dendritic spines in the hippocampus and elsewhere in the brain (Irwin et al. 2005). These immature spines are rescued by crossing the KO mouse with an mGluR5-deficient mouse so that the offspring have reduced mGluR5 activity (Dolen et al. 2007). This finding has had important therapeutic implications for patients with FXS in that mGluR5 antagonists can reverse the neurobiological findings of immature dendritic spines, in addition to epilepsy and behavioral and cognitive abnormalities in animal models (Levenga et al. 2010; Yan et al. 2005; de Vrij et al. 2008; McBride et al. 2005; Dolen et al. 2010). Currently, there are three mGluR5 antagonists that have been used in human trials: fenobam which was successful in a single dose in 12 adults with FXS (Berry-Kravis et al. 2009); AFQ056, developed by Novartis and which has completed a European trial with positive significant effects only in those with FXS and methylated *FMR1* alleles (Jacquemont et al. 2011) with no significant response in those without methylation; and R04917523, developed by Roche and currently in a multicenter controlled trial in the USA. It is hoped that both behavioral and cognitive benefits will occur in those with FXS who are treated with mGluR5 antagonists, but we have to wait for further studies to understand the impact of these targeted treatments.

Silverman et al. (2010) studied the autism mouse model BTBR which has social deficits and repetitive and restrictive behavior of repetitive self-grooming. The BTBR mouse was treated with MPEP, an mGluR5 antagonist that has reversed the fragile X neurobiological abnormalities in the KO mouse. They found reversal of the repetitive self-grooming behavior without sedation in the BTBR mouse with MPEP treatment suggesting that mGluR5 antagonists may be helpful in those with autism without the *FMR1* mutation (Silverman et al. 2010).

Other pathways important for behavior and cognition are also negatively impacted by the loss of FMRP in FXS. The GABA system is generally downregulated in the absence of FMRP (D'Hulst et al. 2009; D'Hulst and Kooy 2007; El Idrissi et al. 2005), and use of Arbaclofen, a $GABA_B$ agonist, has been both helpful in the KO mouse and most importantly in children and adults with FXS (Berry-Kravis et al. 2010b). This medication is the R isomer of Baclofen, which has been available for years in the treatment of cerebral palsy. Ganaxolone is a $GABA_A$ agonist with potential utility in FXS since the $GABA_A$ receptors are significantly lowered in the disorder (D'Hulst et al. 2009). Preliminary studies in the KO mouse have shown positive effects on behavior (Kooy et al. 2010).

Recently, Olmos-Serrano et al. (2010) documented deficits in inhibitory transmission in the amygdala of the KO mouse including dramatic reduction in the frequency and amplitude of phasic inhibitory postsynaptic currents, in tonic inhibitory currents as well as a reduction in the number of inhibitory synapses. There were significant alterations in GABA availability both intracellularly and at the synaptic cleft. This led to hyperexcitability in the principal neurons of the amygdala which was rescued by treatment with a GABA agonist, Gaboxadol. This animal evidence is further support of the need for GABA agonists in the treatment of patients with FXS. Work by Suvrathan et al. (2010) in the amygdala of the KO mouse demonstrated that the mGluR5 antagonist MPEP only rescued the decrease in the miniature excitatory postsynaptic currents and MPEP did not rescue the deficit in long-term potentiation or surface expression of the AMPA receptor in the amygdala. Therefore, it is likely that combined psychopharmacologic interventions with targeted treatments will be needed in FXS.

Recently, Bilousova et al. (2009) reported that matrix metalloproteinase 9 (MMP9), a protein important for maintenance of synaptic plasticity, was significantly elevated in the CNS of the KO mouse. The authors demonstrated that treatment of the neonatal mouse for a 1-month period with minocycline, which lowers MMP9, led to significant maturation of the dendritic spines and improvements in behavior and cognition in the KO mouse. This report generated much interest in the families of children with FXS because minocycline is available currently by prescription. However, minocycline used before 8 years of age can lead to significant graying of the permanent teeth. A survey of 50 patients with FXS who were treated for longer than 2 weeks demonstrated that families perceived a positive effect, particularly in language and behavior, in 70% of patients (Utari et al. 2010). Side effects were minimal, although one patient had graying of his fingernails and toenails and another was thought to have graying of the teeth. Although the positive results of the minocycline trial were likely influenced by a placebo effect, the appropriate controlled trial is currently underway to determine whether this treatment is effective in children (Wang et al. 2010). An open trial of minocycline reported by Paribello et al. (2010) in 20 patients with FXS ages 13–32 years demonstrated benefit in the Aberrant Behavior Checklist, the Clinical Global Improvement Scale, and the visual analog scale for behavior with minimal side effects. Recent studies of minocycline also show enhancement of the EIF4AI translation factor (Hashimoto and Ishima 2010) which may be of benefit in patients with FXS. In addition, minocycline is a neuroprotective agent, and it is highly effective in blocking or inhibiting cytochrome C release from mouse liver mitochondria both in vitro and in vivo (Wang et al. 2008). Screening of 1,040 compounds by the Neurodegeneration Drug Screening Consortium of NINDS found that minocycline was the second most effective drug to block cytochrome C release in response to Ca++ stimulation (Wang et al. 2008). There is significant oxidative stress in the cells from those with FXS (de Diego-Otero et al. 2009) in addition to cells from those with premutation involvement (Ross-Inta et al. 2010) and idiopathic autism (Giulivi et al. 2010). Therefore, stabilization of mitochondria is likely an important issue (and therapeutic target) across neurodevelopmental, one that requires further study.

Other potential therapeutic approaches, including the use of PIK3 inhibitors (Gross et al. 2010), GSK3β antagonists including lithium (Berry-Kravis et al. 2008), or miRNA targeting (Muddashetty and Bassell 2009), are also being considered as therapeutic approaches for FXS. It is likely that more than one targeted treatment will be needed for the reversal of both the cognitive and behavioral problems in those with FXS. Furthermore, the use of enhanced learning techniques such as computer and assistive technology will be needed as the synaptic connections are strengthened to maintain and augment these improvements (Wang et al. 2010).

Involvement in carriers of premutation alleles

There is a broad spectrum of involvement of developmental and neurological problems with aging that can occur with the premutation (Fig. 1). For many years, those with the premutation were thought to be unaffected, except for FXPOI, which was first reported by Cronister et al. in 1991 (Cronister et al. 1991). Mothers who bring their children into clinic are usually without apparent clinical involvement cognitively, although anxiety and depression are common (Roberts et al. 2009). When the mothers themselves are carriers of full mutation alleles, learning and emotional problems are more common than in carriers of premutation alleles (Bennetto and Pennington 2002).

Males with the premutation were once called normal transmitting males and therefore, by definition, were considered normal. However, in 1996, cognitive and behavioral problems with the premutation were first reported in three boys who presented with learning disabilities or ADHD, which were thought to be related to a mild decrease of FMRP (Hagerman et al. 1996). Reports of premutation involvement became more common after the premutation was associated with elevated levels of *FMR1* mRNA in 2000 (Tassone et al. 2000a). Elevated mRNA in the premutation was also confirmed by other groups (Kenneson et al. 2001; Peprah et al. 2010; Allen et al. 2004). Multiple case reports of premutation involvement,

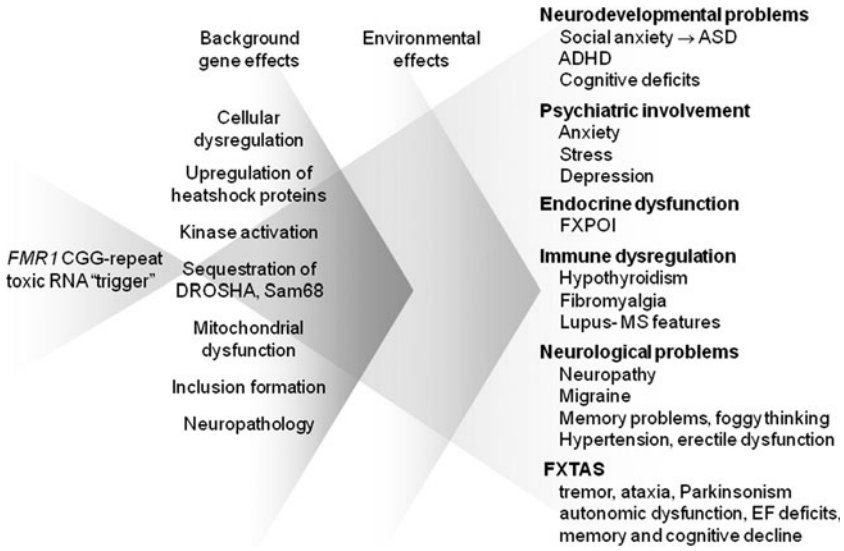

Fig. 1 This depicts the molecular changes and the spectrum of clinical involvement in premutation carriers

mainly in males, included reports of autism and ASD (Farzin et al. 2006; Aziz et al. 2003; Clifford et al. 2007; Tassone et al. 2000b; Goodlin-Jones et al. 2004); nevertheless, only a subgroup of premutation carriers had developmental problems. Specifically, these reports include ten boys who were assessed for fragile X, including eight who were tested because of developmental or behavioral problems and two who were siblings of boys with FXS (Aziz et al. 2003). Of these ten cases, four had repeats less than 50 CGGs (in the gray zone), three of whom were found to have ASDs. Of the remaining six, one had Asperger's syndrome with a 56 CGG repeat. In the Clifford et al. (2007) study, there were seven males with the premutation; two were probands in fragile X families, and one of these had ASD (14% overall). In the study of Goodlin-Jones et al. (2004), there were four boys and two girls with ASD and the premutation, and they were compared with premutation patients without ASD. Although the CGG repeat number did not differ between the two groups, the level of FMRP was significantly lower in the premutation carriers who had ASD (Goodlin-Jones et al. 2004). These case reports suggest that those who present clinically with the premutation were more likely to be diagnosed with ASD. The subsequent study by Farzin et al. (2006) was designed to test this hypothesis. This study (Farzin et al. 2006) included 14 boys (probands) with the premutation who presented clinically, 13 boys (nonprobands) with the premutation who were identified with the premutation through cascade testing of the family once a proband was diagnosed, and 16 boys who were siblings but did not have the premutation (controls). After a detailed assessment, utilizing standardized testing to assess ASD and ADHD, it was found that 13 of 14 (93%) of the probands had ADHD, and all were receiving medication to treat this problem. This was significantly increased from 6 of 13 (38%) of the nonprobands with ADHD and 2 of 16 (13%) of the controls. Regarding a diagnosis of ASD, 10 of 14 (71%) of the probands had an ASD diagnosis compared with 1 of 13 (8%) of the nonprobands and 0 of 16 controls. This study demonstrates that probands are at higher risk for both ADHD and ASD, but the overall prevalence of these problems will require a longitudinal follow-up study of premutation carriers diagnosed at birth to eliminate any residual bias. The assessment of more than 1,000 families by web questionnaires demonstrated a prevalence of autism or ASD of 13% in boys with the premutation and 1% in girls with the premutation (Bailey et al. 2008).

It is not known why some children with the premutation have developmental problems whereas most do not. ASD and perhaps other developmental problems in premutation children are more common in boys, and the study of Goodlin-Jones et al. demonstrated a 50% rate of seizures in the affected premutation carriers in addition to lowered FMRP compared with the comparison premutation carriers without ASD. It is likely that seizures (perhaps related to lower FMRP) further interfere with brain connectivity in the vulnerable patient during development, and therefore, the child would be more likely to develop an autism spectrum disorder (Brooks-Kayal 2010). Also recent animal studies of the premutation mouse demonstrate lowered levels of FMRP in addition to elevated *FMR1*-mRNA in many brain areas, particularly the amygdala, hippocampus, and cortex, when compared with controls without the premutation (Qin et al. 2011). It is likely that both of these changes cause significant problems with cognition and behavior, particularly in those with a premutation over 150 repeats.

There is also evidence that brain connectivity may be different in some carriers compared with controls in brain imaging studies. In young adult males with the premutation,

there is a deficit of amygdala activation on MRI to fearful faces compared with their brothers who do not have the premutation (Hessl et al. 2007). In carriers, amygdala activation to non-fearful faces was normal compared with their brothers, suggesting that the deficit may be specific to emotionally activating stimuli. In addition, a recent fMRI study by Hashimoto et al. (2010) has demonstrated that activation of the right ventral inferior frontal cortex and left premotor/dorsal inferior frontal cortex is significantly deficient in adult male carriers without neurological disease compared with age-matched controls. There was a negative correlation between the right ventral inferior frontal cortex activation and the level of *FMR1*-mRNA. The authors suggest that these alterations in the prefrontal cortex may underlie both the executive function and memory deficits that are seen in older carriers, even those without FXTAS. In MRI studies of gray matter loss, the region of interest analysis in asymptomatic premutation carriers demonstrated gray matter loss in the anterior subregions of the cerebellar vermis compared with age-matched controls (Hashimoto et al. 2011a). There was a negative effect of the CGG repeat size on the gray matter density in the dorsomedial frontal regions in carriers. In addition, diffusion tensor imaging changes in premutation carriers without neurological problems compared with age-matched controls demonstrated elevations in axial diffusivity and redial diffusivity in the middle cerebellar peduncles (MCP) compared with controls (Hashimoto et al. 2011b). This area of the MCP becomes more significantly involved with severe white matter disease if a carrier develops FXTAS as described below.

Although the imaging studies suggest early subclinical involvement in the brain of carriers without FXTAS, a large study by Hunter et al. (2008) did not show neuropsychological deficits in carriers compared with non-carriers in those under the age of 50 years. There were 506 women and 138 men between the ages of 18 to 50 years, but these numbers included only 30 males with the premutation, although 298 females had the premutation. There were no neuropsychological deficits in the males, but in the larger cohort of females, there were significantly more severe symptoms associated with ADHD than the non-carrier females. There was also an association of repeat length with a factor of self-reported inattention and impulsivity, suggesting again that the higher the CGG repeat within the premutation range, the greater the clinical involvement. In comparing the Hunter study to other centers that have carried out neuropsychological studies of premutation carriers, there is a difference perhaps in ascertainment and in the measures utilized since the other centers have seen problems in a subgroup of carriers. The Cornish et al. (2008) found executive function deficits in adult male carriers that worsened over time from just inattention to dysinhibition with age. Similar findings were seen by Moore et al. (2004) and from studies by Grigsby et al. (2006, 2008) and Brega et al. (2008). Further collaborative work utilizing sensitive measures to early executive function deficits should clarify whether carriers are at greater risk for ADHD and executive function deficits as they age.

Recent studies of hippocampal neurons with the premutation in culture have demonstrated changes that support the developmental problems described in children. Chen et al. (2010) reported that premutation neuronal cultures demonstrate reduced dendritic complexity with shorter dendritic lengths and fewer branches between 7 and 21 days in vitro compared with wild-type (WT) neurons. Synaptic structure was also different in premutation neurons in that the size of puncta labeled with synapsin (presynaptic vesicle protein) and postsynaptic puncta labeled with phalloidin were larger compared with WT at 14 and 21 days in culture. In addition, premutation neurons die more easily in culture by 21 days compared with normal neurons, suggesting that they may be more vulnerable to environmental trauma or toxicity (Chen et al. 2010). The premutation neurons also demonstrate elevations of stress proteins and their mRNAs, including heat shock proteins (Hsp27 and Hsp70) and αB-crystallin, which have been reported in fibroblasts from premutation carriers (Garcia-Arocena et al. 2010). These changes are also consistent with the cellular loss and brain atrophy seen in patients with FXTAS and with the neurodevelopmental problems reported in some children with the premutation. Perhaps the neuronal cell loss that was demonstrated in vitro can be exacerbated by the stress of seizures, making it more likely for ASD to develop in premutation carriers with seizures.

Further support for neurodevelopmental problems in the premutation comes from studies of the migration of premutation neurons. Cunningham et al. (2010) have reported altered embryonic neocortical development in the premutation mouse compared with WT. They found orientation deficits of migrating neurons and a decrease in the neural precursor cell marker Tbr2 in these mice. They also detected a 42% reduction in FMRP levels in the premutation embryonic telencephalon. The knock-in (KI) premutation mouse model has also been helpful in detecting early deficits in learning. By 12 weeks, the premutation mouse was unable to detect a change in the distance between two objects, and at 48 weeks, they could not detect a transposition of objects (Hunsaker et al. 2010).

Premutation alleles can also lead to significant neurotoxicity with aging, specifically FXTAS which occurs in approximately 40% of males and 10–16% of females who are older than 50 years (Jacquemont et al. 2004; Coffey et al. 2008; Rodriguez-Revenga et al. 2009). The premutation also leads to toxicity in the ovary, such that approximately 20% of women with the premutation experience early

ovarian failure or insufficiency (FXPOI) before age 40 (Sherman et al. 2007; Wittenberger et al. 2007). FXTAS was first reported in 2001 (Hagerman et al. 2001) with five case reports of the onset of an intention tremor initially followed by ataxia and evidence of brain atrophy and white matter disease on T2 imaging. Further studies documented involvement of autonomic function including impotence, hypertension, orthostatic hypotension, and eventually urinary and bowel incontinence, neuropathy symptoms (particularly pain), parkinsonism, executive function deficits, cognitive decline with eventual dementia in 50% of people, and emotional difficulties including irritability, apathy, and depression (Grigsby et al. 2008; Jacquemont et al. 2003, 2007; Bacalman et al. 2006; Seritan et al. 2008; Berry-Kravis et al. 2007; Allen et al. 2008; Soontarapornchai et al. 2008; Aguilar et al. 2008; Leehey et al. 2007). MRI features include global brain atrophy and white matter disease with spongiosis involving the MCP sign, periventricular regions, subcortical regions, and pons (Adams et al. 2007, 2010; Cohen et al. 2006; Brunberg et al. 2003). Females are less affected than males, both radiologically and cognitively (Coffey et al. 2008; Adams et al. 2007; Hagerman et al. 2004), but they often have autoimmune problems including hypothyroidism and fibromyalgia (Coffey et al. 2008) with the rare occurrence of multiple sclerosis (Zhang et al. 2009).

Although psychiatric involvement is not seen in the majority of children with the premutation, psychiatric symptoms of depression and/or anxiety can occur in mid-adulthood. A detailed study by Roberts et al. (2009) of 93 women with the premutation demonstrated a mood disorder in 47%, which was significantly higher than the comparison group from the National Comorbidity Survey Replication data set. Similar results were seen in a study of 85 premutation carriers (both men and women), including lifetime mood disorder in 65% of those with FXTAS and 42% in those without FXTAS and lifetime anxiety disorder in 52% of those with FXTAS and in 47% of those without FXTAS (Bourgeois et al. 2011).

The RNA toxicity can involve not only the limbic but also the endocrine system with either FXTAS, FXPOI, or other neurological problems (Gokden et al. 2009; Greco et al. 2007). Impotence is commonly seen before the onset of tremor and ataxia and inclusions eventually develop in the Leydig cells of the testicles that produce testosterone (Greco et al. 2007). The involvement from the premutation is depicted in Fig. 1, and it is far broader and more common than involvement from the full mutation. Coffey et al. (2008) found a higher rate of muscle pain, fibromyalgia, thyroid disease, neuropathy symptoms, and hypertension in 141 female carriers compared with age-matched controls. A study by Rodriguez-Revenga et al. (2009) found similar problems in over 280 female carriers with chronic muscle pain in 24.4%, thyroid disease in 15.9%, and FXPOI in 18.6%. Hunter et al. (2010) studied 334 women with the premutation and 37 men with the premutation compared with controls between the ages of 18–50 years. Men with the premutation did not report any medical condition at higher rates than non-carriers. However, women with the premutation reported mental health disorders including ADHD, anxiety, and depression significantly more often that non-carriers, although after adjusting for covariates these increased rates were not significant. However, women with ovarian insufficiency, as manifested by irregular cycles, reported higher rates of thyroid problems and depression/anxiety compared with controls. Clearly further studies are warranted regarding the medical, particularly autoimmune problems, such as thyroid disease and fibromyalgia, in carriers compared with controls.

Because the increased rate of autoimmune problems in parents of children with FXS is similar to what has been found in parents of children with autism (Atladottir et al. 2009), Chonchaiya et al. (2010) studied the FXS children (n=61) of mothers who have autoimmune disease compared with the children (n=97) of mothers without autoimmune disease. The hypothesis was that the children of mothers with autoimmune disease would be more likely to have autism. The odds ratio (OR) for ASD was 1.27 which was not significantly different, but the OR for seizures was 3.81 (p=0.031) and the OR for tics was 2.94 (p=0.019) so they were significantly increased in children of mothers with autoimmune disease. This suggests that there is an intergenerational effect of autoimmune disease in those with the premutation on their offspring, similar to what has been reported in a subgroup of patients with autism (Ashwood et al. 2006; Enstrom et al. 2009; Martin et al. 2008). Ashwood et al. (2010) have also studied the cytokine and chemokine profile of those with FXS both with and without autism compared with controls. They found significant differences in plasma protein levels of a number of cytokines including increased in IL-1α with a decrease in the chemokines RANTES and IP-10 between FXS and typicals. Those with FXS without autism had higher levels of IL-6, eotaxin, and MCP1α but lowered levels of RANTES compared with FXS with autism. The alterations of these profiles are likely to create meaningful changes in neurodevelopment, and this has been reviewed in autism (Enstrom et al. 2009).

RNA toxic gain of function as a basis for premutation disorders

The unique neuropathological finding in FXTAS is the presence of intranuclear inclusions in neurons and astrocytes throughout the brain, particularly in the hippocampus

and limbic systems (Greco et al. 2002, 2006). These inclusions are also found in the premutation KI mouse in both neurons and astrocytes (Wenzel et al. 2010). The inclusions are tau and synuclein negative but are positive for *FMR1* mRNA. The toxicity of the premutation is thought to relate to sequestration of important proteins by the expanded-CGG-repeat mRNA (Sellier et al. 2010b; Garcia-Arocena and Hagerman 2010). These proteins include splicing proteins of other RNAs in addition to Drosha, which regulates miRNAs (Kenneson et al. 2001). The sequestration may develop slowly over time such that symptoms of neurodegeneration are not seen until late adulthood, as is reported with the development of inclusions over time in the premutation mouse (Wenzel et al. 2010; Brouwer et al. 2007).

Most recently, Ross-Inta et al. (2010) demonstrated mitochondrial dysfunction in fibroblasts and brain samples in premutation carriers both with and without FXTAS. Mitochondrial dysfunction in carriers included uncoupling between electron transport and synthesis of ATP in addition to decreased levels of mitochondrial proteins such as the ATPase β-subunit from complex V, cytochrome c oxidase subunit IV from complex IV, and MnSOD as part of the mitochondrial antioxidant defense. These findings were most severe in those with FXTAS, but they were also present in carriers without FXTAS. Patients with FXTAS gradually become very weak as their disease progresses, which is consistent with a worsening of mitochondrial function. What is not known is whether young children with the premutation, particularly those who are affected with an ASD, also demonstrate mitochondrial problems. Mitochondrial deficits have been found in a subgroup of children with idiopathic autism (Giulivi et al. 2010; Oliveira et al. 2005), but the types of defects vary. In the premutation, the levels of the mitochondrial proteins correlated inversely with the CGG-repeat numbers in the premutation range. These protein changes increase oxidative stress, increase oxidatively modified mitochondrial proteins, and activate the unfolded protein response and phosphorylation of the α subunit of the heterotrimeric eukaryotic translational initiation factor 2 (eIF2α), resulting in a decrease in protein translation (Ross-Inta et al. 2010). Further studies are needed to develop effective interventions for the premutation symptomatic carrier with either neurodevelopmental or aging problems.

Conclusion

There are two ways in which the *FMR1* mutation can lead to autism or ASD. The full mutation that causes FMRP deficiency leads to dysregulation of both GABA and glutamate systems, which creates an imbalance of inhibitory and stimulatory systems in addition to problems with synaptic plasticity and connectivity in the brain. Many of the proteins that are dysregulated in the absence of FMRP are themselves associated with autism when their genes are mutated. The premutation can lead to autism or ASD through a process of RNA toxicity causing miRNA dysregulation, early cell death, and mitochondrial abnormalities and in some cases somewhat lowered levels of FMRP which also affect brain connectivity. It is essential to order *FMR1* DNA testing in any individual who presents with ASD of unknown etiology. The identification of the full mutation can lead to the use of targeted treatments, and new interventions are currently being studied in those with the premutation (Hagerman et al. 2008).

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