FRAGILE X SYNDROME

From Genetics to Targeted Treatment

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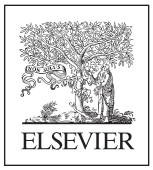

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Foreword

In 2016 it was 25 years since the identification of the *FMR1* gene and its new mutation mechanism. This book will give an overview of what has been achieved since then and gives an overview of the present knowledge of fragile X syndrome (FXS) and the gene involved. But let's go back first for a short visit into history.

In 1943 Martin and Bell described a pedigree of mental defect showing sex linkage. They showed in two generations of this family 11 males with imbecility. This term was typical for those days, but has since been evaluated into mental retardation and in this century into intellectual disabilities. In 1986 John M. Opitz described the burden in the families as follows:

> And then as always, one stops to recollect with total astonishment and great reverence the massive burden of pain carried so patiently by the mothers, fathers, sibs, grandparents and the many others involved so closely on a daily basis with the apparent failure, defect, handicap, disability, and disappointment in the many thousands of Martin-Bell syndrome families throughout the world. **Am J Med Genet 23:1–10**

In 1969 Lubs noted a secondary constriction, referred to as a fragile site, which has been used to describe the syndrome as FXS. The presence of the cytogenetic expression of the fragile site was implemented as a diagnostic criterium but this was not a very reliable tool, in particular in the identification of carriers.

The development of recombinant DNA technology around 1980s made the cloning and identification of disease genes possible. The close association between the syndrome in males and the fragile site at Xq27.3 indicated that the gene involved must be located at, or near to, the fragile site. Accordingly, the efforts of many different laboratories have been aimed at obtaining probes and fragments as close as possible to this fragile site, with the ultimate goal cloning the gene involved in FXS and the mutation in the disease gene.

The discovery by Verkerk and coworkers in 1991 that the disease is caused by a large-scale expansion of a highly unstable trinucleotide repeat in the *FMR1* gene has elucidated a new mutation mechanism of heritable unstable DNA. The gene was named *FMR1* (fragile X mental retardation 1), assuming that this was the first of an unknown number of future genes that might be isolated from the X chromosome involving fragility and mental retardation. The protein missing in fragile X patients was subsequently named FMRP. In the subsequent years more than 10 diseases with unstable repeat genes have been identified, all involved in neurological disorders. The presence of an unstable repeat in the *FMR1* gene has helped in direct testing in fragile X families and the identification of FRAX patients because the mutation is almost exclusively of the same type and there is an extremely low occurrence of other mutations in the disease. Since the identification of the unstable repeat in the *FMR1* gene much effort has been spent to get an answer to the following questions: What is the mechanism of repeat instability? What is the timing of repeat instability during embryonal life? What is the function of the repeat in the disease gene? What are the

functions of the (normal) gene product, with special focus on the brain?

Clues to the mechanisms that cause the abnormalities observed in FXS were limited. Initially research progress has been slow which is in part due to the lack of brain material of patients. To gain more insight in the pathological and physiological processes, researchers focused on animal models. No natural occurring animal models for FXS have been described. Therefore transgenic mouse models for FXS have been generated in Rotterdam and Houston. These mice show characteristics of FXS and have been made available to the research community. These mice might help to learn more about the function of the *FMR1* gene and the effect that the lack of the protein has on brain functioning. Furthermore, animal models might help in studying the timing and mechanisms of the repeat amplification. These mice have been instrumental in research to the understanding of the pathogenesis of FXS. Many chapters in this book are describing experiments using these animal models. Also models for FXS has been generated in flies, zebra fish, and rats. All of these have there own advantages and disadvantages. Rats (an animal widely used by the pharmaceutical industry) might be useful in testing drugs while flies and zebra fish can be used for screening drugs.

The different contributions cover the progress in research in the field of FXS very well. They are subdivided into three different sections:

1. Clinics, diagnosis, epidemiology, molecular mechanisms, and models
2. Pathways involved
3. Clinical trials

Although we have learned a lot in the last 25 years, it will be clear to the readers that there is still more exciting work to do. In my view we still have to gain more insight in the pathological and physiological processes both at the level of (lack of) protein and the mechanism of repeat instability. So far progress in treatment of patients has been limited, not to say disappointing despite the fact that preclinical studies in mice were successful. We need to understand better differences within patient groups and we need better instruments to study the effect of treatment.

I hope this book will encourage readers and researchers to extend our knowledge further with regard to FXS.

Rotterdam, January 2016
Ben A. Oostra

Preface

Why a book? This is the first question you will ask upon seeing the nearly 500 pages of this book on Fragile X Syndrome. Isn't a book something very much of the past, something we used to be proud of since the invention of typography many centuries ago, an art now replaced by cybergraphy, providing us continuously with information free of charge wherever we are, even when watching a movie or relaxing on a sunny beach. Those who are satisfied with the information provided this way, please stop reading here. We are very much aware that even a simple search with the phrase "Fragile X Syndrome" on even the most amateurish of all search engines on any computer, laptop, or smartphone will result in thousands of hits within milliseconds, each of which will guide you through blogs, fora, papers, essays, etc. on this, or, in fact on any other topic. We felt however, that without the proper background on this complicated neurodevelopmental disorder, the "cyber only" reader would be congested with information within seconds and it will be impossible for him or her to filter out the relevant and reliable information. A classic example of too much of a good thing, that is, somewhat equivalent to going to a university without ever having seen inside of a secondary school. By providing the information on this genetic disorder in a highly structured and relevant format, our book is meant to serve as an anchor point for those in search of information.

Why on the Fragile X Syndrome? The simple fact that we choose this topic because both of us have been working on it for so long is not the answer, not at all. The reason why we selected this topic is that the disorder keeps surprising us time after time after time by creating novel insights, by unraveling cellular mechanisms, and by its involvement in yet another molecular pathway. In the early years, immediately following the discovery of the gene, at that time novel mutational mechanism raised much attention. The many functions of the *FMR1* gene are another still not completely resolved mystery. Rather than a single function, the gene appears to play a role in a multitude of cellular processes and molecular pathways in the cell. Most interesting, several of these pathways are amendable to treatment with drugs that have been for many years on the shelf of various pharmaceutical companies that are eager to collaborate with the academic world to improve the condition of the patient. As such the Fragile X Syndrome has become the lead example of a monogenetic disorder that paved the way for the targeted intervention studies in many related neurodevelopmental disorders.

This book provides the state of the art in Fragile X Syndrome research, with an emphasis on the pathways amendable to treatment. It includes with an overview of the current clinical trials and reflects on those (What have we learned?). Of course we have not forgotten the input from the patients and their parents. The book, without exception written by long-term experts in the field, will appeal to a broad readership and is meant as a point of reflection for the "Nestors" in the field and at the same time as a point of inspiration for novel investigators that are eager to enter the field. For medical doctors,

patients, caregivers, and relatives it is meant to provide a realistic overview of what scientific research has achieved and what can be expected in the near future.

No book should ever be written without acknowledgments. We thank all contributors for their commitment and their eagerness to transform their expertise in written language. Only in retrospect this is easy. A specific thanks to our reviewers, many of whom felt they could have contributed to the contents of the book as well (and rightly so!), who dedicated their time to improve the chapters for little more than this anonymous reward. Their efforts are immensely appreciated. And of course we thank our collaborators, students, and colleagues, for continuous inspiration over a long, long period of time. Finally we thank each other for our almost perfectly complementary expertises, professional networks, and characters. Together we made it work and it was fun to do so!

Rob and Frank

CHAPTER

1

The Clinical Phenotype of the Fragile X Syndrome and Related Disorders

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INTRODUCTION

This chapter deals with the clinical aspects of the fragile X syndrome (FXS) and, more briefly, of the fragile X–related disorders: fragile X tremor ataxia syndrome (FXTAS) and fragile X premature ovarian insufficiency (FXPOI). Although related through the same causal gene, *FMR1*, the three conditions have different clinical presentations and underlying pathophysiology, FXS being caused by lack of transcription, while FXTAS and FXPOI are caused by excessive transcription of the gene. This vast topic has been the object of countless scientific articles, only a minority of which are cited here, and of several excellent reviews, including, just to cite a few, those by Penagarikano, Mulle, and Warren (2007), Garber, Visootsak, and Warren (2008), Hersh and Saul (2011), Kidd et al. (2014), van Esch (2015), Hall et al. (2014), Hagerman and Hagerman (2002, 2015).

These disorders, for which we coined the name FRAXopathies (Pirozzi, Tabolacci, & Neri, 2011), fall within the class of conditions caused by microsatellite instability, recently reviewed by Brouwer, Willemsen, and Oostra (2009) and by Nelson, Harry, Orr, and Warren (2013).

THE FRAGILE X SYNDROME

Background

Historically, two independent descriptions of FXS existed, before it was so named. The first of these was authored by Martin and Bell (1943), the second by Lubs (1969). Understandably, for many years the condition was referred to as the Martin–Bell syndrome, even though Martin and Bell did not describe a "syndrome" sensu stricto, but rather a case of pure intellectual disability (ID), or mental deficiency, as it was then called, without

Fragile X Syndrome. http://dx.doi.org/10.1016/B978-0-12-804461-2.00001-9

accompanying physical manifestations. The paper, more often quoted then read, has two parts, one dealing with the clinical description of several affected members in a large family, the other interpreting the heritability of the condition. The clinical description is brief and straightforward. The eight examined males "show a severe degree of dementia, their mental ages being between two and four years. No peculiar features, either mental or physical, have been recognized which would serve to distinguish the disease that afflicts this family from other forms of dementia." The sexual development was reported normal and no mention of macroorchidism was made. Two females were recognized as "backward," probably carriers, in retrospect, of a full mutation of the *FMR1* gene. The interpretation of the pedigree correctly concluded for X-linked inheritance, allowing for the exceptionality of two transmitting males, in whom the manifestation of the mental impairment "was suppressed by the presence of some controlling factor." Looked at from hindsight, the distribution of cases within the family tree is in full agreement with the so-called Sherman paradox, consisting of an increase in the number and proportion of affected individuals in successive generations.

Lubs' report of a family with X-linked ID is mainly focused on the description of a marker X, that is, an X chromosome with a breakage near the end of the q arm, later to become known as the fragile site FRAXA, where the causative gene *FMR1* is located. The clinical condition is recognized as syndromal, given that the propositus presented with a combination of "mental retardation and multiple minor anomalies." However, the described phenotype is quite bland, with mention of prominent maxilla and low-set, large ears. Interestingly, the growth parameters of the propositus (height, weight, and head circumference) are below the 3rd centile, different from what is usually seen in a child with FXS. On the other hand, in the two photographs depicting the propositus and his older brother, the *gestalt* of FXS is easily recognizable. The fact that Lubs described the same condition as Martin and Bell should not be doubted. Richards, Sylvester, and Brooker (1981) were able to retrieve seven members of the Martin–Bell family and five of them proved to carry a fragile X chromosome. These authors also recognized the presence of physical features, including macroorchidism, and proposed the eponymic designation "Martin–Bell syndrome." Nonetheless, the designation FXS eventually prevailed.

The description that follows is meant to represent the typical phenotype in a male patient, whose most common physical and behavioral traits are listed in Table 1.1. However, it should be understood that FXS makes no exception to the rule that syndromes of ID and multiple

TABLE 1.1 Major Physical and Behavioral Findings in Fragile X Syndrome (FXS)

Physical	Behavioral
- Elongated face	- Anxiety
- Large ears	- Hyperactivity
- Highly arched palate	- Attention deficit
- Macroorchidism	- Emotional lability
- Mitral valve prolapse	- Gaze avoidance
- Hyperlaxity of joints	- Stereotypic movements
- Connective tissue dysplasia	- Echolalia
- Muscular hypotonia	- ASD

ASD, Autism spectrum disorder.

congenital anomalies tend to have variable expressivity, sometimes rendering the clinical diagnosis difficult to reach. Another aspect that will be touched upon is the changing of the phenotype with increasing age. In infancy, the physical manifestations tend to be less marked. However, this should not justify a missed diagnosis beyond the age of 2 years, when the delay in developmental milestones is already evident. A very brief description will also be given of the clinical presentation of affected females.

The FXS phenotype is caused by inactivation of the *FMR1* gene, which consists, in more than 95% of the known cases, in an expansion above 200 units (full mutation) and subsequent methylation of the CGG triplets and CpG island in the promoter region of the gene (Verkerk et al., 1991). Point mutations and deletions have been reported only rarely, even though wider application of NGS and MLPA techniques may show that such mutations are not as rare as presently thought (De Boulle et al., 1993; Wang, Lin, Lin, Li, & Li, 1997; Wells, 2009; Collins et al., 2010; Gronskov, Brondum-Nielsen, Dedic, & Hjalgrim, 2011; Myrick et al., 2014). The corresponding phenotypes may not always be typically FXS. For instance, the case described by De Boulle et al. (1993) displays a much more severe and complex clinical picture, while the case of Myrick et al. (2014) only shows ID and seizures.

Physical Manifestations

The physical phenotype of FXS is manifested mostly in males, and it tends to be subtle, consisting of minor anomalies that can be explained, to a large extent, as secondary to muscular hypotonia and a connective tissue dysplasia (Opitz, Westphal, & Daniel, 1984), that gives the skin a velvety texture. A typical male will present with relative macrocephaly, long and narrow face, tall forehead, hypotelorism, prominent jaw, large and anteverted ears, and high arched palate (Figs. 1.1 and 1.2). An accurate and helpful description of the craniofacial characteristics of FXS can be generated by a 3D analysis of facial photographs of patients, both male and female (Heulens et al., 2013). The hands are characterized by spatulate fingers, deep palmar and interphalangeal creases, hyperextensibility of the wrist, and interphalangeal

FIGURE 1.1 **A boy of about 10 years, with a typically elongated face, midface hypoplasia, and large anteverted ears.**

FIGURE 1.2 **Three boys aged between 6 and 10 years, demonstrating that the typical facial traits may be minimally expressed or lacking altogether.** Nonetheless, the FXS "gestalt" is obvious.

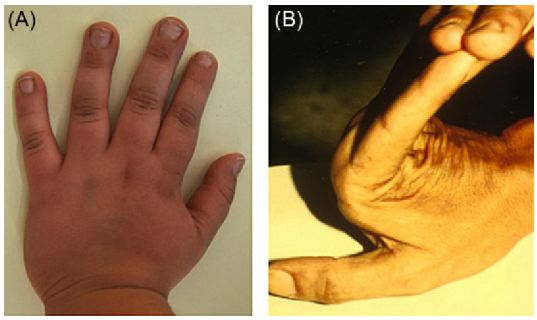

FIGURE 1.3 (A) A view of the hands show spatulated fingers with redundant skin and (B) hyperextensibility of joints.

joints (Fig. 1.3). Droopy shoulders, pectus excavatum, valgism of elbow and knee, and flat feet are also commonly present, consequent to loose ligaments and muscular hypotonia. The most typical finding is represented by enlarged testes (macroorchidism), up to a volume of 50 mL (normal values range from 20 to 30 mL in a young adult). Macroorchidism, present in nearly all adults, is usually not seen in children, becoming evident at puberty. Light and electron microscopic studies on testicular biopsies showed interstitial edema, an increased amount of lysosomal inclusions in Sertoli cells, and disturbance of spermatid differentiation (Johannisson, Rehder, Wendt, & Schwinger, 1987). Although limited, there is evidence indicating that spermatogenesis is deranged, with consequent reduced fertility (Hersh & Saul, 2011). Except for macroorchidism, genitalia are normal.

As already mentioned, the phenotype tends to become more marked with age (Fig. 1.4). The affected newborn may look entirely normal and some degree of reduced muscle tone can be the only indicator, insufficient to establish a specific diagnosis. At this age, feeding problems with frequent gastroesophageal refluxes and chronic otitis media are relatively common. Delayed motor milestones and speech development are further diagnostic indicators before the appearance of the physical traits listed earlier. The stature tends to be taller than average until

FIGURE 1.4 **The changing facial phenotype between the ages of 6 and 26 years in the same individual.**

growth decelerates, ending in a final stature that is on average below the mean. Some children will develop excessive craving for food and become obese, with small extremities and apparent microgenitalism, requiring a differential diagnosis with the Prader–Willi syndrome. It has been suggested that this specific subgroup has lowered expression of a gene in the 15q11–q13 region, encoding the cytoplasmic *FMR1*-interacting protein 1 (CYFIP1) (Nowicki et al., 2007). In older subjects, the facial phenotype, together with hypotonia and joint laxity, becomes more accentuated, facilitating the clinical diagnosis (Fig. 1.5). Mitral valve prolapse, when present, is usually seen in adults, probably a manifestation of connective tissue dysplasia. There are essentially no major malformations to be reported as component manifestations of FXS. Internal organs are normal.

MRI of the brain shows a significantly decreased size of the posterior cerebellar vermis and increased size of the fourth ventricle, as well as significantly increased size of the caudate nuclei (Reiss, Aylward, Freund, Joshi, & Bryan, 1991; Gothelf et al., 2008). These areas of the brain are involved in the regulation of impulse control and attention, and their reported changes may be related to the lack of impulse control and attention deficit commonly present in individuals with FXS. More recent morphofunctional studies showed that FXS individuals have reduced functional connectivity in multiple cognitive and affective brain networks, as

FIGURE 1.5 **Two young men in their early 20s, with more pronounced facial traits, and an older man with full facial manifestations: tall forehead, elongated face, midface hypoplasia, long philtrum and chin, large ears.** The accentuation of the phenotype with age largely depends on the persistent muscular hypotonia.

FIGURE 1.6 **A typical female of approximately 20 years.** The face is elongated due to a tall forehead and a prominent chin.

well as different sets of interconnected subnetworks, compared to control individuals (Hall, Jiang, Reiss, & Greicius, 2013; Bruno et al., 2016).

In postmortem brains of FXS individuals, cortical dendritic spine morphology appears immature, with long, thin spines much more common than the stubby and mushroom-shaped spines that are typical of normal development. In affected individuals there is also a higher density of spines along dendrites, suggesting a possible failure of synapse elimination. While variously misshapen spines are characteristic of a number of ID syndromes, the overabundance of spines seen in FXS is remarkable (Irwin, Galvez, & Greenough, 2000).

The physical phenotype of FXS females, if at all present, is not particularly distinctive. It is similar to that described in males, although usually less marked (Figs. 1.6 and 1.7). Elongated, hypotonic face and large ears are typical traits, when present (van Esch, 2015).

Individuals with FXS are usually in good general health. In addition to epilepsy (see further) and the already mentioned mitral valve prolapse, an increased frequency of ear infections, gastroesophageal reflux, constipation, refractive errors, strabismus, and sleep problems have been reported. These and other emerging medical issues were recently reviewed by Kidd et al. (2014).

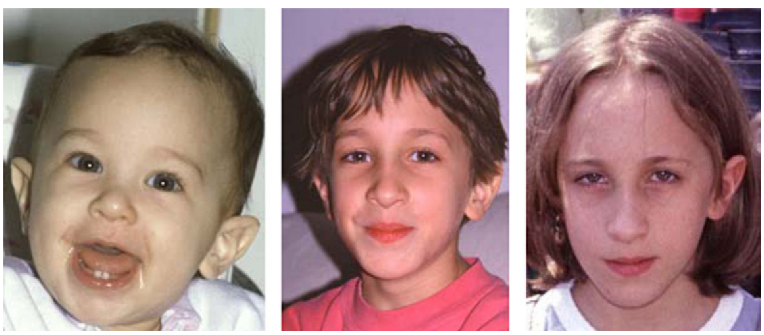

FIGURE 1.7 **A female at the age of 5 months, 8 years, and 13 years, respectively.** In the neonate, the only hint of the syndrome is given by the large ears. Later on, the face becomes elongated and hypotonic.

Epilepsy

The first formal reports on epilepsy in FXS (Sanfilippo, Ragusa, Musumeci, & Neri, 1986; Musumeci et al., 1988) described a characteristic EEG pattern originating during sleep from the temporal lobe. In subsequent, more comprehensive studies based a on larger series of patients and literature review, Musumeci et al. (1999) estimated the prevalence of epilepsy in FXS at approximately 20%. Seizures are age related, usually appearing between 4 and 10 years and disappearing after puberty, with some exceptions (Sabaratnam, Vroegop, & Gangadharan, 2001), and are easily controlled by single-drug antiepileptic therapy. The most common presentation is that of complex partial seizures that involve the temporal and frontal lobes, followed by generalized tonic–clonic seizures and, less frequently, simple partial seizures. The EEG is characterized by a pattern of paroxysmal discharges activated during sleep, as seen in epilepsy with centrotemporal spikes. EEG abnormalities can be observed in a proportion of FXS boys who do not have seizures (Berry-Kravis, 2002). A recent survey showed that epilepsy in FXS is significantly associated with autism, as a cooccurring condition (Berry-Kravis et al., 2010). Epilepsy in FXS is generally benign, with a tendency to resolve spontaneously, even though discontinuation of therapy must be considered with caution because it may lead to recurrence of seizures in some cases.

Cognition

ID, including language delay, is the norm in males with FXS, on average of moderate degree, with IQ ranging between 35 and 55 and a mean around 40, according to Merenstein et al. (1996). Cognitive impairments may affect working and short-term memory, as well as visual–spatial ability. However, there is ample variability, from borderline normal to severe ID, due to a number of modifying factors, yet to be fully understood. The main factor is probably mosaicism for variable states of the *FMR1* mutation, due to its inherent instability. Mosaicism can refer either to size or to methylation. Size mosaicism occurs when some cells contain a premutation (PM) [and are therefore capable of producing fragile X mental retardation protein (FMRP), the protein product of *FMR1*] rather than a full mutation. Methylation mosaicism means presence of cells in which the full mutation remains unmethylated. Such cells are also capable of producing FMRP (deVries, Halley, Oostra, & Niermeijer, 1998). Clearly, a residual production of FMRP, due to the presence of one or the other kind of mosaicism, can attenuate the cognitive delay, explaining the mild ID in so-called high-functioning males (Hagerman et al., 1994). This is further demonstrated by rare individuals of normal intelligence, carriers of a totally unmethylated full mutation (Smeets et al., 1995; Pietrobono et al., 2005; Tabolacci et al., 2008).

Incidentally, it should also be mentioned that Aziz et al. (2003) described 10 boys with *FMR1* premutations, who had clinical manifestations of FXS, including social impairment, speech and language delays, hyperactivity, and autistic features.

Another important modifier is likely represented by the genetic background of each individual, where an abundance of sequence variants, currently of unknown significance, can be expected to influence the level of intellectual proficiency. Lastly, environmental stimuli are of great importance. Early intervention in the areas of psychomotor stimulation, speech therapy, schooling, and social integration are all factors of the utmost importance to improve

cognition. Personal experience with clinical trials experimenting new drugs indicated that one significant obstacle against attaining statistical significance of the observed improvement in the tested behaviors was the placebo effect, suggesting that special attention given to participants in a trial is by itself an ameliorating factor.

The intelligence level in female carriers of a full mutation is even more variable than that in males, ranging from normal to mildly delayed with some learning disability in the majority of cases, being severely delayed only in a small minority of cases. Behavioral problems can also be present. Depression and anxiety are the most common of these, affecting more than 50% of cases, often accompanied by phobias, shyness, and difficulties in establishing social interactions (Roberts et al., 2009b). This ample variability is correlated with the level of FMRP protein being produced, and hence with the status of X inactivation (Willemsen et al., 2003). Inactivation of the normal X in a majority of cells will tilt the balance toward increasingly severe delays and vice versa.

The pathophysiology of intellectual delay in FXS continues to be elusive. Synaptic function and, more generally, brain connectivity, are areas of intense investigation, hopefully leading to a better understanding of this crucial point (Bear, Huber, & Warren, 2004; van der Molen, Stam, & van der Molen, 2014)

The Behavioral Phenotype

The behavioral phenotype of FXS is more typical than the physical phenotype and in a child or adolescent it can provide a better clue toward the clinical diagnosis. Such typical phenotype consists of short attention span, hyperactivity, gaze avoidance, perseverative speech, echolalia, impulsivity, hypersensitivity to sensory stimuli (such as light and sound), emotional lability, stereotypies (such as hand flapping or other repetitive movements), and occasional tantrums (Cornish, Turk, & Hagerman, 2008; Harris et al., 2008; Grefer, Flory, Cornish, Hatton, & Roberts, 2016). Many of these behaviors result from a high underlying level of anxiety and poor adaptability to unexpected situations. Anxiety is, indeed, a most serious problem, interfering heavily with daily activities, and it may be associated to some extent with high parenting stress and lower optimism, as suggested by a recent study (Tonnsen, Cornish, Wheeler, & Roberts, 2014).

On the other hand, and more so in a relaxed and familiar setting, affected boys can sustain a simple conversation, show a good sense of humor, and a behavior generally appropriate to the circumstances. They tend to be affectionate and like to receive attention from others. They have a sense of responsibility and can be engaged in simple, supervised jobs.

It is usually said that autistic features are present in a large proportion of FXS individuals (reviewed by Budimirovic & Kaufmann, 2011). FXS is also reported as the most common monogenic cause of autism, accounting for approximately 5% of subjects ascertained for autism (Schaefer & Mendelsohn, 2008). This is certainly true if autism is intended as autism spectrum disorder (ASD), according to the definition given by the *Diagnostic and Statistical Manual of Mental Disorders* (*DSM-5*). There are reports indicating that ASD comorbidity correlates with lower IQ, poorer adaptive behavior skills, and greater receptive language delays (Hatton et al., 2006; Harris et al., 2008; Roberts, Bailey, & Mankowski, 2009). This concept should be clearly explained to parents, for whom autism continues to be a scary word, evoking Kanner's historical description of children lacking affection and unable to relate themselves

to others, as if they were closed in a shell (Kanner, 1943). In any case, the neurobiological basis underlying the autism–FXS comorbidity is yet to be fully elucidated (Abbeduto, McDuffie, & Thurman, 2014). ASD can also be seen in premutation carriers. Chonchaiya et al. (2012) determined the prevalence of ASD in 50 boys with the *FMR1* premutation compared with their siblings and a control population. Twenty-five boys (probands) were found to carry a premutation after direct referral for developmental issues and another 25 (nonprobands) were found to be carriers after testing, following identification of a family member with either the full mutation or a premutation. ASD was significantly more prevalent among probands compared with both nonprobands and controls, while cognitive and adaptive functioning in nonprobands was similar to that of controls.

The behavioral phenotype of female carriers of a full mutation mimics that of males, although in a milder form. Affected females can be described as shy, anxious, moody, tending to depression, and exhibiting social avoidance.

Treatment

FXS defines a subtle and yet complex phenotype, with many nuances that generate a continuity from the milder cases, bordering with normality, to the more severe ones. The factors modulating the degree of severity were briefly mentioned. Their full clarification is not merely of academic interest, but it can be expected to have important practical implications for the treatment of affected persons. Given that a customized treatment, designed on the needs of each individual, is likely to be more effective than a generic one, it is extremely important that the phenotype of each individual be finely dissected, above and beyond the result of the molecular test, exposing all of its physical, intellectual, and behavioral manifestations.

Until a specific and effective treatment becomes available, the management of FXS persons will continue to rely on traditional methods, according to the symptoms and needs of each individual. Such methods include special educational programs, to be applied preferably within the mainstream of general education with the aid, as needed, of speech/language therapy, occupational therapy, physical therapy, and psychological or counseling services.

Several approved drugs are available for symptomatic medication, as needed, and with special attention to possible side effects. Selective serotonin reuptake inhibitors can be prescribed for anxiety and methylphenidate for attention deficit/hyperactivity disorder. For more serious problems, such as aggressive and self-injurious behaviors, antipsychotic medications can be an option. Consensus guidelines for educational and medical treatments can be found at https://fragilex.org/treatment-intervention/consensus-on-clinical-practices/.

FRAGILE X TREMOR ATAXIA SYNDROME

FXTAS is caused by a premutation in the *FMR1* gene. Premutation alleles contain a number of CGGs that varies between 55 and 200, and are unstable. Their transcription is above normal, with correspondingly elevated levels of mRNA, while translation appears to be less efficient than normal, thus producing lower amounts of FMRP (Tassone et al., 2000, 2010; Kenneson, Zhang, Hagedorn, & Warren, 2001). The prevalence of premutation allele carriers in the general population has been variously estimated at 1/113–259 among females and

1/260–800 among males (Hunter et al., 2014; Hagerman, 2008; Dombrowski et al., 2002; Toledano-Alhadef et al., 2001). Although premutation carriers are usually unaffected intellectually, there are reports suggesting that these individuals may be at risk for cognitive and emotional morbidity (Hunter et al., 2008a; Hagerman, 2006). In these studies, a decrease in hippocampal volume was reported in both males and females, in association with memory deficits (Jäkälä et al., 1997). A smaller whole brain volume was found only in females (Murphy et al., 1999). However, according to a more recent study, there is no firm evidence for an altered neuropsychological profile among premutation carriers under the age of 50 years (Hunter et al., 2008b).

Fifteen years ago, Hagerman and coworkers described FXTAS, a condition that can affect premutation carriers, mostly males above the age of 50 (Hagerman & Hagerman, 2004). It was estimated that for premutation male carriers the risk of developing FXTAS by the age of 50 is approximately 30% (Jacquemont et al., 2004), with a tendency to increase with age. The risk is lower (between 8% and 16.5%) for premutation females (Coffey et al., 2008; Rodriguez-Revenga et al., 2009). FXTAS is a late-onset neurodegenerative disorder, whose major clinical manifestations include progressive intentional tremor, ataxia, parkinsonism (Leehey, 2009; Jacquemont et al., 2003). As recently reviewed by Hagerman and Hagerman (2015) and by Hall et al. (2014), the minimal diagnostic criteria of FXTAS include a premutation of *FMR1*, plus one or more of the following: intentional tremor, cerebellar ataxia, and white matter disease in the middle cerebellar peduncles.

Associated features are cognitive decline, often progressing to impairment of executive functions and dementia (Seritan et al., 2008). Peripheral neuropathy with disautonomia was also reported (Coffey et al., 2008; Seritan et al., 2008). The age of onset of tremor and ataxia was shown to correlate with the CGG repeat length and with the degree of brain atrophy, documented by brain imaging (Adams et al., 2007). Actually, the phenotype in females can be even more complex, including a variety of dysfunctions (Roberts et al., 2009b). Coffey et al. (2008) evaluated 146 female premutation carriers with and without core features of FXTAS (tremor and gait ataxia), and 69 age-matched controls. Compared with controls, carriers with definite or probable FXTAS had greater medical comorbidity, with increased prevalence of thyroid disease, hypertension, seizures, peripheral neuropathy, and fibromyalgia, while the non-FXTAS premutation group had more complaints of chronic muscle pain, persistent paresthesias of extremities, and a history of tremor. An increased prevalence of thyroid disorder was not confirmed by other studies (Hundscheid, Smits, Thomas, Kiemeney, & Braat, 2003; Hunter, Rohr, & Sherman, 2010).

Immunocytochemical staining of postmortem brain tissue from FXTAS patients showed the presence of eosinophilic, ubiquitin-positive intranuclear inclusions both in neurons and astrocytes. Similar inclusions were found in tissues outside of CNS, that is, testes and peripheral nerve ganglia. The number of inclusions correlates with the CGG expansion length (Greco et al., 2002, 2006). According to current interpretation, these inclusions consist of proteins sequestered by expanded *FMR1* mRNA, which is thought to exert a deleterious gain-of-function effect by impairing the normal function of the sequestered proteins (Hagerman & Hagerman, 2015).

Recently it was shown that CGG repeats trigger repeat-associated non-AUG–initiated (RAN) translation of a cryptic polyglycine-containing protein, FMRpolyG. FMRpolyG accumulates in ubiquitin-positive inclusions in *Drosophila*, cell culture, mouse disease models, and FXTAS patient brains, contributing to the development of neurodegeneration that is typical of FXTAS (Todd et al., 2013).

THE FRAGILE X PREMATURE OVARIAN INSUFFICIENCY

Up to 20% of female premutation carriers develop premature ovarian insufficiency (POI), that is, menopause before the age of 40 (Allingham-Hawkins et al., 1999; Wittenberger et al., 2007). Welt (2008) proposed the acronym FXPOI to describe the condition. An analysis of the hormonal profile of female premutation carriers showed increased levels of follicle stimulation hormone and decreased expression of anti-Müllerian hormone (Welt, Smith, & Taylor, 2004; Rohr et al., 2008). Consequences of FXPOI are reduced fertility and early estrogen deficiency, causing increased risk of low bone density, early onset of osteoporosis, and increased risk of coronary disease. The relationship between expansion length and risk to develop FXPOI is not linear, with maximum risk confined to premutation carriers with an expansion of 80–100 CGGs (Sullivan, Welt, & Sherman, 2011; Ennis, Ward, & Murray, 2006). There has been a debate about the hypothesis that a parent-of-origin effect could influence the duration of reproductive life in female premutation carriers, starting with a study by Hundscheid et al. (2000), in which 28% of carriers of a paternally inherited premutation developed FXPOI, versus only 3.7% with a maternally inherited premutation, supposedly due to a paternal imprinting effect. However, this assumption was not supported by another study (Murray, Ennis, & Morton, 2000), in which the analysis of 116 female premutation carriers does not provide evidence that premutation alleles are subjected to paternal imprinting. The molecular basis of FXPOI is not fully clarified, even though there is some evidence suggesting RNA toxic gain of function as one of the possible pathophysiologic mechanisms (Buijsen et al., 2016; Elizur et al., 2016). Further studies on the endocrine profile and, ideally, ovarian histopathology, are warranted. Animal models provide some useful insights, as recently reviewed by Sherman et al. (2014).

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CHAPTER

2

Fragile X Syndrome Genetics

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SETTING THE STAGE

The discovery of the *FMR1* gene and its associated unstable CGG trinucleotide repeat mutation in May 1991 marked a watershed moment in genetics (Oberle et al., 1991; Verkerk et al., 1991; Yu et al., 1991). Along with the subsequent recognition of expansion of unstable repetitive elements as causative mutations in dozens of additional human genetic disorders, the molecular basis for fragile X syndrome (FXS) provided long-sought answers for genetic questions that had defied explanation for many decades. That this fundamental discovery of a genetic principle occurred through study of a human condition reflects the intriguing specificity of the phenomenon, one that is so far unknown in the other animal species that have typically been used to define our understanding of genetic principles. While many questions remain regarding repetitive DNA sequences and their contributions to normal and abnormal phenotypic variation, remarkable progress in the intervening years has led to a rich understanding of the biology of *FMR1* and FXS, allowing consideration of rationally designed therapeutic interventions in this common condition of intellectual disability.

As in other fields of biology, human genetics was revolutionized by the rapidly improved methods for manipulation and detection of DNA that began in the 1970s with the discovery of restriction enzymes. The genetic, phenotypic, and cytogenetic descriptions of FXS were well advanced by the mid-1980s, and the disorder's unusual genetic characteristics along with its apparently high frequency made it a prime target as the methods for positional cloning were developed.

The ability to analyze chromosomes using stains to define banding patterns and to identify anomalies during the 1960s and 70s allowed individuals with common abnormal phenotypes to be categorized. In some cases, these studies suggested potential chromosome locations that might be involved. In the case of FXS, the 1969 discovery of an anomaly in the distal end of the long arm of the X chromosome, which could be elicited by several chemical treatments (typically reduced folate levels) of lymphocytes cultured from patients' blood, allowed collection of individuals who had similar phenotypic features with a potentially common genetic etiology.

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FIGURE 2.1 **The fragile X chromosome.** A Giemsa-stained X chromosome from a patient with fragile X syndrome (FXS) showing the characteristic fragile site *(arrow)*.

Thus, individuals with an apparently X-linked form of intellectual disability accompanied by a set of physical features first described by Martin and Bell (Martin & Bell, 1943) could be classified as carrying a marker (later termed fragile) X chromosome (Lubs, 1969). Efforts by Sutherland (1977) allowed consistent methods for producing the fragile site in cells from affected individuals (Fig. 2.1). This provided the ability to collect and analyze families where the FXS was segregating.

Sutherland found many chromosomal fragile sites in the human genome. Some of these were common in the population, but the fragile site at Xq27.3 associated with FXS was of the rare, folate-sensitive variety. As the first fragile site described on the X chromosome, the locus was designated FRAXA. Numerous studies of individuals with intellectual disability followed rapidly. These found a high frequency of the FRAXA site in the cognitively disabled and a rate in the general population estimated to be as high as 1/1200 individuals (Webb et al., 1986; Turner et al., 1986). A high false-positive rate for the chromosome test was later found to have skewed this estimate, and the true frequency is approximately 1/3500 (Crawford et al., 2002; Hagerman & Hagerman, 2002). While there are some isolated populations with a higher frequency due to a founder effect (Falik-Zaccai et al., 1997), this estimate is consistent across the world.

GENETIC ODDITIES

The high frequency of FXS, coupled with the observation that most males affected by FXS do not reproduce, led to the concept that the disorder must be due to an astonishingly large rate of new mutation. In X-linked genetic conditions that lead to reduced or absent reproduction, approximately one-third of the pool of X chromosomes carrying the mutation is lost at each generation. Thus, the frequency of new mutation can be estimated as that required to replenish the lost chromosomes to maintain the frequency of the disorder. The observation of a high disease prevalence suggested an anomalously high mutation frequency for FXS ($\sim 7 \times 10^{-4}$), thousands of times the estimates for the average locus (Sherman et al., 1984). This was another curious feature of FXS that spurred interest in understanding the genetics that underlay this common disorder.

Studying over 200 families carrying FRAXA site and the FXS phenotype, Sherman, Jacobs, and coworkers noted unusual genetic transmission of the disorder (Sherman et al., 1984; Sherman et al., 1985). Their work extended that of Martin and Bell, who described incomplete penetrance of what they presumed to be an X-linked recessive mutation in the large original family (they described both mildly affected females and males who transmitted the disorder without being affected). Sherman et al. were able to quantify the increasing likelihood of intellectual disability in later generations transmitting the fragile X chromosome, with probabilities based on the affected status of close relatives and which parent contributed the mutant X chromosome. As noted by Martin and Bell and others, many families had unaffected males without the FRAXA site who had clearly transmitted their X chromosomes to their daughters who had then gone on to have sons with FXS and the FRAXA site. The presence of "normal transmitting males" suggested a suppression of the physical and chromosomal phenotypes rather than new mutations with germline mosaicism because in some families siblings transmitted in a similar fashion. Another feature apparent in the pedigrees was that affected females (who were present at a rate lower than males) never inherited their disorder from normal transmitting males, only from mothers. Thus, daughters of normal transmitting males had no risk of being affected with cognitive disability. Affected individuals were only found in sibships where the mother transmitted the mutant X chromosome, whether she was affected or not. Sherman et al. (1985) suggested the notion that a "premutation" might account for the presence of unaffected male carriers, with conversion to a "full mutation" requiring passage through the female germline. The discovery of the unstable CGG repeat allowed validation of this hypothesis, where repeat instability proved to be different in cells of the male and female germline.

These observations of reduced penetrance, variable expressivity and risk that is dependent on position in a pedigree segregating FXS became known as the "Sherman paradox," a short-hand to describe the complicated behavior of the mutation as it passed through the generations. Thus, fragile X families had characteristics reminiscent of the long-debated phenomenon of genetic anticipation, largely defined by features of myotonic muscular dystrophy (DM), where subsequent generations in this autosomal dominant condition showed more severe forms and earlier age-of-onset of the disorder.

Fierce debate among geneticists about the concept of genetic anticipation spanned the 20th century. The debate began in the age of eugenics with ideas about families where members became less fit with each generation due to underlying degenerate "genes" and DM was held up as a prominent example. Eminent geneticists, such as Lionel Penrose countered that families showing anticipation as in DM were simply ascertained in a biased fashion, and that there was no known mechanism for such a phenomenon. Agreeing with Penrose, Curt Stern argued in his classic 1973 text *Human Genetics* that:

> The concept of anticipation does not readily fit in the system of genetic facts and interpretation.…Indeed, anticipation seems a statistical rather than a biological phenomenon. (Stern, 1973)

Stern anticipated a possible explanation: in the same section of the text, referring to the possibility that an unstable mutation might underlie the phenomenon of anticipation, he wrote:

> It is therefore not reasonable to explain the high variability of certain phenotypic effects by a low stability of the genes that control them. (Stern, 1973)

The discovery of an unstable sequence as the causative mutation in FXS demonstrated a mechanism that could explain the intergenerational variability in both this disease and possibly also in DM. The 1992 description of an unstable CTG repeat where length of the expansion could account for disease severity in DM further validated the concept (Shelbourne & Johnson, 1992). These early findings paved the way for understanding additional similar effects on severity and age of onset in other disorders that showed variation dependent on parent of origin and position in the pedigree. For example, it had long been known that in the highly penetrant, autosomal dominant, late-onset neurodegenerative disorder Huntington disease, children of fathers who carry the mutation typically showed significantly earlier onset of symptoms. With the appreciation of an unstable CAG repeat as the cause of the disorder, this could be largely explained by the enhanced instability of the repeat when transmitted by fathers and by the propensity for longer expansions of the repeat to cause earlier onset of the disorder.

Today, at least 24 genetic disorders are caused by unstable simple repeats (Fig. 2.2). The causal repeat may be located within any portion of a gene with its location often resulting in its downstream effects. For example, the most abundant class expands a CAG repeat located within a coding exon thus resulting in the increased length of a normal polyglutamine tract. Some repeats located in noncoding portions of a gene, most notably DM1 and DM2, which have a 3′ UTR CTG or intronic CCTG repeat respectively, appear to exert their effect via an RNA-mediated process where RNA-binding proteins appear sequestered on the repeat sequence, limiting their normal function. In other expanded repeats, such as FXS and Friedreich ataxia, increased length can trigger an epigenetic silencing of the affected gene. Hence the discovery of the unstable repeat in *FMR1* in 1991 has led to a major, and still human-specific, class of disease-causing mutations.

POSITIONAL CLONING OF FRAXA AND *FMR1*

As the FRAXA site was rare in the population and segregated completely with the disorder in families, it was reasonable to assume that the genetic lesion responsible for the syndrome was also causing the fragile site. Linkage analysis with genetic markers in the region confirmed that DNA markers this portion of the X chromosome were linked to the disorder (Carpenter, Thibodeau, & Brown, 1991; Dahl et al., 1989; Goonewardena et al., 1991). Refinement of the location on the genetic map was possible with the ongoing enhancements of DNA markers. By the late 1980s, the genetic interval was small and methods for isolating DNA fragments had advanced to the point where multiple teams of investigators set out to isolate DNA from the region.

One of the principle tools that all the groups employed in this effort was a panel of somatic cell hybrids developed by the Warren group. Developed in the 1970s, somatic cell hybrid mapping was a popular method for assigning enzymes and DNA markers to chromosomes. The method involves the isolation and selection of chromosomes or chromosome fragments in cell lines of a different species after cell:cell fusion. For mapping human markers, it was possible to fuse human cells with rodent (typically hamster) cell lines that tended to eliminate the human chromosomes after fusion. By using a rodent partner that was deficient for an essential enzyme, it was possible to select for desired human chromosomes carrying the

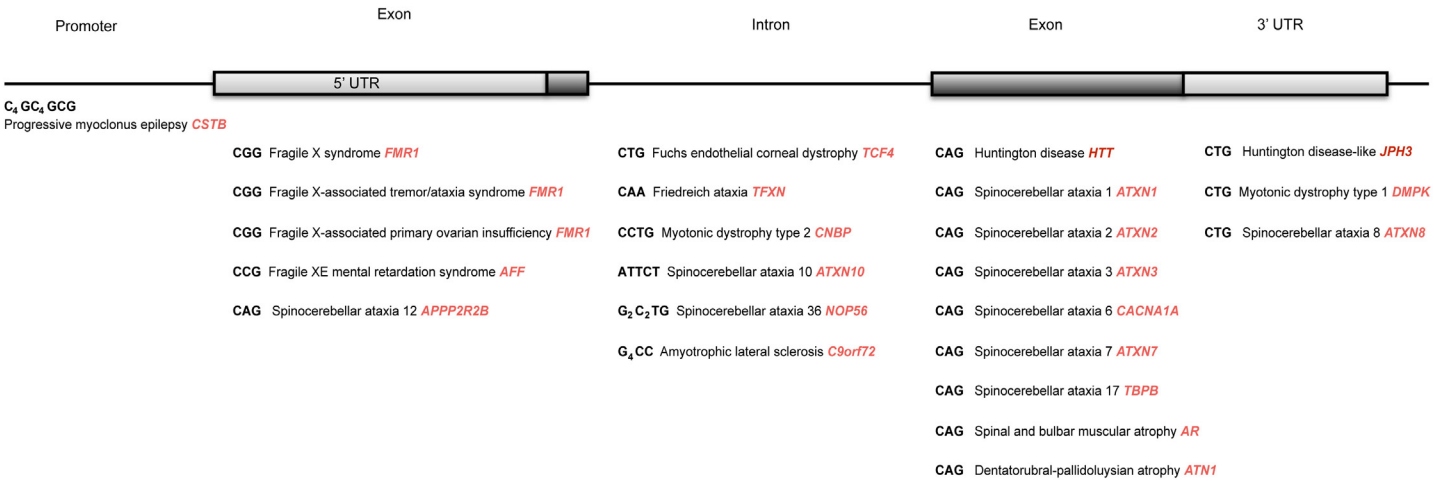

FIGURE 2.2 **Twenty four disorders due to simple repeat expansion.** A generic gene structure showing the location of the unstable repeats *(bold)* followed by the disease name and gene *(red)*.

complementary enzyme. The presence of the X chromosome could be readily selected for in hypoxanthine-guanine phosophribosyl transferase (HPRT) deficient rodent cells using media that prevented growth of cells lacking the enzyme. Warren developed a series of cell lines that isolated X chromosomes from a fragile X individual and demonstrated that the fragile X chromosomes remained sensitive to alterations in folate in the medium. He further showed that he could induce and select for breakage events that frequently occurred at or near the FRAXA site. These events allowed him to develop a panel of somatic cell hybrids where each line carried a human fragile X chromosome that was broken and fused to a random hamster chromosome. This panel allowed for testing of DNA fragments from the region of the fragile site, physically ordering them relative to the breakpoints, and allowing their positions relative to the FRAXA site to be determined. These efforts to place DNA markers used in the genetic map onto a physical map helped to further narrow the interval where the FRAXA site was located. The panel was also a vital tool for validating the fragments at the FRAXA site once they were identified.

Parallel efforts to develop a restriction fragment based physical map were ongoing in several groups (Bell et al., 1991; Hirst et al., 1991; Vincent et al., 1991). These studies took advantage of pulsed-field gel electrophoresis (PFGE), which provided the ability to separate the very large (100–1000 kb) restriction fragments of DNA that result from digestion with restriction enzymes that recognize infrequently occurring DNA sequences. Southern hybridization of restriction fragments derived from several enzymes using probes from the Xq27.3-Xq28 region allowed an additional map to be assembled and compared with the genetic and somatic cell hybrid maps. As many of the rare-site restriction enzymes are also sensitive to DNA methylation, and because this method can be applied to DNA samples from affected individuals, it became apparent that there were differences between the maps produced from fragile X individuals and those from the general population. The differences appeared to demonstrate a region near the FRAXA site that had increased levels of methylation, marked by absence of digestion with certain restriction enzymes, in FXS individuals. This fueled speculation that alterations in methylation of CpG sites near FRAXA could be a feature of FXS, possibly signifying alterations in gene expression (Oberle et al., 1991).

Another critical methodology that allowed identification of the FRAXA site was the development of methods for large-insert DNA cloning. The ability to isolate megabasepair length fragments of human DNA in yeast artificial chromosomes (YACs) offered the opportunity to characterize large regions without cumbersome methods to extend from one cosmid length insert (40 kb) to the next. Nelson et al. (1991) developed libraries of YACs from the human X chromosome using somatic cell hybrids carrying either the whole X or a fragment from Xq24-Xqter. Employing additional hybrid cell lines, it was possible to define the locations of the inserts in the YAC clones on the X chromosome. Using this approach and the Warren panel of hybrids with breakpoints at the FRAXA site, one YAC clone was found to be the closest distal marker to the FRAXA site to be identified. This led Nelson and Warren to collaborate to identify the FRAXA site and mutation. They engaged the groups of C. Thomas Caskey and Ben Oostra in the effort. Oostra's group in Rotterdam provided access to a library of larger YAC clones developed by the CEPH group in Paris. The identification of the 450 kb YAC 209G4 provided a DNA fragment that spanned the FRAXA site as defined both by breakpoints in the Warren hybrid panel and also by in situ hybridization to intact fragile X chromosomes. Refinement using cosmid subclones of the YAC allowed the FRAXA site to be narrowed to

a region that was rich in CpG dinucleotides and heavily methylated specifically in fragile X chromosomes. Increased length and familial instability of restriction fragments isolated from the DNA of fragile X individuals pointed to a region of containing CGG repeats as the likely source of both length and methylation differences. Screening of cDNA libraries with DNA fragments from the region yielded evidence for transcription of a gene that contained the CGG repeats in its 5′ untranslated region (Verkerk et al., 1991). Termed *FMR1* (Fragile X Mental Retardation-1), this transcript initiated from a CpG island that was slightly proximal to the CGG repeats and extended for some 40 kilobasepairs to the distal direction. Its sequence suggested no function; it was unlike any gene described at the time.

Work in the laboratories of Grant Sutherland and Rob Richards and of Jean-Louis Mandel also yielded DNA fragments from YACs that detected the unstable DNA with anomalous methylation using Southern blot hybridization (Oberle et al., 1991; Yu et al., 1991). Both groups later described CGG repeats as the basis for the instability. The Caskey, Nelson, Oostra, and Warren group subsequently demonstrated the loss of expression of *FMR1* that resulted from repeat expansion and methylation (Pieretti et al., 1991). Thus the basis for FXS appeared to be loss of function of *FMR1*. Efforts to understand the function of this gene are ongoing and several chapters in this volume discuss their status in much more detail.

FMR1 STRUCTURE AND FUNCTION

FMR1 is expressed in most mammalian tissues (Devys et al., 1993), and is particularly abundant in the brain and testes (Devys et al., 1993; Hinds et al., 1993). In the brain, *FMR1* is expressed primarily in neurons, and its protein (termed FMRP) can be found in the cell body, as well as in more distal compartments, such as dendrites (Devys et al., 1993; Feng et al., 1997a; Weiler et al., 1997). A small amount of fragile X mental retardation protein (FMRP) can be detected in the nucleus. FMRP expression begins early in development and continues throughout life (Hinds et al., 1993). Due to the focus on cognitive deficits in FXS, functions of FMRP in cells other than neurons has received less attention, although a role for glial cells in fragile X phenotypes is increasingly likely (Gholizadeh, Halder, & Hampson, 2015; Pacey & Doering, 2007; Wang et al., 2004).

Human *FMR1* is encoded in 17 exons that span 38 kb of Xq27.3 (Eichler et al., 1993) and has been well conserved in evolution, with homologs found in all vertebrates and some invertebrates. In most vertebrates, including all mammals, two well-conserved paralogous genes, *FXR1* and *FXR2*, have been described (Siomi et al., 1995; Zhang et al., 1995). These are coexpressed in many tissues. There is evidence that they can compensate for loss of *FMR1* function in some instances and that they have additional functions that *FMR1* does not (Bontekoe et al., 2002; Cook et al., 2014; Darnell et al., 2009; Mientjes et al., 2004; Xu et al., 2011).

Fig. 2.3 shows the generation of several alternatively spliced mRNAs that encode several isoforms of FMRP in humans (Ashley et al., 1993a; Verkerk et al., 1993) and mice (Ashley et al., 1993a). The well-conserved fruit fly (*D. melanogaster*) dFMR1 produces long and short isoforms (Banerjee et al., 2010) that may have important differences in expression and function. The short dFMR1 lacks a glutamine/asparagine (QN)-rich protein interaction domain in the C-terminus of the protein; deleting the long isoform revealed that the short isoform,

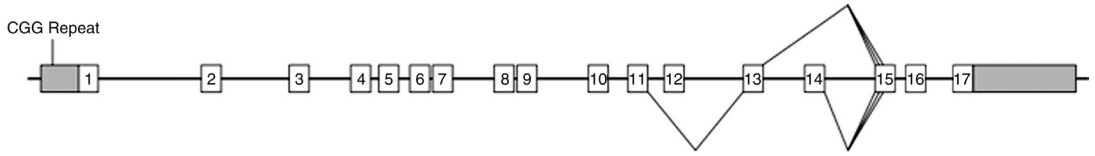

FIGURE 2.3 **Gene structure and splicing pattern of *FMR1*.** *FMR1* is composed of 17 exons. The alternative splicing at the 3′-end of the gene results in 12 mRNA isoforms. The CGG repeat located in the 5′-untranslated region (UTR) is the site of the common expansion mutation in fragile X syndrome. Boxes, exons; straight lines, introns; angled lines, alternative splicing; grey boxes, untranslated regions.

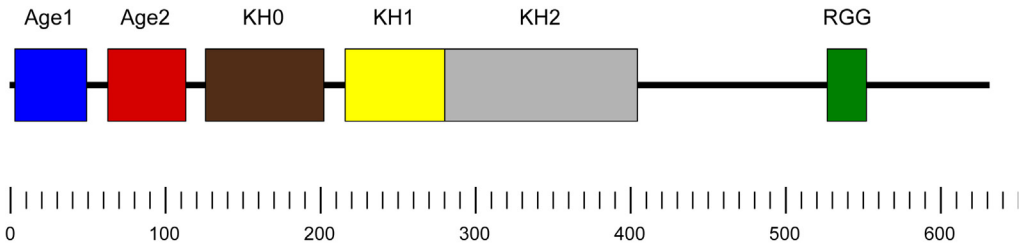

FIGURE 2.4 **Major protein domains of FMRP.** KH0, KH1, KH2, and RGG domains are involved in RNA-binding. The Agenet *(Age)* domains are similar to other protein domains known to interact with histones and trimethylated peptides. FMRP also contains NES and NLS sequences (not shown).

without the QN domain, is insufficient for flies to properly form short- or long-term memories (Banerjee et al., 2010). The corresponding C-terminal region in human FMRP mediates interaction with kinesin and dendritic transport (Dictenberg et al., 2008). Most studies have utilized human or mouse isoform 1, which is the full-length, so-called canonical transcript including all exons. However, Brackett et al. (2013) showed that, at the mRNA level, isoform 7 is typically the most abundant isoform. It remains unclear what function individual isoforms might have in mammals.

The main mammalian isoform of FMRP is a 71-kDa protein containing several conserved functional domains (Fig. 2.4). FMRP has four RNA-binding motifs, including three K homology (KH) domains (KH0, KH1, and KH2) and a region enriched in arginine-glycine-glycine residues termed an RGG box (Myrick et al., 2015a). Evidence from several studies suggests that FMRP binds RNAs in a sequence-specific manner mediated by these domains. Arginine methylation in the RGG box has been found to regulate FMRP's affinity for certain RNAs (Blackwell, Zhang, & Ceman, 2010). FMRP also contains nuclear localization and export signals (NLS and NES) (Eberhart et al., 1996), which assist its movement into and out of the nucleus (Feng et al., 1997a). FMRP also contains two tandem Agenet domains at its N-terminus (Maurer-Stroh et al., 2003). The Agenet domain is one member of a proposed "Royal Family" of protein domains that also includes the Tudor, MBT, and Chromo domains (Maurer-Stroh et al., 2003). Agenet domains have been shown to bind trimethylated lysine residues and show structural similarity to the UHFR1 protein, which is believed to interact with methylated histone H3K9 (Adams-Cioaba et al., 2010).

FMRP AND mRNA METABOLISM

FMRP is a selective RNA-binding protein, found in early studies to bind 4% of the mRNA in the mammalian brain by weight (Ashley et al., 1993b). Microarray and yeast 3-hybrid assays identified some 400 mRNAs that associate with FMRP (Brown et al., 2001; Dolzhanskaya et al., 2003; Miyashiro et al., 2003; Zou et al., 2008), although only 14 were validated with direct biochemical interaction. RNA secondary structures, the best studied of which is the G-quadruplex, mediate binding of FMRP with its target RNAs. A G-quadruplex consists of two to four G-quartets/tetrads stacked on top of each other, with each G-quartet made of four guanines in a planar conformation interacting via cyclic Hoogsteen-type hydrogen bonds (Joachimi, Benz, & Hartig, 2009). FMRP's C-terminal RGG box recognizes G-quadruplexes in vitro (Darnell et al., 2001), and several of FMRP's target mRNAs are predicted to assume G-quadruplex structures. Biochemical assays confirmed that G-quadruplexes mediate the interaction of FMRP with *Fmr1*, *MAP1b*, and *Sema3F* mRNAs (Menon & Mihailescu, 2007 Menon, Mader, & Mihailescu, 2008; Schaeffer et al., 2001).

Additional RNA secondary motifs have been identified among FMRP targets. Multiple U-rich pentamers were found in both coding and 3′-UTR regions of some FMRP target mRNAs (Chen et al., 2003), and one study used UV cross-linking and mutagenesis assays to show that FMRP binds a U-rich region in the 5′-UTR of hASH1 (Fahling et al., 2009). A secondary structure referred to as a "kissing complex" has also been reported to bind FMRP's KH2 domain in vitro (Darnell et al., 2005). Finally, a recent study also showed that FMRP binds to superoxide dismutase 1 (*Sod1*) mRNA through a novel RNA structure termed Sod1 Stem Loops Interacting with FMRP (SoSLIP) (Bechara et al., 2009). SoSLIP consists of three stem-loop structures separated by short stretches of single-stranded RNA and acts as a translational activator (Bechara et al., 2009). SoSLIP interacts with FMRP's C-terminal region, which includes the RGG box, and competes for binding with the G-quadruplex structure (Bechara et al., 2009).

Many of the mRNAs targets of FMRP have been found to localize to dendrites in neurons. Among these targets, fluorescence in situ hybridization (FISH) demonstrated the dendritic localization of *RGS5*, *GABA-Aδ*, *SAPAP3/4*, and *eEF1A* mRNAs (Huang, Chotiner, & Steward, 2005). *Map1b* mRNA was also found to be located in dendrites localized with FISH (Antar et al., 2005); it and *Arc/Arg3.1* mRNA both copurify when FMRP is isolated in brain extracts using specific antibodies (Zalfa et al., 2003). In addition, *Arc/Arg3.1* and *CamKIIα* mRNAs have been reported to be present in dendrites (Bramham & Wells, 2007). Finally, the mRNA encoding the abundant scaffold of the dendrite, *PSD-95*, has been shown to directly associate with FMRP in dendrites both in vitro and in vivo (Muddashetty et al., 2007; Zalfa et al., 2007). Together, these findings support a model in which FMRP binds and regulates a subset of dendritic mRNAs. This lends support to the notion that FXS results from aberrant regulation of dendritically translated proteins due to absence of FMRP.

FMRP is found to cosediment with polyribosomes in both neuronal and nonneuronal cells (Feng et al., 1997b; Khandjian et al., 2004; Stefani et al., 2004). This association with polyribosomes supports the hypothesis that FMRP acts as a translational regulator for its mRNA targets. This association is eliminated by puromycin, which disrupts actively translating polyribosomes, demonstrating that FMRP is associated with actively translating polyribosomes (Stefani et al., 2004). In addition to interacting with polyribosomes, FMRP can be found in stress granules, structures that sequester mRNAs whose translation is being suppressed, in

mRNA-protein complexes (Didiot et al., 2009). Thus FMRP is thought to dynamically regulate translation of its mRNA partners.

In most studies, FMRP is found to inhibit translation of its target mRNAs. FMRP has been shown to reduce translation of numerous mRNAs in rabbit reticulocyte lysate, *Xenopus laevis* oocytes, and immortalized cells from an *Fmr1* KO mouse (Mazroui et al., 2002; Laggerbauer et al., 2001). In the reticulocyte assay, removal of the FMRP binding site from the *MBP* mRNA abolished FMRP's ability to repress its translation, confirming that FMRP binding was necessary for translation suppression (Li et al., 2001). Biochemical and genetic assays also indicate that *D. melanogaster* dFmr1 represses translation of the *Map1B* ortholog *futsch* (Zhang et al., 2001). In vivo assays demonstrated that the target proteins Map1B, Arc/Arg3.1, and CamKIIα are increased in abundance in the brains of *Fmr1* KO mice, consistent with the loss of FMRP-mediated repression (Zalfa et al., 2003; Lu et al., 2004). To specifically interrogate FMRP's effect on translation at synapses, synaptoneurosomes (a brain preparation enriched in synaptic structures, SNS) from *Fmr1* KO mice were examined. Increased levels of Map1B, CamKIIα, and Arc/Arg3.1 proteins were found (Zalfa et al., 2003). Subcellular fractionation of *Fmr1* KO SNS also revealed a shift of CamKIIα, PSD-95, and GluR1/2 mRNAs onto actively translating polyribosomes, consistent with these mRNAs being derepressed (Muddashetty et al., 2007). Surprisingly, FMRP was found to increase translation of *Sod1* mRNA by strengthening SoSLIP's ability to activate translation (Bechara et al., 2009) and *hASH1* through an as yet unknown mechanism (Fahling et al., 2009). In general FMRP has been found to repress translation of targets, but it can also activate translation of some transcripts. The details of mechanisms of RNA association and regulation of translation, the cell and animal models that have been essential to discovering FMRP's functions, the numerous biochemical pathways affected by FMRP's absence along with possible avenues for treatment can be found in other chapters in this volume.

The function(s) of *FMR1* discovered so far have significant appeal to understanding the pleiotropic nature of the clinical presentation of individuals with FXS, but numerous examples of unexpected roles for this gene and protein suggest caution. For example, Alpatov et al. (2014) demonstrated that the Agenet domain of FMRP binds methylated histones and modulated the DNA damage response. Similarly, Myrick et al. (2015b) demonstrated that the amino terminus of FMRP binds BK channels and modulates the action potential in the axon. After a quarter century of many groups' efforts to understand the consequences of FMR1's absence, there remains much to be understood.

RESOLVING THE SHERMAN PARADOX

Understanding the dynamics of the unstable CGG repeats in *FMR1* was greatly aided by the large numbers of families and researchers that had been assembled during the race to identify the gene and mutation. Over the remainder of 1991, the American-Dutch, French and Australian groups compared their data with several other groups at three meetings: the Cold Spring Harbor Genome Mapping and Sequencing meeting in May, the biennial International Fragile X and X-linked mental retardation conference held in Strasbourg in August and at a special session organized for the International Congress of Human Genetics in Washington DC in October. By August, the American-Dutch group demonstrated that large expansions

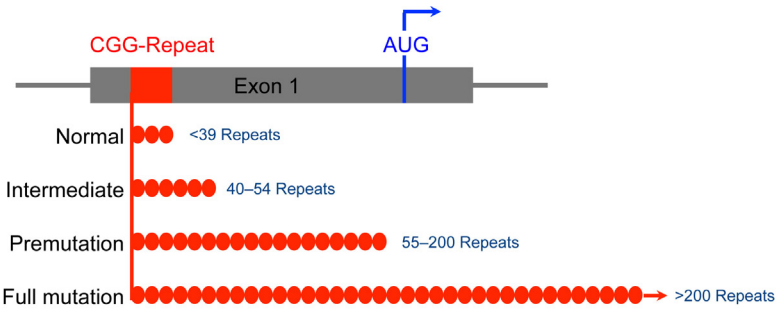

FIGURE 2.5 **Allelic classes of *FMR1*.**

of the CGG repeat were associated with absence of the *FMR1* transcript (Pieretti et al., 1991). Characterization of the association of repeat length and propensity for instability and FXS was limited in resolution until methods for PCR-based amplification of the repeat lengths in the normal and premutation ranges were established. This allowed the refinement of the length thresholds for categories of the repeats' effects and the resolution of the Sherman paradox (Fu et al., 1991).

Today we recognize that the *FMR1* repeat has four allelic classes (Fig. 2.5). Alleles found in the general population can range in length from 5 to 44 triplets, with the most common alleles harboring 29 or 30 repeats. There are interrupting AGG triplets in these alleles. These are typically found at the ninth or tenth repeat, and again at the 19th or 20th triplet. Numerous studies point to AGG interruption as having a stabilizing influence on the repeat (Eichler et al., 1994; Kunst & Warren, 1994), Alleles in individuals with FXS, referred to as *full mutation* alleles, typically measure well over 200 repeats, often as many as several hundred. They are usually mosaic in length within the individual, indicating instability during development, and appear to have few, if any interruptions of the CGG triplet. Full mutations derive in all cases from *premutation alleles*, which are defined by lengths between 55 and 200 repeats. In contrast to alleles in the normal range, premutation alleles are meiotically unstable, and can expand to full mutation lengths during maternal transmission. All full mutations are derived from maternal premutations. The fourth allelic class is termed *intermediate alleles*, with lengths between 45 and 54 repeats. While repeats in this length category are not known to expand to full mutations, and pose no risk to offspring, they can be unstable within families, and their instability can depend on the presence and pattern of AGG interruptions. They often mutate in a single transmission by one or two repeats (Zhong et al., 1996). Many mechanistic studies have begun to unravel the basis for instability of longer *FMR1* CGG repeats and these are described in another chapter of this volume.

Unaffected males carrying premutation length alleles comprise the normal transmitting males noted by Sherman et al. (Sherman et al., 1985). The Sherman paradox was resolved with the recognition that the length of the maternal premutation predicts the risk of an allele's expansion into the full mutation range during transmission to offspring (Fu et al., 1991). For example, a woman with a premutation of 60–69 repeats has a 2% chance of the allele expanding beyond 200 repeats, compared to a woman with 90–99 repeats, who has a 94% risk (Nolin et al., 2011), with an escalating risk for increasing repeat lengths. Further improvement of

PCR surveillance of repeat length has allowed refinement of the risk of having an affected child by characterizing the presence of AGG triplets in a mother's premutation allele (Nolin et al., 2011; Yrigollen et al., 2012).

When the CGG repeat in *FMR1* expands into the full mutation range, epigenetic alterations are triggered, resulting in widespread methylation of the gene that is correlated with its loss of transcription (Pieretti et al., 1991). Indeed, the methylation changes described earlier in early PFGE mapping of the FRAXA locus resulted from this effect of the full mutation. This methylation imprint occurs early in embryogenesis (Malter et al., 1997; Sutcliffe et al., 1992) and leads to histone changes that reflect the gene silencing (Coffee et al., 1999, 2002). Thus the full mutation is a null mutation, and the absence of its encoded protein, FMRP, is responsible for the disorder (Meijer et al., 1994). Several groups have explored the mechanisms for silencing and the potential for reactivation of the locus, and these are discussed in other chapters of this volume.

Although they are fewer than might be expected, conventional mutations of *FMR1* have been described. Most are clear loss of function mutations that disrupt expression by deletion, nonsense, or splice site mutations. These lead to physical and cognitive phenotypes that fit well into the definition of FXS (Coffee et al., 2008; Gronskov et al., 2011; Lugenbeel et al., 1995; Wang et al., 1997). These null mutations establish that the phenotype clinically recognized as FXS is the sole consequence of the loss of *FMR1* function but do not add to our understanding of FMRP function. Single base variants can often reveal aspects of protein function. Some missense mutations identify amino acids critical to maintaining tertiary structure, for example I304N and G266E both disrupt FMRP structure leading to a fragile X-like phenotype (De Boulle et al., 1993; Myrick et al., 2014). An amino terminus missense mutation (R138Q) maintains the canonical FMRP function of RNA-binding and translational regulation while failing to bind the BK channel, leading to a prolonged action potential (Myrick et al., 2015b). This finding suggests that FMRP has independent presynaptic and postsynaptic functions. Okray has shown that a guanine insertion (14579insG) alters the carboxy terminus of FMRP, losing both the RGG box and nuclear export signal (Okray et al., 2015). Suhl recently identified a variant (c.*746T > C) in the 3′ untranslated portion of *FMR1* in a patient with developmental delay and moderate intellectual disability (Suhl et al., 2015). It was shown that this variant results in the loss of synaptic translation of FMRP in an activity dependent manner by abrogating HuR binding to the *FMR1* message. Thus, small variants of *FMR1* may be quite informative regarding FMRP function but are not typically identified clinically as genetic testing focuses largely of the repeat expansion and not *FMR1* sequencing. Hopefully, with increasing clinical whole exome sequencing, additional variants will come to light and reveal additional functions.

PREMUTATION DISORDERS

Premutation alleles carry the risk of having a child with FXS (in females), but were initially thought to be benign for the health of men and women who carried them. Nearly a decade after the initial description of the FMR1 repeat, it became clear that premutations could result in phenotypes that do not resemble FXS. Women who carry the premutation allele are at ~25% risk of primary ovarian insufficiency, referred to as ***F***ragile ***X***-associated ***P***remature

Ovarian ***I***nsufficiency, or FXPOI (Sullivan et al., 2005; Conway et al., 1998). In these carriers, menopause prior to 40 years of age, and sometimes considerably earlier, has a major impact on their reproductive futures, as well as their risk for earlier health sequelae following menopause. While the mechanism behind FXPOI is poorly understood, the greatest risk appears among women with premutation alleles in the middle of the premutation range (Sullivan et al., 2005). Premutation males, on the other hand, are at substantial (greater than 50%) risk of ***F***ragile ***X***-associated ***T***remor ***A***taxia ***S***yndrome, or FXTAS, a late-onset neurodegenerative disorder characterized by tremor, ataxia, and cognitive decline. Individuals with FXS are not at risk for these disorders; they appear to result from expression of mRNAs carrying the expanded CGG repeats, which have toxic effects in cells. The focus of this volume is on FXS and we refer the reader to the following reviews for details of studies of FXTAS and FXPOI (Hagerman & Hagerman, 2016; Loesch & Hagerman, 2012; Pastore & Johnson, 2014).

ORIGINS OF FXS

A chapter on the epidemiology of FXS is offered in this volume. While the demonstration of premutations that can pass for many generations without causing FXS offered an explanation for the astonishingly large rate of mutation, the origins of the premutation remains a puzzle. Inspection of nearby markers can define chromosomal origins of premutation alleles. Some haplotypes defined by nearby markers are increased in frequency in chromosomes carrying premutation alleles. Some of these apparently predisposed haplotypes exhibit unusual patterns of AGG interruption in the repeat (Eichler et al., 1994) and might suggest mechanisms for becoming unstable. Alternatively, other, closely linked sequence variation could play a role. It is likely that premutation length alleles can persist for dozens or hundreds of generations before expanding into full mutations. This reduces the need to invoke a high new mutation rate, but raises questions about how premutations came to be at their high current abundance in the population. Estimates suggest that 1/151 women carries a premutation allele (Seltzer et al., 2012). Due to this high frequency of premutation, most diagnoses of FXS occur in families with no prior history and it is increasingly common for women contemplating pregnancy to undergo testing for their *FMR1* repeat length.

CONCLUSIONS AND PERSPECTIVES

As described earlier, the recognition that mutations in unstable repeat sequences caused FXS provided an explanation for the long-standing phenomenon of anticipation in human genetic disease. It allowed for improved understanding of how a mutation segregating through a family could manifest very differently because it relaxed the requirement for the mutation to be identical from individual to individual. This conceptual advance allowed us to define the molecular underpinnings of parent of origin effects, such as the requirement for the fragile X premutation to be passed by mothers in order to expand into the full mutation range and cause FXS, or the earlier onset of symptoms in children of fathers carrying the Huntington disease repeat expansion compared with those inheriting the mutation from their mothers.

Many of the mechanistic aspects of the behavior of the CGG repeats at the *FMR1* locus remain poorly understood, and there are many challenges that remain to completely understand the basis for the instability and downstream effects of expansions of these and other disease related expansion. Other chapters in this volume address *FMR1*, consequences of its absence in humans and model organisms, and the potential for treatments to alleviate symptoms. Investigations into the detailed mechanisms of instability, epigenetic silencing, and parent of origin effects have been stalled by difficulties recapitulating these phenomena in model organisms, particularly the mouse. Results from mouse models generated to study repeat instability and chromatin alterations have been disappointing (Peier & Nelson, 2002). CGG repeat lengths that are highly unstable when passed between generations in humans have proven to be quite stable in the mouse. With careful selection of ever-longer repeats at each generation, two groups have developed *Fmr1* knockin mice with CGG repeats at or above the ~200 threshold for methylation and silencing of the gene, but found neither methylation nor transcriptional silencing (Brouwer et al., 2007; Entezam et al., 2007). In general, it has been found for other loci and other repeat motifs that repeat lengths that are quite unstable in humans are not in mouse models, hampering mechanistic studies. A much more detailed analysis of the status of these investigations can be found in the chapter authored by Karen Usdin. Ben-Hur and Benvenisty detail efforts to understand the events leading to transcriptional silencing while Chiurazzi and Tabolacci discuss the potential for reactivation in their chapters.

The expanded CGG repeat mouse models have borne fruit in the study of mechanisms of the *FMR1* premutation disorders FXTAS and FXPOI. For FXTAS, there is abundant evidence for a toxic gain of function of the premutation through production of mRNA carrying CGG repeats. While the bulk of studies point to effects on RNA binding proteins through aberrant interactions with the riboCGG sequences (Iwahashi et al., 2006; Jin et al., 2007; Sellier et al., 2010, 2013; Sofola et al., 2007), recent evidence suggests that some of the pathology may be due to repeat associated non-ATG (RAN) translation of peptides through the expanded CGGs (Oh et al., 2015; Todd et al., 2013). In other disorders resulting from expanded repeats, such as C9orf72-associated amyotrophic lateral sclerosis, RAN translation of dipeptides clearly has a major effect on pathology. Studies of DM have provided the most compelling evidence for the role of altered functions of RNA binding proteins due to production of toxic mRNA (in the case of DM, carrying a very large CUG repeat in the 3′ untranslated region of the mRNA). Muscle phenotypes in DM and DM2 (resulting from expansion of a noncoding CCUG repeat) have been well explained by effects on alternative splicing of transcripts due to altered availability of RNA binding proteins, such as those in the muscleblind family (MBNL1, 2, and 3) (Kanadia et al., 2003, 2006; Ho et al., 2004). What fraction, if any, of the phenotypes that result from CAG repeat expansions in coding sequences might result from mRNA effects (either aberrant protein binding or production of RAN products) remains unclear. The focus in those disorders has been on gain of protein function due to the expanded polyglutamine sequences (Shao & Diamond, 2007; Weber et al., 2014), but RAN products have recently been detected in HD (Banez-Coronel et al., 2015), some evidence for mRNA-based effects has been reported (Hsu et al., 2011; Li et al., 2008; Wang et al., 2011). Studies of disorders caused by unstable repeats has contributed to more than genetic mechanisms: they have revealed pathological mechanisms that were previously unimagined.

It is intriguing to note that the unstable repeats associated with human disease were discovered during studies of disorders that follow Mendelian patterns of inheritance, albeit with

interesting deviations in penetrance or expression. For instance, DM and HD were clear examples of autosomal dominant inheritance, often used as examples in teaching. The conceptual advance that instability of a mutation can lead to variation in phenotype within a single pedigree has been a significant one. An additional, admittedly more extreme conceptual leap might be anticipated: Instability of simple repeats could be responsible for phenotypes that do not follow any clear pattern of inheritance, yet have an underlying genetic cause. For example, HD segregates in families as a highly penetrant dominant disorder, yet the expanded repeat is usually transmitted unstably. The threshold length for the HD CAG repeats to cause the disorder is typically expressed as a range from 36 to 40 repeats, with all individuals carrying 40 repeats and above being affected. Due to the correlation of repeat length with age of onset, those individuals with repeat lengths below 40 have the possibility of outliving the symptoms and being classified as nonpenetrant. In most HD families, repeat lengths measure in the 40s, with occasional larger expansions that can result in juvenile onset, but most transmissions result in small changes that are biased toward larger repeats. These increases sometimes account for families showing apparently new cases, where the parent had a subclinical repeat length (27–35 repeats). Due to the bias toward expansion, the HD CAG repeats present as a highly penetrant dominant disorder.

Interestingly, if the HD CAG repeats were equally prone to reductions in length, it would be much more difficult to discern an inheritance pattern. The repeats associated with human disease have been identified because they fit the pattern of Mendelian inheritance (with variation). If other repeat sequences exist that have similar phenotypic effects while mutating both into and out of the pathogenic repeat lengths, they would not appear as simple Mendelian alleles and would be much more difficult to identify. Such loci could contribute significantly to phenotypes (both pathologic and within normal variation) but have been overlooked in the genetic analyses carried out to date. Studies such as genome-wide association studies (GWAS) and next-generation based whole exome and whole genome sequencing (WES and WGS) are unlikely to have captured repeat-associated phenotypes.

GWAS is dependent on variations in the frequencies of single-nucleotide variants (SNV) to describe differential inheritance of genomic segments between case and control populations. SNVs used in GWAS are typically frequent (minor allele frequencies 1% or greater) in order to have discriminatory power between cases and controls. They can be used to define larger segments of SNVs in close proximity that are typically inherited as a block (haplotype). SNVs or haplotypes that differ in frequency between cases and controls are thought to carry genetic lesions that predispose or protect individuals for the phenotype of interest. There are clear examples of predisposing haplotypes at many of the genes carrying unstable repeats responsible for disease. Indeed, the identification of the HD mutation relied on the recognition of a shared haplotype (Huntington's Disease Collaborative Research Group, 1993). However, if a repeat that mutated into and out of the pathogenic range was involved, association would likely be missed due to similar frequencies in the case and the control populations.

Next generation sequence analyses of whole exomes or of whole genomes present another challenge to identification of unstable repeats associated with phenotypes. The underlying technology is challenged by repeating sequences and is prone to errors, particularly deletions. Thus, accurate sizing of repeat lengths in a normal range with next generation sequencing methods is difficult, and many of the pathogenic lengths exceed the typically read lengths of the methods. Thus, employing WES or WGS to define variation that is common

among individuals with a specific phenotype is unlikely to reveal genetic disorders caused by changes in repeat length. Other methods, such as the Complete Genome sequencing approach have some promise to alleviate this shortcoming, but cost has remained an obstacle for these methods. As an example of the limitations of next generation sequencing, the 2011 discovery of the massive GGGGCC repeat expansions in an intron of C9orf72 in families segregating ALS required Southern blot hybridization after extensive sequencing of the region failed to uncover the repeat (DeJesus-Hernandez et al., 2011).

Could there be additional repeat sequences that contribute to human disease and/or normal variation? The appreciation of mechanisms involved in the known repeat expansion disorders, particularly those based on unusual mRNAs, suggests that there could be abundant phenotypic variation that depends on repeat variation. How much might cumulative repeat sequence in mRNA contribute to subtle alterations in splicing patterns that could affect growth, development or function? How might the presence of RAN products and their variation dependent on polymorphic repeat lengths at numerous loci lead to changes in cellular function or aging? In a curiously similar manner to the situation in the years leading up to the era of positional cloning, additional technological advances will be required in order to carry out the characterization of large numbers of individuals that will allow these concepts to be investigated at a reasonable cost. It is likely that additional phenotypic impacts of simple repeat sequence variation remain to be discovered.

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CHAPTER

3

Molecular Diagnostics and Genetic Counseling in Fragile X Syndrome and *FMR1*-Associated Disorders

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FRAGILE X SYNDROME

Fragile X syndrome (FXS) (FXS #MIM300624; ORPHA 908) is the most common inherited cause of inherited intellectual disability (ID) and developmental delay, and it is the most commonly known monogenic cause of autism spectrum disorders (ASD). The *FMR1* gene (fragile X mental retardation 1 gene) is inherited as an X-linked dominant trait with a reduced penetrance of 80% in males and 30%–50% in females. The estimated prevalence is 1 per 5000 men and 1 per 4000–6000 women (Coffee et al., 2009; Hill, Archibald, Cohen, & Metcalfe, 2010).

FXS is generally caused by an expansion and hypermethylation of an unstable CGG trinucleotide repeat located in the 5′UTR of the *FMR1* gene (Verkerk et al., 1991; Yu et al., 1991).

In the general population the number of repeats is polymorphic and normal alleles range from 5 to 44 CGGs. A second class of alleles, with a CGG number between 45 and 54 repeats (Maddalena et al., 2001), is known as the gray zone or intermediate alleles (IA) and, depending on the presence of the AGG interruptions, they can be more or less unstable and have the propensity to expand into a premutation (PM) allele in subsequent generations (Nolin et al., 2013; Yrigollen et al., 2014a). The clinical implications of IA remain unclear, as a number of phenotypes observed in PM carriers have also been observed in individuals carrying these alleles (Hagerman & Hagerman, 2015; Madrigal et al., 2011). Alleles within

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TABLE 3.1 Molecular Measures of the fragile X mental retardation (*FMR1*) Gene and Correlation With the Phenotype

Allele	CGG repeat number	Methylation status, CpG island, CGG	mRNA	FMRP	Phenotype
Normal	5–39	None	Present	Present	Normal
IA	40–54	None	Present	Present	"Normal" more studies are needed
PM	55–200	Small percent	Increased; 2- to 8-fold	Slightly reduced	FXTAS, FXPOI, and other associated PM disorders
FM	>200	Yes	No	No	Classic FXS; 100% males and 30%–50% females

FM, Full mutation allele; FMRP, fragile X mental retardation protein; IA, intermediate allele; PM, premutation allele.

the 55–200 CGG repeats range are known as PM. Generally unmethylated, PM alleles are transcribed and present with elevated *FMR1* mRNA levels and decreased fragile X mental retardation protein (FMRP) expression levels (Allen, He, Yadav-Shah, & Sherman, 2004; Kenneson, Zhang, Hagedorn, & Warren, 2001; Peprah et al., 2010; Primerano et al., 2002; Tassone et al., 2000). PM alleles are unstable and tend to increase the CGG repeat number through generations. Full mutation (FM) alleles harbor greater than 200 CGG repeats and are generally methylated, which leads to silencing by inhibition of transcription with the consequent absence of the encoded protein, FMRP (Table 3.1). It is the lack of FMRP that causes FXS. Males carrying FM are always affected with FXS, while only 30%–50% of females with the FM are affected. However, >40% of individuals with FXS can present with either size or methylation mosaicism or both (Nolin, Glicksman, Houck, Brown, & Dobkin, 1994). Size mosaics are those who have alleles both in the FM range and in the PM range, while methylation mosaics present with alleles both methylated and unmethylated; the latter can span the FM range or can span the entire range from normal/PM/FM. Mosaicism is caused by somatic instability of the FM in early embryogenesis that can lead to retraction of the expanded CGG repeat. Mosaicism allows the expression of the *FMR1 mRNA* and of FMRP, which, in some cases, has been associated with milder ID in males (Pretto et al., 2014).

AGG interruptions are interspersed and present within the CGG tract in the *FMR1* gene. They are found in normal, intermediate, and PM range alleles and commonly occur after 9 or 10 uninterrupted CGG repeats [$(CGG)_9AGG(CGG)_9AGG(CGG)_n$]. It is believed that the biological function of these interruptions is to stabilize the gene during transmission and, therefore, decrease the risk of DNA polymerase slippage during DNA replication. The presence or absence of AGG interruptions does not correlate with the transcriptional or translational activity of the gene, and their distribution patterns can vary greatly between populations, and are largely inherited without change (Ludwig et al., 2009; Peprah et al., 2010; Tassone et al., 2007; Yrigollen et al., 2012, 2014b).

In majority of the cases, FXS is due to a CGG repeat expansion in the *FMR1* 5'UTR, but other *FMR1* mutations (such as point mutations or deletions), leading to a loss of function of the gene, may also cause FXS or an FXS-like phenotype. As standard molecular testing does not include sequencing of the *FMR1* coding region, the prevalence of point mutations causing FXS is not well known, although it seems that missense mutations in the *FMR1* gene might

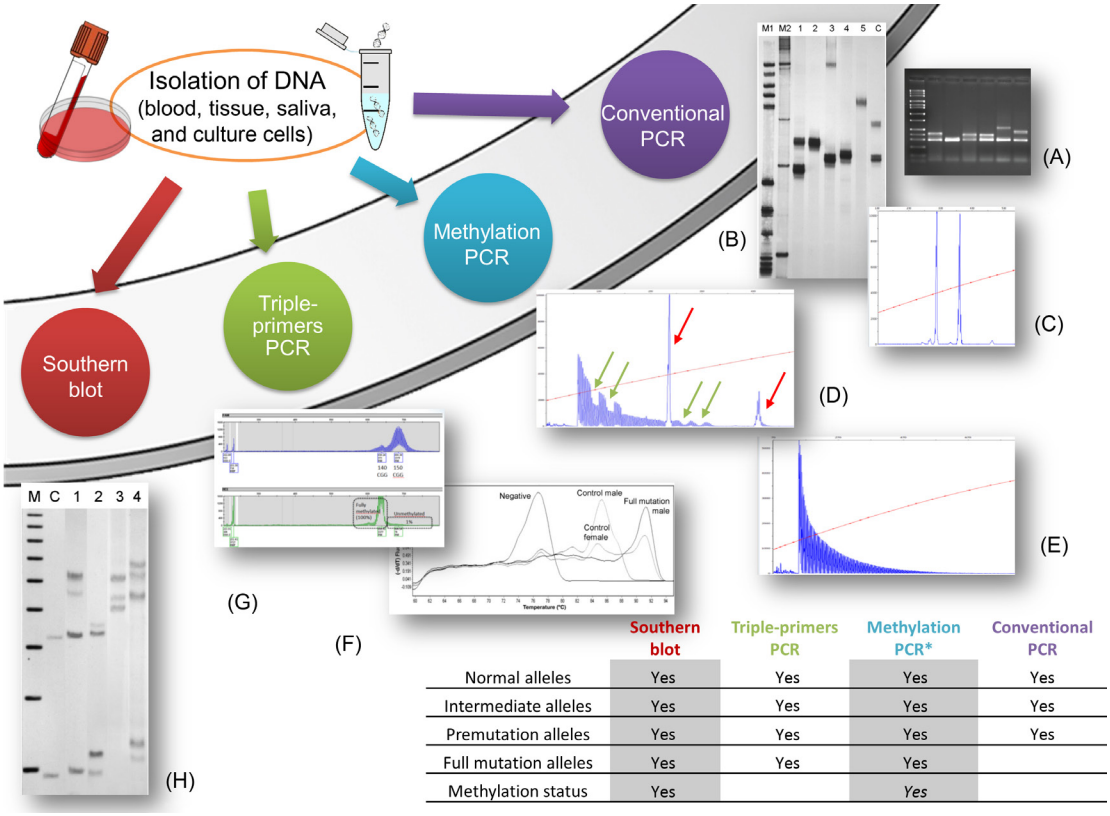
account for a proportion of cases in males with FXS-related symptoms, such as those linked to ID and developmental delay (Handt et al., 2014; Myrick et al., 2014; Wells, 2009). At present, the estimated frequency of point mutations is 1%–2% of the cases of FXS (Handt et al., 2014). The use of techniques, such as next generation sequencing (NGS), should provide knowledge about the prevalence of these mutations in the near future.

THE DIAGNOSIS OF FRAGILE X SYNDROME

The molecular diagnosis of FXS is based on CGG repeat sizing, as well as the methylation status of the CpG island located in the promoter region of the gene. After the *FMR1* gene was cloned in 1991, Southern blot analysis became the gold standard DNA diagnostic technique. Southern blot is performed by DNA digestion using two restriction enzymes: one aimed at determining the size of the expansion and the other at the methylation status. The enzymes most commonly used are EcoRI (directed to determine the size of the expansion) and EagI or NruI (to determine the methylation status). The digested DNA is then separated on an agarose gel, transferred to a charged nylon membrane, and hybridized with the *FMR1*-labeled specific genomic probe StB12.3, Ox1.9, pP2, or Pfxa7. This approach provides a characteristic mutational pattern for each individual status as the ones showed in Fig. 3.1.

To date, a combination of polymerase chain reaction (PCR) (particularly useful for CGG sizing within the PM range) and Southern blot analysis (for sizing larger alleles and for determining their methylation status) still represents the gold standard DNA methodology for the diagnosis of FXS. However, Southern blot analysis presents some inconvenience: it is laborious, time consuming, and requires large amounts of DNA. In addition, it provides an imprecise number of CGG repeats particularly in the normal and PM range. On the other hand, conventional PCR does not always obtain CGG amplification, likely due to the high CG content and to the tendency to form undenaturable secondary structures. In the PCR approach, fluorescent labeled primers surrounding the CGG region are used (Fu et al., 1991) and the product is analyzed in an image analyzer to determine the exact number of CGG repeats using small quantities of DNA. The precise number of CGGs is very important to discriminate between normal, intermediate, and PM alleles. In males, the absence of PCR product indicates the presence of a pathologically expanded allele, when an internal control is utilized to ensure the absence of amplification of the *FMR1* allele. Despite its speed and low cost, the conventional PCR has some limitations. It is unable to detect large PMs or FMs; it does not distinguish between a homozygous female (both chromosomes carrying an allele with the same CGG number) and a woman with an allele in the normal range and another allele of over 100 CGGs; and no information is provided about AGG interruptions or methylation.

Thus, in the past decade a number of PCR-based diagnostic strategies have been proposed for the identification of *FMR1* gene repeat expansions. Several reports have described a novel PCR-based approach used to improve the efficiency of the FXS diagnosis mainly based on the use of three primers (Chen et al., 2011; Filipovic-Sadic et al., 2010a,b; Tassone, 2015). The triplet repeat–primed PCR (TP-PCR) method was developed by Warner et al. (1996) to screen for the presence of expanded alleles in myotonic dystrophy. It was subsequently used to amplify expanded alleles of the FMR1 gene into the FM range (Tassone, Pan, Amiri, Taylor, & Hagerman, 2008). This PCR assay uses locus-specific primers flanking the repeat, together with



	Southern blot	Triple-primers PCR	Methylation PCR*	Conventional PCR
Normal alleles	Yes	Yes	Yes	Yes
Intermediate alleles	Yes	Yes	Yes	Yes
Premutation alleles	Yes	Yes	Yes	Yes
Full mutation alleles	Yes	Yes	Yes	
Methylation status	Yes		*Yes*	

FIGURE 3.1 **The gold standard diagnosis for fragile X syndrome (FXS) uses a combination of Southern blot and polymerase chain reaction (PCR) analysis. This figure shows the result from different PCR-based molecular approaches (A–G) and from Southern blot (H) directed to the FXS diagnosis. The table shows which allele category is clearly detected by the different molecular approaches.** The *asterisk* denotes that several methylation-based PCR approaches are not sensitive for the detection for full mutation female and unmethylated expanded allelles.

a paired primer amplifying from multiple priming sites within the CGG repeat. TP-PCR gives a characteristic pattern, enabling the rapid identification of large expanded repeats that may not be amplified using the flanking primers alone. At the same time, this approach provides information on the presence and distribution of the AGG interruptions (Chen et al., 2010) (Fig. 3.1).

On the other hand, triplet repeat–primed methylation-specific PCR (TP-MS-PCR) combines allele-specific methylation PCR and capillary electrophoresis. Methylation status is determined using a methylation-sensitive restriction enzyme and a PCR with two sets of dye-tagged primers, and alleles are sized by capillary electrophoresis. Methylation PCR enables high-throughput, high-resolution, and semiquantitative methylation assessments of *FMR1* alleles, as well as determination of CGG repeat length. It has been proposed to be a more sensitive approach than Southern blot analysis (Chen et al., 2011).

Thus, the combination of two simple PCR methods—TP-PCR and TP-MS-PCR—can provide information about the whole range of expansion, the AGG interruptions, and the

methylation status. It is able to characterize homozygous females from FM females, thereby achieving rapid and reliable prenatal and postnatal FXS diagnosis.

Recently, Aliaga et al. (2016) described a methylation-specific quantitative melt analysis (MS-QMA) approach, targeting 12 CpG sites, 9 of which located within the intron 1 of the *FMR1* gene, to screen for FXS from birth in both genders. Methylation of this region, named the fragile X–related epigenetic element 2 (FREE2) and located on the exon 1/intron 1 boundary of the *FMR1* gene was reported to influence FMRP expression and cognitive impairment in individuals with the FM (Godler et al., 2012). Further, the measurement of methylation at the FREE2 site was proposed to be a suitable methodology for newborn screening for both FXS and sex chromosome aneuploidy (Inaba et al., 2014).

The MS-QMA combines high-resolution melt and high-throughput quantitative real-time PCR standard curve for accurate quantification of DNA methylation in a single assay. MS-QMA is used to estimate the presence of cryptic FM alleles in individuals who also carry normal or gray zone alleles. The authors state that the diagnostic and analytical sensitivity of this technique is higher than that of any TP-PCR or Southern blot analysis and could be used as a first-line screening method.

DNA obtained from many different tissues including saliva, blood, brain tissue, amniotic fluid, or chorionic villi can be used for DNA diagnosis. For prenatal diagnosis, it is preferable to perform the study in chorionic villi, and thus, it should be taken into account that these samples are not fully methylated until after the 14th week of pregnancy. In the case of a female, maternal contamination must be ruled out (Castellvi Bel et al., 1995). However, the choice of technique and tissue depends on the diagnostic strategy according to laboratory availability and the clinical and family characteristics. With the use of an adequate combination of techniques, the sensitivity and specificity will be over 99%, with a minimum number of false positive or negative results, which could be due to a polymorphism in the target region.

An indirect diagnosis could be made with intragene microsatellite markers or microsatellites surrounding the gene, such as FRAXAC1, FRAXAC2, DXS548, and p39, which is a diagnostic approach used sometimes in preimplantation/preconceptional diagnosis (Apessos, Abou-Sleiman, Harper, & Delhanty, 2001).

Finally, a number of methodologies targeting the expression of FMRP levels have been developed. Immunocytochemistry is a test used to detect the presence or absence of the FMRP by using a monoclonal antibody against FMRP. This test is mainly used for the assessment of cases in which sample obtainment is difficult and may be useful in large-scale screening of a male population, as males with a FM have no or a very low expression of FMRP. The test can be performed on blood smears or hair roots (Ravindran, Patel, Adhia, D'Souza A, & Babu, 2005; reviewed in Willemsen & Oostra, 2000). However, this approach is not useful in females or in PM carriers, as the interpretation of the results is complicated by the presence of the second normal X chromosome in females and by the presence of unmethylated alleles, which may present lower FMRP expression, particularly in the upper PM range, in carriers (Kenneson et al., 2001; Primerano et al., 2002; Ramos & Willemsen, 2003; Willemsen et al., 2003). Therefore it is not commonly used for FXS diagnosis.

Using a combination of two different FMRP-specific antibodies (avian and murine), Iwahashi et al. (2009) developed a sandwich enzyme-linked immunosorbent assay (ELISA), which is sensitive and highly specific for the detection of the intact FMRP in peripheral blood

lymphocytes. The authors proposed that this ELISA could be adapted for large-scale screening and used as a tool to study genotype/phenotype correlations in FXS.

Recently, LaFauci et al. (2013) developed a rapid, highly sensitive method for quantifying FMRP from dried blood spots and lymphocytes. This assay uses two FMRP-specific antibodies, a bacterially expressed abbreviated FMRP standard and a Luminex platform to quantify FMRP expression levels. The assay readily distinguishes between samples from males with fragile X FMs and samples from normal males. It also differentiates mosaic from nonmosaic FM male samples. Considering that this capture immunoassay approach is simple, accurate, and inexpensive, it has a great potential for newborn or population screening. Indeed the authors (Adayev et al., 2014) further reported a study on dried blood spots derived from individuals with different *FMR1* mutations, and proposed it as an efficient approach for newborn screening for FXS.

In a different study FMRP levels were quantitatively measured in blood platelet (Lessard, Chouiali, Drouin, Sébire, & Corbin, 2012). While the method was able to correctly discriminate between samples derived from individuals with FXS and normal controls, the distinction between FXS mosaics or FM females and controls was less obvious.

Thus, although all these methodologies, aimed to measure FMRP levels, appear to be quite robust in differentiating FMRP levels between FXS and controls, they do not perform as well in females with FXS, in PM carriers, and FXS mosaics. In addition, these methodologies report large variations in FMRP levels within the normal samples, which represent an issue particularly in the presence of an unmethylated expanded allele. Hence, better technologies need to be developed to accurately measure FMRP expression in both males and females carrying alleles for all ranges of the *FMR1* mutations and methylation status.

Upon the identification of an individual harboring an *FMR1* expanded allele, a cascade family study is required to detect other relatives carrying the PM or the FM. In these cases precise determination of allele size and the presence of the AGG interruption are important to determine the risks of expansion in the carriers. Moreover, the methylation status or the presence of mosaicism is relevant for genotype–phenotype correlations (de Vries et al., 1996; Pretto et al., 2014).

GENETIC COUNSELING IN *FMR1*-ASSOCIATED DISORDERS

Genetic counseling of FXS involves the study of a wide range of clinical manifestations including developmental, neurodegenerative, and reproductive symptoms that may vary with the age of onset and severity. Genetic counselors must have a solid understanding of this genetic condition, including trinucleotide repeat instability and phenotypic variability, as genetic counseling is offered based on the CGG expansion status. Four classes of allele are described depending on the CGG repeat size as indicated in the following subsections.

Normal Range

Alleles in the normal range (5–44 CGG repeats) are generally stable during transmission to the next generation, but instability has been described for the larger alleles (Nolin et al., 2013). The presence of alleles in the normal range, does not involve a risk to offspring, despite a positive family history.

Intermediate Alleles or Gray Zone

Intermediate or gray zone alleles range from 45 to 54 CGG repeats. They are frequent in the general population; indeed the prevalence observed in many studies varies between 1/22–66 in females and 1/42–112 in males (Tassone et al., 2012).

The stability of transmission of these alleles depends on the number of AGGs interruptions (Nolin et al., 2013; Yrigollen et al., 2014a), although no expansions to the FM in only one generation have ever been reported. A lack of interruptions increases instability. Currently, the follow-up of individuals with no AGG interruption is indicated, despite the low risk of expansion to FM in the next generation.

Individuals carrying these alleles do not normally manifest ID, fragile X–associated tremor/ataxia syndrome (FXTAS) or fragile X–associated primary ovarian insufficiency (FXPOI), although some groups have suggested associations between IA with FXTAS (Hall, Tassone, Klepitskaya, & Leehey, 2012; Liu, Winarni, Zhang, Tassone, & Hagerman, 2013), with FXPOI (Bodega et al., 2006), with autism, cognitive disabilities, and parkinsonism (Aziz et al., 2003; Loesch et al., 2007, 2009). However, these findings have not consistently been supported by other studies (Ennis et al., 2006; Madrigal et al., 2011). Thus, larger studies are needed to determine the potential risk of clinical repercussions in individuals carrying these smaller alleles.

Premutation Alleles

Alleles ranging from 55 to 200 CGG repeats are called PM. The prevalence of PM alleles differs in different populations and ranges from 110 to 250 in females and from 260 and 810 in males (Maenner et al., 2013; reviewed by Tassone et al., 2012) varying according to the studied population. PM alleles are highly unstable during maternal transmission and tend to expand to a FM in only one generation. The smaller allele that has been reported to expand from PM to FM in one generation is 56 CGG (Fernandez-Carvajal et al., 2009). Expansion also occurs in paternal transmission, albeit remaining within the PM range. Thus, all the daughters of a PM male are obligated PM carriers.

All affected children have carrier mothers (of a FM or PM) who have a 50% risk of having another affected child in future pregnancies. Table 3.2 summarizes the risk of expansion of the different kinds of *FMR1* alleles based on both the CGG repeat number and the presence of AGG interruptions. A previous study, performed in the general population (Eichler et al., 1994), showed that almost 95% of alleles have one or two AGG interruptions, which are stably transmitted such that the number of AGG interruptions and their position within the CGG tract are likely to correspond in the parent and offspring. In contrast, alleles in FXS families contain no or few AGGs at the 5′-end, and contain long stretches of uninterrupted CGGs at the 3′-end. It was recently shown that maternal alleles with no AGG interruptions confer an increased risk for unstable transmissions to a FM in the following generations, and thus the inclusion of AGG genotype studies would be of benefit in clinical practice (Table 3.3) (Nolin et al., 2013, 2015; Yrigollen et al., 2012, 2014a).

In general, many individuals carrying PM are clinically unaffected; however, several PM-related disorders have been described in the last decades (Hagerman & Hagerman, 2015). Among these, the most prevalent are FXPOI (Sherman, 2000a), present in approximately 20%

TABLE 3.2 Genetic Counseling in *FMR1*

Progenitor	Percent risk of expansion in offspring	Offspring	
		Males	Females
No expansion	0	Normal	Normal
IA carrier male	All females IA range	Normal	Normal
IA carrier female	50% Normal range; 50% IA range	Normal	Normal
PM carrier male	All females PM range	Normal	Reduced penetrance of PM-associated disorders
PM carrier female	50% normal range	Normal	Normal
	50% PM	Reduced penetrance of PM-associated disorders	Reduced penetrance of PM-associated disorders
	FM	100% ID	30%–50% ID
FM male	All females PM range	Normal	Normal, reduced penetrance of PM-associated disorders
FM female	50% normal range	Normal	Normal
	50% FM	100% ID	30%–50% ID

Percentage according to the expansion risk indicated in Table 2.2. Possibility of point mutation or deletions in the *FMR1* is not included in the risk. FM, Full mutation allele; IA, intermediate allele; ID, intellectually disability; PM, premutation allele.

TABLE 3.3 Risk of Expansion to a FM Based on the Maternal CGG Repeat Number Allele and on the Presence/Absence of AGG Interruptions

	Risk of expansion to FM alleles				
	Nolin et al. (2015)		Yrigollen et al. (2012)		
Allele range	$(CGG)_n$	AGG not tested	$(CGG)_n$	With AGG interruptions	Pure CGG
Normal	5–39	0%	5–39	0%	0%
IA	40–54	0%	40–54	0%	0%
PM	55–59	0.5%	<59	1%	3%
	60–64	1.7%	60–69	3%	49%
	65–69	7%	70–79	69%	90%
	70–74	21%	80–89	93%	95%
	75–79	47%	90–99	97%	100%
	80–84	62%	>100	100%	100%
	85–90	81%	—	—	—
FM	>200	100%	>200	100%	100%

of the female carriers (De Caro, Dominguez, & Sherman, 2008; Sherman, 2000b; Sullivan, Welt, & Sherman, 2011), and FXTAS, a neurological disorder characterized by intention tremor, cerebellar gait ataxia, parkinsonism, executive function deficits, neuropathy, and cognitive decline (Hagerman et al., 2001; Tassone & Berry-Kravis, 2010), present in approximately 50% of PM males. In addition, behavioral features, such as impaired executive function, social deficits, ASD, ADHD, anxiety, immune-mediated disorders, chronic pain, chronic migraine, hypothyroidism, hypertension, and sleep apnea, have been observed in *FMR1* PM carriers (Au et al., 2013; Bailey, Raspa, Olmsted, & Holiday, 2008; Coffey et al., 2008; Hamlin et al., 2011, 2012; Leehey, Legg, Tassone, & Hagerman, 2011; Roberts et al., 2009; Winarni et al., 2012).

The fragile X PM is considered to be the most frequent genetic cause of primary ovarian failure. The risk of FXPOI is around 20% (Sullivan et al., 2011), and the average age of menopause is 4–6 years earlier than that of the general population. Thus PM women must be counseled about the risk of developing primary ovarian failure, and it is recommended to program their reproduction before the age of 35 years. The CGG number is important to determine the possible development of FXPOI, with the highest risk being found in women with 80–99 CGG repeats (Sullivan et al., 2005). Smoking reduces the age of menopause in all women, and has a greater effect on PM women (Allen et al., 2007; Spath et al., 2011).

Genetic counseling for men and women carrying PMs should address the risk of developing FXTAS. This syndrome presents with incomplete penetrance; approximately 50% of PM carrier males will develop neurodegenerative symptoms of FXTAS in their lifetime (Rodriguez-Revenga et al., 2009). The first clinical signs of the syndrome typically appear with tremor and ataxia when patients are in their 50s and 60s (Leehey et al., 2007). The risk and severity of the disorder appear to be related to the CGG repeat number, with higher risk in larger repeat numbers (Leehey et al., 2008; Tassone et al., 2007). Nevertheless, a biomarker to predict the appearance of FXTAS or protective factors in asymptomatic carriers has yet to be identified. Other clinical signs associated with PM male carriers are neuroendocrine dysfunction, including testosterone deficiency (Greco et al., 2007), hypertension (Hamlin et al., 2012), or bowel and urinary incontinence (Hagerman, 2008).

Female PM carriers are also at risk for FXTAS. However, the disorder is much less common in females, with as few as only 16% developing FXTAS symptoms. Moreover, the age of onset is later and milder in presentation and cognitive decline is usually not present (Rodriguez-Revenga et al., 2009).

Other pathologies associated with PM carriers are psychiatric disorders, such as depression, anxiety, or mood disorders (Bourgeois et al., 2011; Farzin et al., 2006; Hunter, Sherman, Grigsby, Kogan, & Cornish, 2012; Rodriguez-Revenga, Madrigal, Alegret, Santos, & Mila, 2008), migraine (Au et al., 2013), immune-mediated disorders [particularly hypothyroidism (15.9%)] (Coffey et al., 2008), and fibromyalgia (25%) (Leehey et al., 2011; Martorell et al., 2012; Rodriguez-Revenga et al., 2013); the latter two being more common among women with FXTAS (frequency of 43 and 50%, respectively) (Wheeler et al., 2014).

Full Mutation Alleles

FM alleles contain greater than 200 CGG repeats. Females carrying FM alleles have a 50% risk of transmission to their male and female offspring in the FM range. On the other hand, males carrying FM alleles never transmit the FM allele to their daughters, but rather only

transmit alleles in the PM range. There is loss of the FM in the formation of the sperm and only the PM is transmitted. All males carrying a FM present classical features of FXS, with mild to severe ID, hyperactivity, long face, large or prominent ears, and machroorchidism, and exhibit a variety of maladaptive behaviors overlapping those described for ASD. On the contrary, only 50%–70% of women manifest FXS symptoms (Hagerman & Hagerman, 2002).

In females, a spectrum of clinical features, including physical, cognitive, and behavioral problems, could be present. Although women in general are less affected than men, some may be completely unaffected or exhibit minor neurobehavioral features (Boyle & Kaufmann, 2010; Keysor & Mazzocco, 2002), while others can present with a more severe phenotype. Clinical presentation can vary in relation to residual FMRP expression due to the presence of CGG expansion size or methylation mosaicism, particularly in implicated tissue, and to X-inactivation (de Vries et al., 1996).

Finally, a class of individuals called high-functioning males are individuals carrying the FM, but without methylation of either the CpG island or CGG repeats, and they may not present with ID or are in the moderate ID range. Genetic counseling is the same as for PM males; all their daughters are obligated carriers due to inheritance of a PM allele.

Patients with a strong clinical FXS phenotype or lack of FMRP, but not carrying the CGG expansion in the *FMR1* gene should be screened for *FMR1* point mutations (Myrick et al., 2014, although standard molecular testing does not include sequencing of the *FMR1* coding region. Point mutations can be de novo or inherited from a carrier mother. Males carrying a point mutation or a deletion always have daughters carrying the same mutation, who might be affected depending on X-chromosome inactivation. Carrier females have a 50% risk of transmitting the mutated allele. Within this 50%, all males inheriting the FM allele will be affected and females will be variably affected (30% normal, 25% with severe ID, and the remaining with some impairment). The diagnosis of a FXS patient always requires extending the study to the mother to confirm her carrier status and a cascade screening of all maternal branch family members (McConkie-Rosell et al., 2007).

Genetic counseling should include reproductive options for both PM or FM females. Some couples may decide not to have their own offspring, but rather consider adoption or oocyte or sperm donation. Around 10% of FXS families choose germ line cell donation. In these cases, potential gamete donors should be tested for the CGG expansion in the *FMR1* gene.

Prenatal diagnosis is the option chosen most frequently, generally using chorionic villi, although amniotic fluid is also possible. Prenatal diagnosis should be offered to women carrying a PM or FM, but it is not necessary for male carriers and should not be performed in IA carriers. Before undergoing prenatal testing, it is necessary to inform couples about the possible identification of a female fetus carrying a FM, as around 50% of these fetuses will be clinically affected, and we cannot identify who will or will not be affected yet. Another option is preimplantation or preconceptional genetic diagnosis (PGD). This technique is based on the genetic analysis of an embryo obtained through in vitro fecundation. Nonetheless the risk of FXPOI in PM women may hinder this process. Several scientific societies, such as the European Molecular genetics Quality Network (EMQN), the American College of Medical Genetics (ACMG), the National Society of genetic Counselors (NSGC), and the American College of Obstetrics and Gynecology (ACOG), have published best practice guidelines for molecular genetic testing and diagnosis of FXS and other fragile X–associated disorders (Abrams et al., 2012; American College of Obstetricians and Gynecologists Committee

on Genetics, 2010; Biancalana, Glaeser, McQuaid, & Steinbach, 2015; Finucane et al., 2012; Kronquist, Sherman, & Spector, 2008).

Here is a summary of the recommend *FMR1* testing following these international guidelines:

1. Boys and girls with ID or/and autism. As the phenotypic characteristics are subtle in infancy and are neither specific nor constant, it is recommended to rule out FXS in all cases of ID with unknown etiology, including a wide range of mild to profound ID, as well as developmental delays, autism, hyperactivity, and other behavioral problems.
2. Women with infertility and/or ovarian failure before the age of 40 years, especially in cases with high levels of follicle-stimulating hormone (FSH) and in the absence of other causes, such as ovarian cancer radiation treatment or thyroiditis.
3. Men and women with tremor and ataxia, especially in cases of cerebral ataxia with parkinsonism, intention tremor of unknown cause, and cognitive decline in a person 50 years of age.
4. Relatives of a diagnosed individual harboring an expansion in the *FMR1* gene or family history of FXS. It is also necessary to perform a diagnostic "cascade", that is, family studies arising from a former affected family member, including prenatal diagnosis in cases in which the pregnant woman is a PM or FM carrier.
5. Sperm and ovum donors, due to the high incidence of PM in the general population.

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CHAPTER

4

Epidemiology of Fragile X Syndrome

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INTRODUCTION

Fragile X syndrome (FXS) is the most common known inherited cause of intellectual disability (ID) and single-gene cause of autism spectrum disorder (ASD). In well over 95% of cases, the syndrome is caused by an expansion of a polymorphic CGG repeat region in the 5′UTR of the X-linked *FMR1* gene. Expansion to over 200 repeats, termed the full mutation (FM), leads to hypermethylation of the repeats and the surrounding regulatory region and subsequent silencing of the *FMR1* gene (see Chapter 1 for details). Given FXS is an X-linked disorder, almost all males who carry the FM have overt ID, whereas females have a much wider variation in the expression of symptoms, from no cognitive impairment to profound ID. The severity of symptoms primarily depends on the proportion of the active X-chromosomes carrying the FM in each target tissue. On average, females tend to be less severely affected than males, with 44% of females with a diagnosis of FXS able to live independently compared with 10% of males (Hartley et al., 2011). *FMR1* alleles with 55–200 unmethylated CGG repeats, termed the premutation (PM), have the potential to expand to the FM in one generation. The PM is associated with two late-onset disorders, fragile X-associated tremor/ataxia syndrome (FXTAS) and fragile X-associated primary ovarian insufficiency (FXPOI), but generally not with the symptoms of FXS. The frequency of carriers of the PM is significantly higher than that for carriers of the FM, due to the severity of FXS.

Prior to the identification of the repeat mutation, cytogenetic techniques that detected a fragile site (*FRAXA)* in Xq27 were used to diagnose FXS. Although such methods were novel at the time and important to characterize the phenotype of FXS, they were known to have their limitations with respect to estimating the prevalence of the disorder, both in terms of false positives and false negatives (e.g., J. E. Morton et al., 1997). Once the mutation was identified as a trinucleotide repeat expansion in the *FMR1* gene, Southern blot analysis became the gold standard for the detection of the FM. This type of test, although highly accurate, was too expensive for use in population screening. Over time, PCR-based tests have been developed

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to determine CGG repeat lengths, including those with large FM expansions (e.g., L. Chen et al., 2010; Filipovic-Sadic et al., 2010; Tassone, Pan, Amiri, Taylor, & Hagerman, 2008; Todorov, Todorova, Georgieva, & Mitev, 2010). Other novel technologies have been developed to efficiently screen large numbers of individuals based on attributes of the FM, including methylation and repeat size (e.g., Aliaga et al., 2016; Coffee et al., 2009; Elias et al., 2011; Inaba et al., 2014; Teo, Law, Lee, & Chong, 2012). Methods that quantify the amount of fragile X mental retardation protein (FMRP), the protein produced by *FMR1*, have also been developed as an inexpensive method to screen large populations (Adayev et al., 2014; Ravindran, Patel, Adhia, D'Souza A, & Babu, 2005; Willemsen & Oostra, 2000). Recently Lyons, Kerr, and Mueller, (2015) compared methods developed for screening purposes, which have led to the possibility of screening large numbers of individuals to directly assess the prevalence in the general population, but have only been used in pilot studies to date.

The low frequency of FXS combined with the relatively costly diagnostic testing, has led to difficulties in obtaining prevalence of this disorder with any accuracy. Some studies have focused on screening within target populations, such as ID populations, and then extrapolating the prevalence within these populations to the general population. However, given the variable clinical features of FXS these methods likely provide inaccurate prevalence estimates. Even today, not all health care professions know to refer a child with ID for fragile X testing. Thus, obtaining an accurate prevalence of FXS among males, and especially among females, is a challenge.

It is important to consider why epidemiological studies are necessary. First, FXS leads to considerable reduction in quality of life, not only for the individuals with the syndrome, but for their caregivers as well. The economic burden on the family and the community cannot be underestimated (D. B. Bailey, Jr. et al., 2012; D. B. Bailey, Raspa, Holiday, Bishop, & Olmsted, 2009; Sacco, Capkun-Niggli, Zhang, & Jose, 2013). A strong understanding of the epidemiology of the disorder helps to put into perspective the related burden. Second, results from epidemiologic studies provide the basis for policy-making with issues such as routine screening for FXS or future budget planning. Third, factors associated with the prevalence of the FM (e.g., gender, race/ethnicity) help to gain insight in the dynamics of the mutational mechanism and its population history.

Despite the importance, there has been little consensus about the prevalence of FXS in the general population or among those with specific disabilities (e.g., among those with ID or with ASD). In 2001, a review of the literature was conducted by Crawford, Acuna, and Sherman (2001) to estimate the prevalence of FXS. Based on the published literature from 1991, when the mutation was discovered, to 2000, they estimated that about 1 in 4000 for males and 1 in 8000 for females were affected by FXS. These estimates have been used widely since that time.

In this chapter, we will describe the most recent systematic review and metaanalysis of the literature that was performed by Hunter et al. (2014) to provide estimates of the prevalence of FXS in males and in females. We will also document the studies done among various racial/ethnic groups and geographic regions to show that FXS is present in almost all subpopulations that have been investigated. Lastly, we will review the rare mutations in the *FMR1* gene, both deletions and point mutations, that lead to FXS-associated symptoms. We conclude by suggesting that additional large-scale studies are needed to better define the burden of the FXS globally.

PREVALENCE OF FXS

Turner and her coworkers were one of the first teams to study the prevalence of FXS using cytogenetic diagnostic tools and then following up with DNA tests (Turner, Robinson, Laing, & Purvis-Smith, 1986; Turner et al., 1992). They screened individuals with ID from many different settings with the goal to find families at risk and to follow-up with cascade testing to identify others in the family who carried the expanded *FMR1* mutation. Initially they recognized that the prevalence of 1/1000 males obtained from the first cytogenetic screens was an overestimate in mixed ethnic populations and suggested that 1/4000 may be a more realistic figure (Turner, Webb, Wake, & Robinson, 1996). The first large review of the literature in 2001 also estimated a prevalence of 1/4000 males (Crawford et al., 2001).

The estimate of FXS in about 1/4000 males and about 1/8000 females has been used generally for about 15 years. The figure of 1/8000 among females was never estimated directly, but instead based on the estimate of FXS in males and on two important assumptions: (1) the carrier frequency of the FM among males and females is the same for this dynamic X-linked repeat mutation [first modeled by Winter (1987) and subsequently by others (Ashley & Sherman, 1995; Kolehmainen, 1994; N. E. Morton & Macpherson, 1992)] and (2) only about half of women who carry the FM have overt symptoms of FXS.

The first systematic review of the prevalence of FXS was conducted by Song, Barton, Sleightholme, Yao, and Fry-Smith (2003). Of the studies that directly estimated the prevalence of FXS in the general population, they found the pooled estimate to be 1 in 7,143 (0/4,186 males and 7/45,817 females). The estimated prevalence was 1 in 4425 males, based on studies that screened males with ID and then extrapolated to the general population. As noted by the authors, obtaining accurate estimates from extrapolations is difficult. In general, many studies have small sample sizes and varying inclusion criteria for their target populations (e.g., excluding those with known disorders, including those with a specific level of ID). They also noted that the studies that provided direct estimates based on the general population were many times based on healthy volunteers or based on exclusion of individuals with family history of ID and many did not detect any cases of FXS. These study designs would necessarily lead to an underestimate of the prevalence of FXS.

More recently, Hunter et al. (2014) updated previous estimates of the prevalence of FXS based on a quantitative analysis of the published literature. We will describe this study in detail, as it stands out as the most comprehensive to date and expands the review conducted by Song et al. (2003). Hunter et al. also examined the prevalence of the PM, although we will not present those analyses in detail.

To begin, Hunter et al. conducted a systematic literature review to identify studies that estimated the prevalence of FXS in the general population. They restricted their analyses to studies that used molecular assays to identify the FM and studies with complete information on both the total tested population and the number of mutation carriers. They used similar terms as did Song et al. (2003) to identify such studies, as well as expanded terms to cast a wider net. They separated analyses based on three populations: (1) those that screened the general population without any selection bias; (2) those that screened healthy individuals without any ID, primarily to assess estimates of females with the FM; and (3) those that screened individuals with ID. The last group included studies with various definitions of the affected populations, including children attending special education needs

classes, those with an IQ of less than 70, or those with cognitive, behavioral, or physical assessments.

The primary analysis assessed the frequency of the FM and PM alleles in the total population, and secondary analysis assessed the prevalence of the FM and PM alleles in populations with ID with extrapolation to the general population. For the metaanalysis, the data were modeled to allow for variability in the frequency estimates across the studies and were adjusted for sex, publication year, and geographical region. Twenty-one studies that performed population-based screening without biasing against those with ID (e.g., newborn screening) were included in primary analyses, with results summarized in Table 4.1. The aggregated population used to assess the prevalence of individuals with FM in the total population was about 78,000 males and 75,500 females (Table 4.1). The mean prevalence for males with FXS was estimated to be 1.4 per 10,000, or 1/7,143 (95% confidence interval (CI): 0.1–3.1 per 10,000), with a wide between-study variation (SD, 1.2; 95% CI: 0.1–4.0 per 10,000). For females, the mean prevalence was 0.9 per 10,000, or 1/11,111 (95% CI: 0.0–2.9 per 10,000), with significant heterogeneity between studies (SD, 4.1; 95% CI: 0.5–9.5 per 10,000). There was no significant difference in the estimates of prevalence among males and females when all FM allele frequency data were pooled.

The mean frequency of those with the PM allele among males was estimated to be 11.7 per 10,000 (1/855) (95% CI: 6.0–18.7 per 10,000). There was considerable heterogeneity as indicated by the between-study SD (0.7; 95% CI: 0.3–1.3 per 10,000). Among females, the mean frequency was 34.4 per 10,000, or 1/291 (95% CI: 6.3–83.3 per 10,000). The estimate of PM carriers among females was significantly higher than that for males.

Examination of the estimates based on region of the screening (Europe, Asia, USA/Canada/Australia, other) suggested that estimates of the PM were lower from studies conducted in Asia and in USA/Canada/Australia compared with studies conducted in Europe. Although the pattern was the same for the estimates of the prevalence of the FM, they were

TABLE 4.1 Prevalence of the *FMR1* full mutation (FM) and premutation (PM) estimated from studies that screened the general population.

Population	Number of studies	Identified mutation carriers/*n* tested	Prevalence per 10,000 (ratio)	95% CI (per 10,000)
FM				
Male	13	14/78,104	1.4 (1/7143)	0.1–3.1
Female	7	9/75,539	0.9 (1/11,111)	0.0–2.9
PM				
Male	13	62/45,253	11.7 (1/855)	6.0–18.7
Female	9	539/88,673	34.4 (1/291)	6.3–83.3

CI, Confidence interval.

Data taken from Hunter, J., Rivero-Arias, O., Angelov, A., Kim, E., Fotheringham, I., & Leal, J. (2014). Epidemiology of fragile X syndrome: a systematic review and meta-analysis. American Journal of Medical Genetics A, *164A(7), 1648–1658. Estimates and 95% confidence intervals are based on the random-effects regression model.*

not statistically significant. For the prevalence of the PM, estimates from studies conducted prior to 2000 were significantly lower, which could reflect improvements in genotyping and increased clinical awareness of FXS.

Hunter et al. (2014) also examined studies that assessed populations with ID before extrapolating them to the general population. Of the 15 studies that fit their criteria, 178/7475 individuals with ID had the FM, or about 2.4%. The extrapolated estimates to the general population varied considerably, and depended on the sample size in the study and, most likely the target population screened. For example, Meguid, Abdel-Raouf, Dardir, and El Awady (2007) screened males in Egypt who received special education and extrapolated to a prevalence of 1/1079 FXS in males in the general population. In a study done in Estonia, they extrapolated from those with ID and estimated the prevalence in the general live birth population of males with FXS to be 1/13,947 (Puusepp et al., 2008). Table 4.2 provides a comprehensive list of studies by geographic region that estimated the prevalence of the FM based on the described target population. This table includes only those studies that used molecular assays to screen the target population. Overall, among individuals with some form of ID, the estimated frequency of FM is about 2.5%, although this estimate is greatly influenced by the phenotype of the tested population.

PREVALENCE OF FXS AMONG SUBPOPULATIONS

Once the mutation was identified as a dynamic repeat sequence mutation, investigators examined characteristics of the repeat sequence that may affect its instability and, therefore its risk to expand to the FM. Interestingly, it was soon found that the *FMR1* repeat mutations were in linkage disequilibrium (LD) with flanking genetic variants (e.g., Arinami, Asano, Kobayashi, Yanagi, & Hamaguchi, 1993; Buyle et al., 1993; Haataja, Vaisanen, Li, Ryynanen, & Leisti, 1994; Hirst et al., 1993; Jacobs et al., 1993; Macpherson, Bullman, Youings, & Jacobs, 1994; Malmgren et al., 1994; Oudet et al., 1993a; Oudet, Von Koskull, Nordstrom, Peippo, & Mandel, 1993; Richards et al., 1992). This observation was the first hint that different mutational processes were involved in the multistep path from the initial instability of a repeat allele (initial mutation) to the expansion to the FM.

The repeat structure was also considered as a possible intrinsic factor that may affect stability. Indeed, AGG interruptions within the CGG trinucleotide repeats have been found to play a large role in this expansion process (e.g., Eichler et al., 1994; Kunst & Warren, 1994; Nolin et al., 2013; Snow et al., 1993). In general, the fewer the AGG interruptions and the longer the 3′ uninterrupted CGG repeats, the higher the risk for instability (reviewed in Latham, Coppinger, Hadd, & Nolin, 2014). As with most highly genetic polymorphic sequences, the allele distribution based on repeat length and AGG interruption differs by ethnic/racial group or geographic region (reviewed in Peprah, 2012).

Combining evidence from the repeat structure and the LD patterns, at least three different mutational pathways have been defined (Crawford, Zhang, Wilson, Warren, & Sherman, 2000; Eichler, Hammond, Macpherson, Ward, & Nelson, 1995). Based on these observations, it is straightforward to predict that there may be founder effects resulting in differences in the frequency of *FMR1* expansion mutations in subpopulations. Perhaps the best and earliest example of this phenomenon was reported by Falik-Zaccai et al. (1997) in Israel. They found

TABLE 4.2 Overview of studies that estimated the prevalence of FXS (or FM) in the described population, ordered by country/region and separated by sex. Only studies that performed molecular analyses to detect the FM are included.

Country/region	Source of data	Estimate of FM (identified/n tested)	References
Males			
Afro-Caribbean (Caribbean Island, Guadeloupe, French West Indies)	Moderate/severe ID	6.7% (11/163) Extrapolated to 1/2,381	Elbaz et al. (1998)
Brazil (Maranhão)	ID institutionalized	9.84% (2/238; 1 FM, 1 deletion)	Viveiros et al. (2015)
Brazil	Severe ID institutionalized	0/38	Mulatinho, Llerena, and Pimentel (2000)
Brazil	ID	2% (5/256)	Haddad et al. (1999)
Canada (Quebec)	Newborn screen	1/6,209 (2/12,419)	Levesque et al. (2009)
Canada (Quebec)	Leftover samples from male outpatients	0/10,572	(Dombrowski et al. (2002)
Canada (Manitoba)	Newborn screen	0/778	Dawson, Chodirker, and Chudley (1995)
China	ID	1.1% (5/453)	X. Chen et al. (2015)
China	Mild ID	0.4% (1/243)	Pang et al. (1999)
China	Newborn screen	0/1,000	Chiang, Lee, Wang, and Hwu (1999)
Croatia	ID	2.7% (2/73)	Hecimovic, Tarnik, Baric, Cakarun, and Pavelic (2002)
Denmark	Newborn screening	0/1686	Larsen et al. (2000)
Estonia	ID	3.1% (14/448) Extrapolated to 1/13,947	Puusepp et al. (2008)
Egypt	School-age screened for features of FXS	1/1079 (19/20,500): 6.4% (16/250) ID 2.0% (3/150) LD	Meguid et al. (2007)
France	ID	2.5% (10/403)	Gerard et al. (1997)
Finland	ID institutionalized	4.8% (26/541) Extrapolated to 1,400	Arvio, Peippo, and Simola (1997)
India (New Delhi)	Screened for features of FXS	9.7% (9/93)	Sharma, Gupta, and Thelma (2001)

India (Delhi)	Screened for features of FXS	5.3% (19/360)	Jain, Verma, and Kapoor, (1998)
Hellenic population (Greece and Cyprus)	ID of unknown etiology	1.3% (8/611)	Patsalis et al. (1999)
India	ID of unknown etiology	2.5% (3/118)	Pandey, Phadke, and Mittal (2002)
Japan	ID	1.6% (2/129)	Hofstee, Arinami, and Hamaguchi (1994)
Japan	ID	0.8% (2/256)	Nanba et al. (1995)
Mexico	ID of unknown etiology	3.2% (2/62)	Gonzalez-del Angel et al. (2000)
Netherlands	ID of unknown etiology	1.0% (9/870) Extrapolated to 1/6,045	de Vries et al. (1997)
Pakistan	ID	6.5% (15/229)	Fatima et al. (2014)
Pakistan	ID of unknown etiology	3.5% (10/287)	Kanwal et al. (2015)
Poland	ID institutionalized	2.9% (6/201) Extrapolated to ½,857-1/5,882	Mazurczak et al. (1996)
Singapore	ID selected for FXS features	2.4% (6/255)	Tan, Law, Zhao, Yoon, and Ng (2000)
South African	ID institutionalized	6.1% (9/148): 7.8% (6/77 severe ID) 4.2% (3/71 mild ID)	Goldman, Jenkins, and Krause (1998)
Spain	Newborn screen	1/2,633 (2/5,267)	Fernandez-Carvajal et al. (2009)
Spain (Catalonia)	Newborn screen	1/2,466 (2/4,935)	Rife et al. (2003)
Sri Lanka	ID	1.3% (7/540)	Chandrasekara, Wijesundera, Perera, Chong, and Rajan-Babu (2015)
Taiwan	Newborn screen	1/10,046	Tzeng et al. (2005)
Taiwan	ID	1.9% (4/206)	Tzeng, Tzeng, Sun, Chen, and Lin (2000)
Tasmania	Special education needs	0/1,253	Mitchell et al. (2005)
Tasmania	Newborn blood spots	0/578	Mitchell et al. (2005)

(*Continued*)

TABLE 4.2 Overview of studies that estimated the prevalence of FXS (or FM) in the described population, ordered by country/region and separated by sex. Only studies that performed molecular analyses to detect the FM are included. (*cont.*)

Country/region	Source of data	Estimate of FM (identified/n tested)	References
Thailand	ID of unknown etiology	6.8% (16/237)	Limprasert, Ruangdaraganon, Sura, Vasiknanonte, and Jinorose, (1999)
US (Atlanta, GA)	School-age, special needs classes	0.3% (7/2,471) Extrapolated to: Caucasian: 1/3,717 African American: 1/2,545	Crawford et al. (2002)
US	Newborn blood spots	1/7,312	Tassone et al. (2012)
US	Newborn blood spots	1/5,161 (7/36,124)	Coffee et al. (2009)
US	Preschool with language delay	0.3% (1/379)	Mazzocco et al. (1998)
US	School age with academic difficulties, but not ID	0/673	Mazzocco et al. (1997)
US	Screened (no selection)	0/416	Reiss et al. (1994)
UK	ID	2.2% (4/180)	Jacobs et al. (1993)
UK	School age with special needs	0.5% (20/3738) Extrapolated: 1/5,530	Youings et al. (2000)
UK	ID institutionalized	0.7% (1/138)	
Yugoslavia	ID of unknown etiology	2.6% (2/78)	Major et al. (2003)
Females			
Afro-Caribbean (Caribbean Island, Guadeloupe, French West Indies)	Moderate/severe ID	0/85	Elbaz et al. (1998)
Brazil	ID institutionalized, severe	0/47	Mulatinho et al. (2000)
Canada	Newborn screen	0/735	Dawson et al. (1995)
Canada (Quebec)	Newborn screen	0/12,032	Levesque et al. (2009)
Canada (Quebec)	Mothers of newborns	0/21,411	Levesque et al. (2009)
China	ID testing referral	0/87	Chen et al. (2015)
China	Mild ID	1.0% (1/81)	Pang et al. (1999)

Croatia	ID	2.4% (1/41)	Hecimovic et al. (2002)
India (New Delhi)	ID selected for FXS features	2.7% (1/37)	Sharma et al. (2001)
India (Tamil Nadu)	Screening	0/353	Indhumathi et al. (2012)
Estonia	ID	0/68	Puusepp et al. (2008)
Israel	Screened women without fam hx of ID	1/4,778 (3 asymptomatic/14,334)	Toledano-Alhadef et al. (2001)
Israel	Screened women without fam hx of ID, DD, ASD	1/36,483 Combining 3 studies from Israel: 1/15,000 (4/60,477)	Berkenstadt, Ries-Levavi, Cuckle, Peleg, and Barkai (2007)
Japan	IDD	2.4% (7/296)	Hofstee et al. (1994)
Korea	Women of reproductive age	0/5,829	Kim et al. (2013)
Japan	Normal	0/370	Otsuka et al. (2010)
Netherlands	ID of unknown etiology	0.3% (2/685)	de Vries et al. (1997)
Pakistan	ID	0.9% (1/104)	Fatima et al. (2014)
Pakistan	ID of unknown etiology	2.8% (3/108)	Kanwal et al. (2015)
Sri Lanka	ID	0/310	Chandrasekara et al. (2015)
Taiwan	Pregnant women	0/1,002	Huang et al. (2003)
Taiwan	ID	0.9% (1/115)	Tzeng et al. (2000)
UK	ID	0/74	Jacobs et al. (1993)
US (referral labs)	Pregnant women	0/29,103	Cronister, DiMaio, Mahoney, Donnenfeld, and Hallam (2005)
US	Newborn blood spots	0/6,895	Tassone et al. (2012)
US (Atlanta, GA)	School-age, special needs classes	0/1,061	Crawford et al. (2002)
US	Preschool with language delay	1.3% (2/155)	Mazzocco et al. (1998)
US	School age with academic difficulties but not ID	0/341	Mazzocco et al. (1997)
US	Screened (no selection)	0/56	Reiss et al. (1994)
Yugoslavia	ID of unknown etiology	0/19	Major et al. (2003)

FM, Full mutation; ID, intellectual disability.

that about 26% of the apparently unrelated families with FXS were of Tunisian Jewish descent; at that time, the Tunisian Jews made up only 2%–3% of the general Israeli population. Thus, Tunisian Jews appeared to have an allele distribution that was predisposed to the *FMR1* expansion mutation. Further investigation showed a high proportion of alleles (20%) with no AGG interruptions among the normal Tunisian Jewish population. Also, the proportion of alleles with greater than 35 uninterrupted 3′ CGG repeats was 5% compared with 0.7% among non-Tunisian Jewish controls in Israel. In addition, the haplotype on which these alleles exist accounted for all the observed cases of the FM among X chromosomes of the Tunisian Jewish families. Other examples of possible founder effects of protective or susceptible alleles have been reported, including isolated Basque groups in Spain (Arrieta et al., 2008; Penagarikano et al., 2004) and women with Ashkenazi ancestry (Weiss et al., 2014).

Investigators from Tehran asked an interesting question about whether to screen families with consanguinity and ID for FXS (Pouya et al., 2009). Typically, a clinician will consider screening such families for autosomal recessive disorders. Pouya et al. screened 508 families with ID referred to a genetic research center in Tehran and stratified families by whether relatives were consanguineous or unrelated. They found 3.4% (13/384) and 15.3% (19/124) had FXS, respectively. The authors noted that the higher rate among unrelated families was due to the fact that most families had at least two affected family members that led to the clinical referral. Importantly, the authors emphasized that families with consanguinity and ID should not only be screened for autosomal recessive genes, but also for FXS.

Lastly, with respect to differing rates among admixed ethnic/racial populations, no study has had a large enough sample size to determine whether the incidence of the FM differs among subpopulations. Coffee et al. (2009) screened 36,124 newborn males in a diverse US population and, although the point estimates suggested a lower incidence among African Americans and Hispanics compared with Whites, the confidence intervals overlapped and thus were not statistically different (Table 4.3). Tassone et al. (2012) conducted a pilot screening study of 14,207 newborns (7,312 males and 6,895 females) in the United States using a PCR-based method to determine repeat size. One male with the FM was identified. Due to the increased frequency of the PM allele, they could examine whether rates differed among the three major ethnic/racial groups (mother's self-report). Their sample included 4161 Whites,

TABLE 4.3 Incidence of FM carrier males among newborns using blood spots in a diverse admixed US population.

Race/ethnicity	Identified FM carriers/*n* tested	Incidence per 10,000 (ratio)	95% CI (per 10,000)
White	4/16,252	2.5 (1/4,036)	0.9–6.3
African American	2/10,979	1.8 (1/5,490)	0.5–6.6
Hispanic	1/5,396	1.9 (1/5,396)	0.3–10.5

CI, Confidence interval; FM, full mutation.
Data taken from Coffee, B., Keith, K., Albizua, I., Malone, T., Mowrey, J., Sherman, S. L., & Warren, S. T. (2009). Incidence of fragile X syndrome by newborn screening for methylated FMR1 DNA. American Journal Human Genetic, *85(4), 503–514.*

3069 African Americans, and 3493 Hispanics. Using the findings based on female newborns who carried the PM, the most frequent group, they observed that the incidence of carriers who were African American was higher compared with females who were Hispanic (1/168 vs. 1/570, respectively), but the difference was not statistically significant ($P = 0.08$). Neither differed from the incidence of carriers self-reported as Whites (1/201). Thus, the determination of differences in the incidence of the *FMR1* expansion mutations awaits larger screening studies.

It is important to recognize that there may be additional reasons for differences in the prevalence of the disorder among racial/ethnic groups. Differential access or use of the health care system may lead to the lack of or difficulty obtaining a diagnosis (Visootsak, Charen, Rohr, Allen, & Sherman, 2011). Reproductive options for family building may differ once a diagnosis is made. Thus, beyond the population dynamics of the *FMR1* repeat mutation, there may be cultural reasons for different prevalence estimates of FXS.

FACTORS RELATED TO VARIATION IN CLINICAL PRESENTATION AFFECT THE ABILITY TO ESTIMATE PREVALENCE

Above, we mentioned that the severity of FXS depends on the sex of the individual, as FXS is an X-linked condition. Thus, females with the FM are essentially mosaics; they have some cells with the X-chromosome with the FM active and others with it inactive. The proportion of the active X-chromosome carrying the normal *FMR1* allele (defined as the X-chromosome activation ratio, XAR) is associated with severity of outcome in females: those with a higher XAR are more mildly affected than those with a lower XAR on average (e.g., Abrams et al., 1994; de Vries et al., 1996; Martinez et al., 2005). Similarly, other types of mosaicism are also associated with clinical outcomes in individuals with the FM. Two general categories of mosaicism exist: methylation mosaicism (i.e., some cells have an unmethylated FM while others have the typical hypermethylated FM) and repeat size mosaicism (e.g., typical hypermethylated FM allele in some cells and smaller repeat alleles in others). Most often, repeat size mosaicism includes FM and PM alleles. Less common are mosaics involving the FM allele and an allele with part of the *FMR1* gene deleted, sometimes reducing the number of repeats (e.g., de Graaff et al., 1996; Fan et al., 2005; Gasteiger, Grasbon-Frodl, Neitzel, Kooy, & Holinski-Feder, 2003). As expected, the resulting phenotype depends on the level of mosaicism and the alteration resulting from the deleted region. Individuals, who carry a mosaic genotype, on average, present with a milder phenotype, as some expression of FMRP is restored (e.g., Kaufmann, Abrams, Chen, & Reiss, 1999; Pretto et al., 2014). For example, Pretto et al. (2014) studied 18 individuals with FXS, including 13 mosaics, for which peripheral blood cells and primary fibroblast cells were available. They showed that for both cell types, *FMR1* mRNA and FMRP expression were directly correlated with the percent of methylation of the *FMR1* allele. They also administered cognitive tests and found that Full Scale IQ scores were inversely correlated with the percent methylation and positively correlated with higher FMRP expression. These findings, along with other studies (e.g., Hagerman et al., 1994) point to the correlation of cognition with the level of FMRP produced due to the presence of an unmethylated FM or presence of PM or smaller repeat alleles. This correlation is further supported by rare males who carry a fully unmethylated FM (e.g., Smeets et al., 1995; Z. Wang,

Taylor, & Bridge, 1996). These males are most often identified through other members of the family who are diagnosed with FXS and carry a typical hypermethylated FM, because the manifestation of the unmethylated FM is mild—not only with respect to cognition, but with respect to characteristic facial features (Haberlandt, Zotter, Witsch-Baumgartner, Zschocke, & Kotzot, 2014). Although such males are rare, the resulting variable phenotype associated with the FM suggests that studies that estimate prevalence from testing a target population who manifest some aspect of FXS (e.g., ID or autism) and then extrapolate to the general population underestimate the frequency of the FM.

DELETIONS AND POINT MUTATIONS LEADING TO FXS

For the vast majority of individuals, FXS is due to the large expansion and subsequent hypermethylation of the > 200 CGG repeats. However, about 1% of males with the FXS phenotype have a deletion that removes the *FMR1* gene or its promoter region (e.g., Albright et al., 1994; Coffee et al., 2008; Hammond, Macias, Tarleton, & Shashidhar Pai, 1997) or a point mutation that disrupts the function of FMRP (e.g., Collins et al., 2010). Due to the expense of sequencing the *FMR1* gene, most studies estimating the frequencies of these mutations select individuals with a phenotype similar to that of males with FXS, but are known to not carry the FM. Here, we note some of the larger studies to emphasize the important contribution of such mutations to the cause of FXS and ID.

The largest sequencing study to date was done by Collins et al. (2010), using pooled-template massively parallel sequencing. They tested 963 males who had tested negative for *FMR1* repeat expansion at a genetics diagnostic laboratory over a 5-year period. These children presented with the more general diagnosis of developmental delay, not necessarily those with a FXS-like phenotype. Among these 963 males, 130 novel *FMR1* sequence variants were identified. One mutation identified, a novel missense change, c.413G > A (R138Q), altered a conserved residue in the nuclear localization signal of FMRP. In addition, three promoter mutations were found, all significantly reducing in vitro levels of *FMR1* transcription (their effect on FMRP levels was not assessed). Additional variants reported included 10 noncoding variants in the introns and 3'untranslated region of *FMR1* that may have functional significance, including two predicted splice site mutations.

Other studies of relatively large samples are also notable. Luo et al. (2015) sequenced 60 pediatric cases with FXS symptoms and no FM. They identified one deletion of the *FMR1* gene and detected c.879A > C mutation. This point mutation was reported to alter neighboring splicing, but no effect on the exon junction was noted. Gronskov, Hallberg, and Brondum-Nielsen (1998) screened 118 males referred for FXS testing but who lacked the FM. They found no pathogenic mutations. They did identify three silent mutations that had no apparent effect. Lastly, Handt et al. (2014) screened 508 males with FXS-associated features and no FM and identified three missense mutations—one with p.Gly482Ser and two unrelated with p.Arg534His that may be involved in the ID.

Although it is difficult to generalize the phenotype of individuals with *FMR1* deletions and point mutations because of the selection criteria for studies (i.e., selection for FXS-associated phenotypes), some observations can be summarized. For deletions, the FXS phenotype can be more severe than that resulting from the FM, because some deletions can remove additional

genes when large (e.g., Moore et al., 1999; Probst et al., 2007; Quan, Grompe, Jakobs, & Popovich, 1995; Quan et al., 1995b; Wolff et al., 1997) while other deletions only involve the *FMR1* gene and have a typical FXS presentation (e.g., Gedeon et al., 1992; Meijer et al., 1994; Parvari et al., 1999; Wiegers, Curfs, Meijer, Oostra, & Fryns, 1994). It has been speculated that the *FMR1* flanking region is a hotspot for deletions, primarily due the CpG rich area (de Graaff et al., 1995; Garcia, De Diego, Oostra, Willemsen, & Mirta, 2000; Nichol Edamura & Pearson, 2005).

The study of the physical and molecular phenotypes associated with missense mutations can provide important insights into the pathophysiology resulting from the disruption of *FMR1* function. Missense mutations occurring in the RNA binding domains of FMRP result in the loss of RNA binding and polyribosome association [e.g., p.(Ile304Asn) (De Boulle et al., 1993; Zang et al., 2009) and p.(Gly266Glu) (Myrick et al., 2014)]. Both patients with these mutations presented with physical, cognitive and behavioral characteristics similar to FXS. Two individuals with truncating mutations in the N-terminal half of the gene, potentially leading to nonsense-medicated decay of the mRNA, had the typical FXS phenotype (Lugenbeel, Peier, Carson, Chudley, & Nelson, 1995). Gronskov, Brondum-Nielsen, Dedic, and Hjalgrim (2011) found a substitution in exon 2 of *FMR1* (c.80C > A) causing a nonsense mutation p.Ser27X in patient with classical clinical symptoms of FXS. The patient's mother carried this mutation and had mild ID. Three unrelated patients positive for FXS phenotype were studied and found to have a point mutation that led to exon 10 being skipped. This resulted in the joining of exons 9 and 11 and causing a frameshift and premature termination of translation that removed the conserved region encoding the KH2 and RGG box domains of FMRP (Y. C. Wang, Lin, Lin, Li, & Li, 1997). These mutations suggest that the inability of FMRP to regulate protein synthesis is a critical part of FXS pathophysiology.

An important follow-up study of the patient identified with the missense mutation (c.413G > A (R138Q) in the study by Collins et al. (2010) has helped to dissect the presynaptic and postsynaptic functions of FMRP (Myrick et al., 2015). First, the R138Q mutation is located at the amino-terminal domain of FMRP, not within an RNA binding domain. Expression studies indicated that the postsynaptic translation regulation capabilities of FMRP were retained in the presence of this R138Q mutation, but that presynaptic functions were disrupted. The phenotype of the patient with this partial-loss of function mutation presented with global developmental delay, ID, and intractable seizures, but no other behavioral, neurological, or dysmorphic features commonly associated with FXS. These results suggest that presynaptic function of FMRP may be specifically connected to ID and seizure pathology in FXS, possibly through the amino-terminal domain. Further *FMR1* screening studies of this specific seizure phenotype and ID may be warranted.

In summary, clearly de novo deletions and point mutations do appear among males with FXS symptoms and also among those with different forms of ID. Collins et al. (2010) estimated the frequency of *FMR1* sequence variants causing developmental delay in their study population of 963 males to be about 0.8%, assuming the identified variants are verified as pathogenic and accounting for their false negative rate. In comparison, the frequency of the FM among those with developmental delay or ID is about 2.5% (Table 4.2). Although only a rough estimate, these data suggest that it is important to continue large-scale screening studies to identify the entire range of mutations in the *FMR1* gene. Such studies will identify the diagnostic yield of systematic screening and will provide insight into the complex function of

this essential gene. Importantly, these should be done in a systematic and unbiased way with respect to phenotype.

CONCLUSIONS

Based on the current literature, estimate of the prevalence of individuals with the FM in the total population is about 1 in 7,000 for males and about 1 in 11,000 for females. The prevalence of carriers with the PM in the total population is about 1 in 850 for males and 1 in 300 for females. These estimates are lower than previously reported, but are based on the most current literature. Among individuals with ID, the estimated frequency of FM is about 2.5%, although this estimate is greatly influenced by the phenotype of the tested population. Because of the dynamic, multistep repeat mutation process to reach the FM allele, prevalence of FXS has been seen to vary among isolated populations due factors such as founder effects and genetic drift. No screening study has been large enough as yet to determine whether the incidence of the FM varies in admixed populations, although there is evidence that the overall repeat length distribution does differ by race/ethnicity. Larger screening studies of unbiased populations are warranted and are now feasible due to significant advances in methods to detect the FM and other *FMR1* sequence variants. Such studies will help expand the phenotype description of carriers of *FMR1* mutations and will better estimate costs associated with this clinically significant mutation that has been identified in every geographical that has been studied.

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CHAPTER

5

Mechanisms of Repeat Instability in Fragile X Syndrome

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INTRODUCTION

Fragile X syndrome (FXS) is the most common single-gene cause of inherited intellectual disability. As discussed in more detail in other chapters, FXS is a member of the fragile X-related disorders, a group of human genetic conditions that also includes fragile X-associated tremor/ataxia syndrome (FXTAS) (Hagerman & Hagerman, 2015) and fragile X-associated primary ovarian insufficiency (FXPOI) (Sullivan, Welt, & Sherman, 2011). FXS was the first of this group of disorders to be identified back in 1943 (Martin & Bell, 1943). However, it was not until almost 50 years later that the X-linked gene responsible *fragile X mental retardation 1 (FMR1)* was identified (Verkerk et al., 1991). This work identified not only the affected gene but also demonstrated that the most common responsible mutation was an expansion or increase in the number of repeats in a CGG repeat tract located in the 5′ untranslated region (5′ UTR) of the *FMR1* gene. We now know that this repeat tract is polymorphic in the human population with four different allele size classes being distinguished (Maddalena et al., 2001). Normal alleles have 5–39 CGG repeats with the most common alleles having 29–30 repeats (Tassone et al., 2012). Intermediate or gray zone alleles have 40–54 repeats. Premutation (PM) alleles, those associated with FXTAS and FXPOI, have 55–200 repeats. The threshold for full mutation (FM) alleles, those that result in FXS, is considered to be 200 CGG repeats, but such alleles can have many hundreds, if not thousands of repeats. Both expansions and contractions are seen in fragile X pedigrees, but a strong expansion bias is apparent. Expansions and contractions could both contribute to the repeat size mosaicism frequently seen in carriers of PM and FM alleles, although the relative contribution of each of these processes to the overall repeat length heterogeneity is unclear.

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Factors That Affect Expansion Risk

PM alleles show both small and large intergenerational expansions. Small expansions are more common on paternal transmission (Sullivan, Crawford, Scott, Leslie, & Sherman, 2002), while large expansions into the FM range are exclusively maternally transmitted (Oberle et al., 1991; Rousseau et al., 1991), making gender a risk factor for the transmission of FXS. However, this is not to say that gender is a risk factor for the expansion itself. Although male PM carriers are not at risk of transmitting a FM allele to their children and male FM carriers only have PM alleles in their sperm (Reyniers et al., 1993), there is reason to think that this is the result of selection against large expansions rather than protection of the male germ line against such expansions (Malter et al., 1997). The fact that the FM is seen exclusively on maternal transmission accounts for the anomalous mode of inheritance in FXS pedigrees that came to be known as the Sherman Paradox: That is, the risk of having FXS is dependent on the position in an affected pedigree, with the male sibs of so-called nontransmitting males not being at risk, while the grandsons and great grandsons are (Sherman, Morton, Jacobs, & Turner, 1984).

Recent evidence demonstrates that maternal age is a risk factor for expansions (Yrigollen et al., 2014). This suggests not only that expansion occurs in the oocyte, but either older oocytes are more prone to the events that result in expansion or expansions accumulate in the oocyte over time. In general, the risk of expansion on maternal transmission increases with increasing repeat length with 94% of PM alleles with >90 repeats undergoing expansion to FM alleles on maternal transmission (Fu et al., 1991; Nolin et al., 2003; Nolin et al., 2011).

AGG interruptions within the CGG repeat tract also affect expansion risk. Such interruptions are seen most commonly at the 10th or 11th and 20th or 21st triplet of the CGG repeat tract and reduce the risk of expansion (Eichler et al., 1994; Nolin et al., 2015; Nolin et al., 2013; Yrigollen et al., 2012). The number of uninterrupted repeats at the 3′ end of the repeat tract is the single known factor that best predicts the likelihood of large maternal expansions (Nolin et al., 2013). It has been suggested that alleles with at least 34 uninterrupted repeats are associated with significant risk of expansion (Eichler et al., 1994). There is evidence that in some cases unstable alleles arise from stable ones by the loss of AGG interruptions (Chiurazzi et al., 1996). In other cases expansion seems to occur via the incremental lengthening of a pure repeat tract at the 3′ end (Kunst & Warren, 1994). Contractions on the other hand do not seem to be sensitive to the presence of AGG interruptions indicating perhaps that they arise by a different mechanism (Nolin et al., 2015).

Only about 23 and 15% of the variance in the repeat length changes of paternally and maternally transmitted alleles, respectively, is explained by consideration of both repeat length and AGG interruption status (Nolin et al., 2013). In addition, it is known that the expansion risk of PM carriers from families that already include a child with FXS is much higher than the risk in the general population (Nolin et al., 2013). Thus other factors likely play a role in expansion risk. A number of high-risk haplotypes have been identified that indicate a contribution of cis-acting factors to expansion risk. For example, genotyping of single nucleotide polymorphisms (SNPs) across a 650 kb region flanking the *FMR1* gene identified a SNP variant ~50 kb centromeric to the gene that is associated with an elevated risk of expansion (Ennis, Murray, Brightwell, Morton, & Jacobs, 2007).

Transcription or transcriptional competence is also important for instability. While methylated alleles are stable, large unmethylated PM and FM alleles can be very unstable (Glaser, Wohrle, Salat, Vogel, & Steinbach, 1999; Wohrle et al., 1998), although whether this instability reflects both expansions and contractions is unclear. A retrospective examination of repeat PCR data from female PM carriers (Chen et al., 2010; Grasso et al., 2014) demonstrates that predigestion of the PCR template with a methylation-sensitive enzyme eliminates all traces of expanded alleles. Thus expansions in women only occur when the PM allele is on the active X chromosome. It is still unclear whether variability in the extent of *FMR1* transcription is a factor that contributes to variability in expansion risk. Work in mouse models suggests that it may not be (Lokanga et al., 2013). Whether transcription is required for contractions is also unknown.

Parallels to Other Related Disorders

While FXS was one of the first disorders shown to result from repeat expansion, it is now known that many more diseases share this novel mutational mechanism. These diseases, known collectively as the repeat expansion diseases, include myotonic dystrophy type 1 and type 2 (DM1 and DM2), Huntington disease (HD), many of the Spinocerebellar ataxias (SCAs), and Friedreich ataxia (FRDA). The repeat unit responsible for these diseases ranges from trinucleotides, such as the CGG repeat responsible for the FXDs, the CTG repeat responsible for DM1, HD and many of the SCAs, and the GAA repeat responsible for FRDA, to pentameric and even dodecameric repeats [see (S. M. Mirkin, 2006) for a more comprehensive review of the repeat expansion diseases].

The repeat instability in all of these diseases is likely to be very different from the generalized microsatellite instability (MSI) seen in many forms of cancer since in MSI repeats are as likely to be lost as gained (Peltomaki, 1997), while in the repeat expansion diseases the repeat tract shows a strong expansion bias. Furthermore, while repeat length changes in MSI are small, in some repeat expansion diseases including FXS, many hundreds of repeats can be added to the repeat tract in the space of a single generation. While it has not been conclusively demonstrated that different repeat expansion diseases share common instability mechanisms, there is good reason to think that they may do so. Thus, we can also look to what has been learnt from the study of instability in various models of other repeat expansion diseases to try and understand what may be responsible for repeat expansion and contraction in the FXDs.

Model Systems to Study Repeat Instability

Expansion in FXS PM lymphoblasts, fibroblasts, induced pluripotent stem cells (iPSCs), and embryonic stem cells (ESCs) occurs very rarely, limiting the use of these cell types to examine instability. However, a variety of bacterial [e.g., (Bowater, Jaworski, Larson, Parniewski, & Wells, 1997; Hirst & White, 1998; Kang, Jaworski, Ohshima, & Wells, 1995; Shimizu, Gellibolian, Oostra, & Wells, 1996)], yeast [e.g., (Balakumaran, Freudenreich, & Zakian, 2000; Freudenreich, Stavenhagen, & Zakian, 1997; Maurer, O'Callaghan, & Livingston, 1996; White, Borts, & Hirst, 1999)], and mammalian [e.g., (Al-Mahdawi et al., 2004; Bontekoe et al., 2001; Bontekoe, de Graaff, Nieuwenhuizen, Willemsen, & Oostra, 1997; Burright et al., 1995;

Entezam et al., 2007; Gourdon et al., 1997; Lavedan, Grabczyk, Usdin, & Nussbaum, 1998; Mangiarini et al., 1996; Wheeler et al., 1999)] models have been developed to study repeat instability in different repeat expansion diseases. Early work on the mechanism of repeat expansion in model organisms focused primarily on the use of bacteria and yeast, into which repeats had been introduced either on episomes or integrated in the genome. These organisms provide a genetically defined and readily modifiable system for such studies and have proved to be valuable in the identification of factors that may play a role in repeat instability in humans. However, instability in these simple model organisms is sensitive to the fact that the repeats impair DNA replication and thus selection for shorter repeat tracts during rapid growth is a possible confounder of the results from such systems. In addition, a variety of chromatin modifiers (Debacker et al., 2012; Dion, Lin, Hubert, Waterland, & Wilson, 2008; Gorbunova, Seluanov, Mittelman, & Wilson, 2004; Jung & Bonini, 2007; Libby et al., 2008) have been shown to affect CAG repeat expansion in different mammalian model systems. This, together with the tissue/cell specificity of instability that is apparent in many repeat expansion diseases, is obviously difficult to model in these organisms.

Several mouse models have been developed to study the expansion mechanism and the pathology associated with CGG repeat expansions in *FMR1*. This includes various transgenic (Bontekoe et al., 1997; Lavedan et al., 1998; Peier & Nelson, 2002) and knock-in (Bontekoe et al., 2001; Entezam et al., 2007) mouse models. Expansions have been most intensively studied in a knock-in FXS PM mouse model with 130 uninterrupted CGG/CCG-repeats (Entezam et al., 2007). This mouse, sometimes referred to as the CGG_{nih} KI mouse, shows both expansions and contractions, with expansions predominating as in humans. This mouse model also recapitulates the requirement that the PM allele be on the active X chromosome for somatic expansion to occur (Lokanga, Zhao, Entezam, & Usdin, 2014). However, most of the intergenerational expansions detected in these animals are small involving 5–10 repeats in animals that are ~12 months of age. A similar lack of large expansions is seen in other PM mouse models (Bontekoe et al., 2001; Brouwer et al., 2008; Peier & Nelson, 2002), as well as a mouse model of DM1, another repeat expansion disease in which large maternally transmitted expansions are also seen (Gourdon et al., 1997). This contrasts with the fact that expansions of 100 repeats or more occurs on almost all human maternal transmissions of alleles with ~90 repeats (Nolin et al., 2011). It is possible that this reflects fundamental differences in the expansion mechanism in mice and humans. However, it is also possible that the larger expansions seen in women reflect the cumulative effect of multiple small expansions occurring in oocytes. Since a woman's reproductive lifespan is >20 times longer than that of a female mouse, there is much more time for her oocytes to undergo repeat rounds of expansion that ultimately result in very large FM alleles. An accumulation of repeats with time is consistent with the fact that the average expansion transmitted in mice is a function of parental age (Zhao & Usdin, 2014) and that the risk of transmission of FM alleles increases with maternal age in humans (Yrigollen et al., 2014).

The large body of literature that has been published in the last 2 decades exploring factors that affect repeat instability in different model systems is impossible to summarize in a single chapter. Since it is difficult to model some events in simple model organisms for the reasons aforementioned, and we now know that the loss of some proteins that are critical to repeat expansion in mammals do not lead to a reduction in expansion events in bacteria (Parniewski, Jaworski, Wells, & Bowater, 2000), yeast (Miret, Pessoa-Brandao, & Lahue, 1997), and flies

(Jackson et al., 2005), we will focus here primarily on lessons learnt from work in mice and in human cells, with a focus on work specifically involving CGG repeats. Since expansions vastly outnumber contractions in humans and data from FXS families suggests that expansion and contraction may involve different molecular mechanisms (Nolin et al., 2015), we will deal with these two phenomena separately.

POTENTIAL MECHANISMS FOR REPEAT EXPANSION

Instability May be Initiated by Secondary Structures Formed by the Repeats

Current models for the expansion mechanism are all based on the observation that the individual DNA strands of all expansion-prone repeats form secondary structures, while stable repeats do not [reviewed in (Mirkin, 2006)]. It is generally thought that these structures may be the substrates upon which the expansion process acts. The FXS repeats form folded structures that include hairpins with a mixture of Watson–Crick and Hoogsteen base pairs and tetraplexes (quadruplexes) stabilized primarily by guanine tetrads (Chen et al., 1995; Fojtik & Vorlickova, 2001; Fry & Loeb, 1994; Kettani, Kumar, & Patel, 1995; Mariappan et al., 1996; Mitas, Yu, Dill, & Haworth, 1995; Nadel, Weisman-Shomer, & Fry, 1995; Patel, Bhavesh, & Hosur, 2000; Usdin & Woodford, 1995; Yu et al., 1997). These structures are thought to form during replication or transcription when the DNA duplex is transiently unpaired. While formation of such structures by CGG repeats has not been demonstrated in vivo, RTEL1, a multifunctional DNA helicase, has been shown to protect against CAG repeat expansion (Frizzell et al., 2014). Additionally cleavage by zinc finger nucleases that target CAG repeat hairpins was found to result in cleavage of CAG repeats in human cells (Axford et al., 2013; G. Liu, Chen, Bissler, Sinden, & Leffak, 2010). Thus it is likely that the even more stable secondary structures formed by the CGG repeats are also able to form, at least transiently, in vivo.

Many of these repeats also form cotranscriptional DNA-RNA hybrids or R-loops (Groh, Lufino, Wade-Martins, & Gromak, 2014; Loomis, Sanz, Chedin, & Hagerman, 2014; Reddy et al., 2014) and since many of these repeats, including those at the *FMR1* locus (Ladd et al., 2007), are also bidirectionally transcribed, they can also form double R-loops (Reddy et al., 2014). In the case of the FXS repeats, the amount of both sense and antisense transcript increases as the repeat number increases with the antisense transcript reaching levels comparable to that of the sense transcript (Ladd et al., 2007). Thus the amount of single and/or double R-loops might also be expected to increase with increasing repeat number. R-loop formation is frequently associated with instability (Sollier et al., 2014) and double R-loop formation was found to increase the frequencies of CAG repeat instability in vitro (Reddy et al., 2014). In part, this instability may arise because R-loop formation leaves the nontemplate strand unpaired and thus able to form hairpins and other folded DNA structures (Duquette, Handa, Vincent, Taylor, & Maizels, 2004). Thus, it may be that R-loop formation accounts for, or contributes to, the transcriptional dependence of repeat expansion by increasing the opportunity for DNA secondary structures to form.

The ability of the expansion-prone repeats to form intrastrand folded structures would be expected to facilitate strand slippage during DNA synthesis, and to favor priming from the slipped position. Slippage of the nascent strand could lead to expansions if replication of the

complementary strand occurred before the slipped DNA could be repaired, while contractions could arise if slippage of the template strand occurred and was not repaired (Kang et al., 1995) as illustrated in Fig. 5.1. These structures are also likely to form in the 5′ flaps generated during strand displacement synthesis. Once formed, they would be resistant to removal by flap endonucleases, such as FEN1 (Spiro et al., 1999; Yang & Freudenreich, 2007). Incorporation of these supernumerary bases into the nascent strand would then generate an expansion when this strand serves as the template for DNA synthesis.

In addition to being prone to strand slippage, all expansion-prone repeats tested impede DNA synthesis in vitro and in vivo (Krasilnikova & Mirkin, 2004; Pelletier, Krasilnikova, Samadashwily, Lahue, & Mirkin, 2003; Samadashwily, Raca, & Mirkin, 1997; Usdin & Woodford, 1995; Voineagu, Surka, Shishkin, Krasilnikova, & Mirkin, 2009; Yudkin, Hayward, Aladjem, Kumari, & Usdin, 2014). This could lead to an increased incidence of strand slippage as the replication complex attempts to proceed along the template. Since the CGG-strand of the repeat forms more stable secondary structures than the CCG-strand (Paiva & Sheardy, 2004), replication fork blockage would be more likely to occur when the CGG-strand was the template for DNA synthesis. While the repeat tract might be more prone to undergo strand slippage and strand displacement when the CGG-strand was the nascent strand.

The presence of AGG interruptions would likely reduce the incidence of any fork stalling, strand slippage, and strand displacement since the interruptions would reduce the stability of secondary structures formed by the repeat (Gacy, Goellner, Juranic, Macura, & Mcmurray, 1995; Jarem, Huckaby, & Delaney, 2010; Weisman-Shomer, Cohen, & Fry, 2000).

The Effect of These Secondary Structures May be Mediated via Mismatch Repair Proteins

The structures formed by both CGG and CCG repeats are bound by the mismatch repair (MMR) proteins MutSα and MutSβ in vitro (Zhao, Lokanga, Wu, Kumari, & Usdin, 2016; Zhao et al., 2015). MutSβ, a heterodimer of MSH2 and MSH3 that is involved in repairing small insertions or deletions, also binds other disease-associated repeats both in vitro and in vivo (Du et al., 2012; Owen et al., 2005). MutSβ is required for almost all intergenerational expansions and all somatic ones in the CGG_{nih} KI mouse (Lokanga, Zhao, & Usdin, 2014; Zhao et al., 2015). MutSα, a heterodimer of MSH2 and MSH6 that is normally involved in repairing single base mismatches, also contributes to repeat expansion but likely does so by facilitating the activity of MutSβ (Zhao et al., 2016). MutSβ has also been shown to be essential for expansion in a number of model systems of other repeat expansion diseases (Du et al., 2012; Foiry et al., 2006; Halabi, Ditch, Wang, & Grabczyk, 2012; Kovalenko et al., 2012; Manley, Shirley, Flaherty, & Messer, 1999; Owen et al., 2005; Savouret et al., 2003; Wheeler et al., 2003). Thus, functional MMR proteins that usually maintain genome stability and prevent MSI, are actually required for repeat expansion.

Work in DM1 and HD mouse models has shown that the MutL proteins, the normal downstream binding partners of MutSβ and MutSα in MMR, are also important players in the repeat expansion process (Gomes-Pereira, Fortune, Ingram, McAbney, & Monckton, 2004; Pinto et al., 2013). Intriguingly, in the case of the HD mouse, loss of the least abundant MutL complex, MutLγ, eliminated all somatic expansions (Pinto et al., 2013). Since mice lacking MutLγ are sterile, the effect on expansion in the germ line could not be examined, but there is reason to think that this complex is essential for these expansions as well. While the function

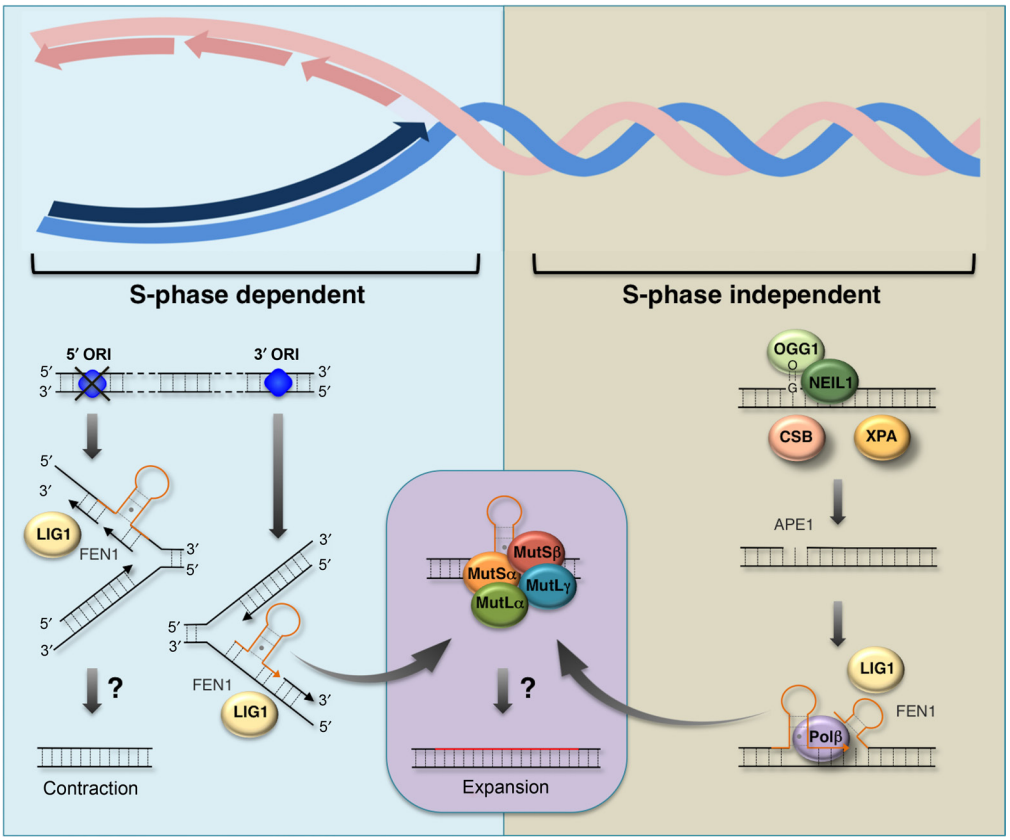

FIGURE 5.1 **S-phase dependent and S-phase independent models for CGG repeat expansions.** The left hand panel illustrates the origin of replication (*ORI*) switch model, one potential S-phase dependent model for repeat expansion. This model is based on the observation that the human *fragile X mental retardation 1 (FMR1)* gene is flanked by two ORIs (Gerhardt et al., 2014a). It is also predicated on the premise that expansions occur as a result of strand-slippage by the nascent CGG-rich strand during lagging strand DNA replication, a phenomenon that is facilitated by the stability of the hairpin formed by CGG repeats. Priming from the slipped position results in the incorporation of additional repeats into the nascent strand. Contractions occur as a result of strand-slippage when the CGG-strand is the lagging strand template when the hairpin is bypassed. In cells where both ORIs are operational, expansions and contractions both occur such that there is no net change in repeat number. However, in the fragile X syndrome (FXS) embryonic stem cells, only the 3′ ORI is active (Gerhardt et al., 2014a). As a result, expansions now outnumber contractions. The right hand panel illustrates one potential S-phase independent expansion mechanism. Repair of oxidative DNA damage within the repeats is initiated by DNA glycosylases, such as OGG1 and NEIL1, which excise the damaged base. CSB and XPA may promote expansions by increasing the efficacy of this step. Next, APE1 makes an incision and removes the abasic site, a step that can also be facilitated by CSB. Repair of the subsequent strand break by the long-patch base excision repair (BER) pathway involves synthesis by Polβ, perhaps together with Polδ, followed by FEN1 removal of the flap-structures formed during the strand displacement synthesis, and ligation by LIG1. Hairpins can form either in the displaced flaps or in the nascent strand due to strand slippage, especially when coordination between Polβ and FEN1 is disrupted. Hairpins formed during replicative or repair synthesis are bound by the mismatch repair (MMR) complexes MutSα and/or MutSβ and in conjunction with the MutL family of proteins result in the generation of expansions. Note that in principle, hairpins formed during any DNA processing pathway could also result in expansion. Proteins that have been implicated in generating expansions in mouse models or human cells are shown in colored spheres. The orange DNA strand represents the hairpins formed by the CGG repeats, the red strand the expansion products.

of MutLγ is not well understood, the fact that it is essential for expansion in the HD mouse suggests that events downstream of MutSβ binding are likely to be vital to the expansion process. While the MMR machinery is frequently coupled to chromosomal replication traveling with the replication fork during S-phase DNA synthesis, it also recognizes a variety of other DNA lesions generated outside of S-phase (Colussi et al., 2002; Stojic et al., 2004b) in some cases acting to activate cell cycle checkpoints and signal apoptosis (Stojic, Brun, & Jiricny, 2004). Since MutSβ's best-known role is to protect the genome against MSI, how MutSβ acts to promote repeat expansion is unclear. Nonetheless, if the mouse models are a good indication of what is happening in humans, then any molecular model for repeat expansion needs to be able to accommodate a role for this complex in the expansion process.

S-Phase Dependent Models for Repeat Expansion

Some models for repeat expansion are based on the idea that expansions arise from repeat-mediated problems with strand slippage, strand displacement or replication fork stalling occurring during chromosomal replication. The expansion frequency in this instance would be sensitive to the direction of replication through the repeats. If replication proceeded from downstream of the repeats, the CGG-rich strand would be the nascent strand during lagging strand synthesis, and thus prone to expansion. In contrast, if replication proceeded from upstream of the repeat, the CGG-rich strand would be the lagging strand template, and thus more likely to give rise to contractions (Mirkin & Mirkin, 2014).

Generation of the expansion substrates during chromosomal replication would also likely be sensitive to the distance from the origin of replication (ORI). This distance may modulate the fidelity and speed of replication through the repeat, and affect whether the repeats fell into the single-stranded Okazaki initiation zone (OIZ), since that would affect the likelihood that secondary structures could form. Work in bacteria, yeast, and episomes transfected into human cells has demonstrated that indeed there is an effect on the orientation of the repeat with respect to the ORI (Cleary, Nichol, Wang, & Pearson, 2002; Kang et al., 1995; Liu et al., 2010; Maurer et al., 1996), as well as an effect of the distance from the ORI (Panigrahi, Cleary, & Pearson, 2002; Rindler, Clark, Pollard, De Biase, & Bidichandani, 2006) that would be consistent with a role of chromosomal replication in repeat instability.

Replication stalling within a repetitive run could also trigger replication fork reversal. This would generate a four-way junction or "chicken-foot" structure with a single-stranded repetitive extension in the nascent leading strand that would then be able to form a secondary structure. Extra repeats may be added to the leading strand when the reversed replication fork is flipped back to restart replication. Evidence in support of this model includes the identification of replication intermediates characteristic of reversed forks in human cells (Follonier, Oehler, Herrador, & Lopes, 2013). The leading strand DNA polymerase could also potentially switch templates during replication through the repeat and continue synthesis using the nascent lagging strand as a template, as demonstrated in a yeast model of GAA repeat expansions (Shishkin et al., 2009). At the end of the Okazaki fragment, the polymerase could switch back to the leading strand template. One appeal of this model is that it could account for the generation of larger expansions in a single step since the typical expansion product would correspond in length to the size of an Okazaki fragment [~200 bp or 66 repeats (Ogawa & Okazaki, 1980)].

Three different replication-based models have been proposed to account for the characteristic timing and organ specificity of expansion that is seen in many repeat expansion diseases. The "origin-switch" model suggests that the propensity of a particular cell to undergo expansions results from a change in ORI usage such that the direction of replication through the repeats is reversed (Mirkin & Smirnova, 2002). An "origin-shift" model has also been proposed based on the observation that simply changing the distance between the ORI and the repeat affects instability in a primate model system (Cleary et al., 2002). A third replication-based model known as the "fork-shift" model is based on the idea that expansion in particular cells arises because of epigenetic events that occur between the ORI and the repeat and affect the proximity of the repeat to an OIZ (Cleary & Pearson, 2005).

Mapping of nascent DNA fragments in human cells has shown that the *FMR1* region contains a number of ORIs including one spanning the FXS repeat in the 5′ UTR of the *FMR1* gene, as well as one located 40–50 Kb upstream and another located downstream of the repeat (Brylawski, Chastain, Cohen, Cordeiro-Stone, & Kaufman, 2007; Gray, Gerhardt, Doerfler, Small, & Fanning, 2007; Yudkin et al., 2014). Analysis of nascent strand abundance shows that replication from the ORI in the *FMR1* gene is reduced in FXS lymphoblastoid cells (Yudkin et al., 2014) consistent with the repeats forming a block to DNA synthesis as originally observed in vitro (Usdin & Woodford, 1995). Single-molecule analysis of replicated DNA (SMARD) shows that replication in normal or PM ESCs proceeds from the ORIs located upstream and downstream of the *FMR1* locus. However, initiation from the upstream ORI is absent in two different FXS ESC lines but not in embryoid bodies differentiated from these cells (Gerhardt et al., 2014a). This has led to the suggestion that the use of the single downstream ORI would result in expansions in ESCs. In contrast, the use of both ORIs in differentiated cells would result in both expansions and contractions such that there was no net change in the repeat number.

The difference in ORI usage has been attributed to a previously identified T/C SNP (ss71651738) (Gerhardt et al., 2014b) that is associated with chromosomes with a high risk of expansion (Ennis et al., 2007). It is unclear how this T-to-C mutation could have such a dramatic effect on ORI usage. Since this region was not more highly methylated than the same region in unaffected cells, it has been suggested that the loss of ORI function may arise because of differences in protein binding or because the mutation affects the flexibility or chromatin accessibility of this region (Gerhardt et al., 2014a). However, it should be noted that the *FMR1* was already expanded and heterochromatinized in these cells. Thus, it is difficult to definitively exclude the possibility that the switch in ORI usage occurs subsequent to chromatin changes associated with expansion/gene silencing. A similar pattern of ORI usage in ESCs with the same SNP prior to methylation, in ESCs from PM carriers with the same SNP or in ESCs containing other expansion prone haplotypes would help substantiate this model.

Since the MMR machinery is frequently found associated with the replication fork, the MutSβ complex, essential for expansion, would be well positioned to access the secondary structures generated during any of these replication-associated events.

The fact that certain inhibitors of DNA synthesis can increase expansion frequencies is sometimes cited as proof of such models (Yang, Lau, Marcadier, Chitayat, & Pearson, 2003). However, whether these inhibitors are affecting chromosomal replication or repair synthesis

is unclear. Similarly, a hypomorphic mutation in DNA ligase I, the protein responsible for ligation of Okazaki fragments during lagging strand DNA synthesis, has been reported to reduce the maternal but not paternal expansion frequency in a DM1 mouse model (Tomé et al., 2011). The same mutation has no effect on the CGG_{nih} KI mouse model (Entezam, Lokanga, Le, Hoffman, & Usdin, 2010). However, this may reflect differences in the point at which this protein becomes rate limiting for expansion in different model systems. In any event, since this ligase is involved in both replication and S-phase independent repair processes like base excision repair (BER), it does not constitute proof of a replication-based model. Similarly, the sensitivity of the expansion frequency to chromosome context in different mouse models [e.g., (Fortune, Vassilopoulos, Coolbaugh, Siciliano, & Monckton, 2000; Gourdon et al., 1997; La Spada et al., 1998; Libby et al., 2003; Zhang, Monckton, Siciliano, Connor, & Meistrich, 2002)] could be seen as providing support for replication-based expansion models. However, chromatin context can be important for expansion independent of ORI activity, including via effects on chromatin conformation and transcriptional activity.

Furthermore, in models of many repeat expansion diseases, including the CGG_{nih} KI mouse model, there is no good relationship between the propensity for somatic expansion and the proliferative capacity of that organ or cell (Gomes-Pereira et al., 2014; Lia et al., 1998; Lokanga et al., 2013). There is also evidence from FXS, as well as other repeat expansion diseases, to suggest that expansion can occur in cells that do not divide. For example, the effect of maternal age on expansion risk in both FXS (Yrigollen et al., 2014) and DM1 (Andrews & Wilson, 1992; Morales et al., 2015) suggests that expansion occurs in the oocyte. In HD there is clear evidence for expansion occurring in mature neurons in both humans and mouse models (Kennedy et al., 2003; Kovalenko et al., 2012). Thus expansion in the repeat expansion diseases can occur in cells that do not carry out genomic duplication and thus where the relationship of the repeat to ORIs would be moot. That is not to say that expansions cannot occur via events arising during normal genomic replication but rather that in the most disease-relevant cell types, expansion occurs independent of S-phase chromosomal replication.

S-Phase Independent Expansion Models

The occurrence of expansions in nondividing cells indicates that the expansion process is initiated by damage to DNA or other events that trigger DNA repair rather than the passage of the replication fork. Since these repair processes also involve DNA synthesis, they are also likely to be affected by the secondary structures formed by the repeats as well as mutations in many of the same DNA processing genes. For example, there is a lot of overlap between proteins involved in DNA repair and those involved in lagging strand DNA synthesis. In addition to an essential role for MMR proteins in promoting expansion, data from mouse and cell models have implicated proteins belonging to two other DNA repair pathways in the expansion process.

BER is the major pathway by which oxidative damage is repaired in mammalian cells and there are a number of lines of evidence that implicate the BER pathway in expansion in different repeat expansion disease models. For example, oxidative damage leads to an increased frequency of intergenerational expansions in the CGG_{nih} KI mouse (Entezam et al., 2010).

Additionally, loss of DNA glycosylases OGG1 and NEIL1, enzymes that remove the oxidized base early in BER, reduce the somatic expansion frequency in HD mouse models (Kovtun et al., 2007; Mollersen et al., 2012). While individual null mutations in these genes do not significantly reduce the germ line expansion frequency, it may be that this reflects some overlap in their function or the contribution of other glycosylases to the removal of the damaged base. It is also possible that some expansions occur as a result of depurination or other sorts of damage that result in the production of either an abasic site or a single-stranded nick in the DNA that is then also repaired by BER. The fact that heterozygosity for a hypomorphic mutation in Polβ, a DNA polymerase essential for BER, leads to a large drop in the expansion frequency in the CGG_{nih} KI mouse (Lokanga, Senejani, Sweasy, & Usdin, 2015), suggests that the contribution of BER to expansions is not limited to the generation of nicks, but requires downstream steps integral to the BER pathway.

Work in vitro has shown that expansion of CAG repeats can occur during repair of 8-oxoguanine lesions via the long patch BER pathway when the normal coordination between Polβ and FEN1 is disrupted (Liu et al., 2009). This has led to a model for CAG repeat expansion in which disruption of the coordination of Polβ and FEN1 by spontaneous strand slippage results in multi-nucleotide gap-filling by Polβ and the formation of a 5′ flap by strand displacement. Hairpin formation by the flap bases prevents FEN1 processing and subsequent realignment of these hairpins allows FEN1 alternate cleavage and generation of an extended 5′ end that now can be ligated (Liu & Wilson, 2012). It has also been suggested, again based on work in vitro, that the retention of hairpins formed by strand slippage during BER is facilitated when repair synthesis involves both Polβ and the more processive polymerase, Polδ, perhaps because Polβ lacks a 3′–5′ proofreading activity and is able to use the 3′ end of the hairpin formed on the slipped strand to prime DNA synthesis (Chan et al., 2013).

In the case of CAG/CTG-repeats it has been shown that hairpin formation increases the risk of oxidative damage (Jarem, Wilson, & Delaney, 2009). This phenomenon is likely structure-specific rather than sequence-specific since many oxidizing agents target guanines in the loops of different hairpins (Jarem et al., 2009). Thus it is possible that hairpins formed by the FXS repeats are also prone to oxidative damage that would predispose the repeat tract to events that ultimately lead to expansion.

A role for BER in repeat expansion is appealing because it would explain the effect of oxidative damage on expansion in the CGG_{nih} KI mouse. It is also in line with emerging evidence that suggests that components of the BER pathway are recruited to regions of open chromatin in response to DNA damage (Amouroux, Campalans, Epe, & Radicella, 2010). Thus BER may also account for the fact that expansion at *FMR1* is only seen on the active X chromosome in mouse and human females.

Transcription coupled repair (TCR), is a form of nucleotide excision repair (NER) that is confined to actively transcribed genes. Loss of Cockayne Syndrome B (CSB), a protein essential for TCR, had no effect on paternally transmitted expansions (Zhao & Usdin, 2014). However, it reduced germ line expansions in older females and somatic expansion in some organs of the CGGnih KI mouse. Similarly, the loss of XPA, a protein involved in both TCR and global genome nucleotide excision repair (GG-NER), had minimal effects on intergenerational expansions in a mouse model of Spinocerebellar ataxia type 1 (SCA1) (Hubert, Lin, Dion, &

Wilson, 2011), but did reduce expansion in neuronal tissues of these animals. This reduction is unlikely to reflect a role of GG-NER since the loss of XPC, a protein involved in GG-NER, had no effect on either germ line or somatic expansion in a mouse model of HD (Dragileva et al., 2009).

Since CSB is essential for TCR but not for expansion it may be that the contribution of TCR proteins to expansions is not via TCR per se, but rather via the participation of these proteins in other DNA repair pathways. The fact that the loss of these proteins does not affect expansion in all cells may reflect the fact that these proteins are acting in an auxiliary capacity, promoting steps in the expansion process for which the key proteins may not always be rate-limiting. For example, CBS is known to regulate BER through its ability to upregulate OGG1 expression (Javeri, Lyons, Huang, & Halliday, 2011) and to promote the incision activities of OGG1 (Tuo, Chen, Zeng, Christiansen, & Bohr, 2002), NEIL1 (Muftuoglu et al., 2009), and APE1 (Wong et al., 2007). It could also potentially facilitate expansions via its ability to modify chromatin and/or increase transcription elongation (Newman, Bailey, & Weiner, 2006; Selby & Sancar, 1997). XPA binds with high affinity to DNA junctions (Yang et al., 2006) and thus may help stabilize the expansion substrates. CSB, together with XPF and XPG, have also been shown to promote R-loop-induced genome instability by promoting the formation of double-strand breaks (Sollier et al., 2014).

It is possible to reconcile the contribution of TCR, MMR, and BER proteins to repeat expansion in a number of ways. For example, as illustrated in the right hand side of Fig. 5.1, oxidative damage to DNA or single stranded nicks generated by other events such as depurination, could initiate BER. Strand slippage and strand displacement during repair synthesis via the long patch BER pathway could result in the formation of hairpins on the nascent strand. The MMR proteins may then bind the hairpins and channel them in an alternate repair process that leads to expansion. The TCR proteins can be accommodated in this model in a number of ways. For example, CSB may be acting via its effect on the BER enzymes while XPA may help stabilize the expansion substrates.

While differences in ORI usage might explain the tissue specificity of expansion in S-phase dependent models, differences in the levels of different DNA repair proteins has been suggested to account for why expansions are more common in some cell types. For example, certain BER proteins, particularly APE1, FEN1, and LIG1, are present at lower levels in the mouse striatum than the cerebellum (Goula et al., 2009; Goula et al., 2012) and microarray analysis of the same two tissues showed an elevated level of expression of *Pcna*, *Rpa1*, *Msh6*, *Fen1*, and *Lig1* mRNAs in the cerebellum (Mason et al., 2014). The reduced expression of these proteins in the striatum has been suggested to explain why CAG/CTG-repeats are more prone to expand in this brain region, since the BER process may be less efficient and thus more prone to events that result in expansions. However, in the CGG_{nih} KI mouse model elevated levels of APE1, FEN1, and LIGI proteins were associated with organs that were the most expansion-prone (Lokanga et al., 2015). There is also some evidence to suggest a role for MMR protein levels in affecting the propensity to expand. In HD and DM1-derived ESCs, the loss of expansions that occurs on differentiation has been attributed to the reduction in MutSβ expression associated with the loss of the pluripotent state (Seriola et al., 2011). Similarly, the high level of expansion observed in FRDA iPSCs relative to differentiated cells was also attributed to the high levels of MutSβ (Du et al., 2012). In the CGG_{nih} KI mouse model the levels of MutSβ did not correlate perfectly with the

most expansion-prone tissues. However, when the levels of MutSα were taken into account, there was a general correlation between the level of these MMR proteins and the extent of expansion (Lokanga et al., 2013).

However, the tissues that are the most expansion prone differ in different repeat expansion diseases and disease models. For example, liver is the most expansion prone organ in the CGG_{nih} KI mouse (Lokanga et al., 2013), while kidney is much more expansion-prone in a DM1 mouse model (Fortune et al., 2000). This suggests that protein levels per se, do not account for the tissue specificity. While there is also no good correlation between the amount of steady state *Fmr1* transcript in the CGG_{nih} KI mouse and the propensity to expand (Lokanga et al., 2013), it may be that the propensity of the repeat to expand is related to some combination of the levels of the proteins involved in generating the expansion and the amount of transcription through the repeats.

POTENTIAL MECHANISMS FOR CONTRACTION AND ERROR-FREE REPAIR

Work in the CGG_{nih} KI mouse model has also identified some of the factors involved in generating contractions. Interestingly, some of these factors also contribute to expansions. For example, loss of MutSβ results in the loss of large contractions but not small ones (Zhao et al., 2015). This suggests that in addition to being essential for expansion, MutSβ also plays a role in generating large contractions. However, since the frequency of contractions increases rather than decreases in mice lacking MutSβ, it might be that there is more than one contraction pathway operating in these mice. Whether MutSβ is acting to promote expansions and contractions via the same mechanism is still unclear.

Work in vitro with CAG/CTG-substrates has demonstrated that BER can also generate contractions. Whether BER of oxidized bases produces expansions or contractions in this assay depends on the location of the oxidized base within the repeat, with expansions resulting from lesions at the 5′ end of the repeat tract and contractions resulting from 3′ lesions (Lai, Xu, Zhang, & Liu, 2013). Contractions arise via a unique lesion bypass mechanism (Xu, Gabison, & Liu, 2013; Xu, Lai, & Jiang, et al., 2014). In addition, repair of oxidative damage to bases in a hairpin loop can also result in contractions via the formation of single-strand breaks that convert the hairpin into 5′ and 3′ flaps that are resolved by FEN1 and the 3′-5′ endonuclease Mus81/Eme1 (Xu, Lai, Torner, et al., 2014). However, whether these processes act at the *FMR1* locus in vivo remains to be seen.

There is also evidence to suggest that there are multiple ways for the error-free repair of the repeat tract (Hou et al., 2011). In addition to promoting CGG repeat expansion, MutSα has also been reported to protect against both intergenerational expansions and contractions in an FRDA mouse model (Ezzatizadeh et al., 2012), to protect against somatic expansions in a DM1 mouse model (van den Broek et al., 2002), and to protect against intergenerational contractions in a HD mouse model (Dragileva et al., 2009). It is also possible that it acts to prevent contractions in a CGG_{nih} KI mouse model (Zhao et al., 2016). The apparently paradoxical ability of MutSα to both promote and protect against expansions may reflect two separate roles for this protein, one mediated via its promotion of MutSβ binding during

BER that results in expansions and one mediated via its role in normal MMR that results in error-free repair.

CSB is another protein that has paradoxical effects on repeat stability. In addition to reducing the extent of somatic expansions (Zhao & Usdin, 2014), loss of CSB also led to an increase in the expansion frequency in CGG_{nih} KI mice that are heterozygous for *Msh2* (Zhao & Usdin, 2015). Since the increased expansions in $Msh2^{+/-}$, $Csb^{-/-}$ mice occurred at the expense of alleles that were of the same size as the parental allele and not at the expense of alleles that had undergone contractions, we hypothesize that CSB is playing a role in error-free repair that only becomes apparent in $Msh2^{+/-}$ mice where the expansion mechanism is less robust. Loss of CSB in a HD mouse model also led to an increase in the somatic expansion frequency when expansion was compromised (Kovtun, Johnson, & McMurray, 2011). Knockdown of CSB as well as ERCC1, XPA, and XPG, three other TCR proteins, also increased the contraction frequency in a reporter assay for CAG/CTG-repeat contractions in human cells (Lin, Dion, & Wilson, 2006; Lin & Wilson, 2007, 2009). Furthermore, XPG can promote CAG hairpin removal in vitro (Hou et al., 2011). TCR is an appealing mechanism for error-free hairpin removal since it involves the generation of incisions at some distance downstream and upstream of the stalled RNA polymerase by XPG and a complex containing XPF and ERCC1 respectively. Its efficiency may thus be less likely to be affected by the presence of secondary structures in the intervening region.

DO CHROMOSOME FRAGILITY AND REPEAT EXPANSION SHARE A COMMON MECHANISM?

In addition to being expansion prone, FXS alleles colocalize with a fragile site, a constriction, gap, or break seen in metaphase chromosomes (Lubs, 1969). This site, sometimes referred to as FRAXA, is one of seven characterized folate-sensitive fragile sites in the human genome, all containing long CGG repeat tracts (Lukusa & Fryns, 2008). It is possible that fragility at FRAXA accounts for the high frequency loss of affected X chromosome (Dobkin, Radu, Ding, Brown, & Nolin, 2009) and the loss of the end of the long arm of the X chromosome that is seen in female FM carriers (Verdyck et al., 2015).

Fragility and expansion share some common features. For example, as with expansions of the CGG repeat tract, fragility is also length dependent, and is observed at high frequency only on FM alleles. In addition, the DNA damage response protein ataxia telangiectasia mutated and RAD3-related (ATR) kinase protects against both types of events (Entezam & Usdin, 2008; Kumari et al., 2009). However, while loss of the ataxia telangiectasia mutated (ATM) kinase increases expansions in mice (Entezam & Usdin, 2009), its inhibition reduces chromosome fragility in FXS lymphoblastoid cells (Kumari et al., 2009). Furthermore, while expansion only occurs on transcriptionally active alleles, fragility is seen predominantly on FM alleles that are silenced (Yudkin et al., 2014). Thus, while expansion and fragility of the CGG tract share many features, the mechanisms involved are likely to be different.

As discussed earlier, the secondary structures formed by the expanded CGG repeats can stall replication fork progression (Voineagu et al., 2009; Yudkin et al., 2014). Folate stress is thought to exacerbate this replication problem by affecting nucleotide pools. This is thought to delay completion of replication of this already late replicating region. The net result is that

chromosome condensation in preparation for anaphase begins before replication of the *FMR1* locus is complete, thus perhaps accounting for the microscopic appearance of the fragile site (Yudkin et al., 2014).

CONCLUDING REMARKS AND FUTURE DIRECTIONS

Many questions related to repeat instability at the *FMR1* locus remain unanswered. For example, whether polymorphisms in any of the genes affecting expansion risk in mice are associated with variable expansion risk in FXS families is unknown. Such an association has been demonstrated for other repeat expansion diseases. Specifically, polymorphisms of *Msh3* (Tomé et al., 2013) and *Mlh1* (Pinto et al., 2013) have been shown to affect expansion risk in mouse models of HD and genome wide association studies (GWAS) have implicated *MLH1* polymorphisms as a factor affecting expansion risk in humans with HD (Consortium, 2015). GWAS has also implicated proteins involved in TCR in expansion risk in humans with SCA3 (Martins et al., 2014). Polymorphisms in the BER, TCR, and MMR pathways may thus contribute to expansion risk in CGG_{nih} KI mice and human PM carriers as well. One can imagine that other factors may also contribute. For example, if oxidative damage increases expansion risk in humans as it does in mice (Entezam et al., 2010), then genetic and environmental factors that modulate the extent of oxidative damage to DNA, could also impact the likelihood of expansion.

Given the current absence of effective ways of ameliorating the consequences of CGG repeat expansion, approaches that reduce the repeat number or the risk of expansion may be worth exploring. Recently a CRISPR-Cas9 approach has been used to remove repeats in FXS iPSCs (Park et al., 2015). Not only was the repeat size reduced, but the DNA methylation associated with the original allele was also eliminated. However, the difficulty of effective delivery of these editing complexes to affected cells like neurons limits the postnatal use of such an approach. Small molecules that decrease the risk of intergenerational expansions or increase the likelihood of contractions are likely to be more easily administered. Such molecules would have to be carefully chosen since inhibition of DNA repair pathways could well lead to the accumulation of deleterious mutations elsewhere in the genome. Since MSH3 is essential for expansion, and its loss is not associated with significantly elevated mutation rates, it has been suggested that inhibition of MSH3 may lead to reduced risks of expansion in the repeat expansion diseases (Dragileva et al., 2009; Gonitel et al., 2008; Halabi et al., 2012; Lopez Castel, Cleary, & Pearson, 2010). Work in DM1 cells has also demonstrated an effect of compounds, such as aspirin in reducing the rate of expansion (Gomes-Pereira & Monckton, 2004) and inhibition of two histone deacetylases HDAC3, and HDAC5 has also been shown to reduce expansions in a plasmid-based tissue culture model of CAG repeat expansions (Gannon, Frizzell, Healy, & Lahue, 2012).

Reducing oxidative damage is also an appealing approach given our demonstration that parental exposure to an oxidizing agent increases expansion risk in a CGG_{nih} KI mouse model (Entezam et al., 2010). The antioxidant XJB-5-131 has been shown to significantly reduce expansion risk and to delay the onset of disease symptoms in a HD mouse model (Budworth et al., 2015; Xun et al., 2012). While it remains to be seen whether such compounds would reduce the rate of FX repeat expansion in vivo, such antioxidants may also have a neuroprotective effect, as well as improve oocyte quality. As such, they may have beneficial effects for PM carriers independent of any direct effect on expansion.

We have known about the unusual mutation that causes FXS for almost a quarter of a century now. However, while we have made progress in our understanding of the incidence of expansion prone alleles and some of the factors that affect expansion risk in humans, we still have a long way to go before we fully understand the molecular mechanism responsible and ascertain whether this process can be effectively and safely limited in humans.

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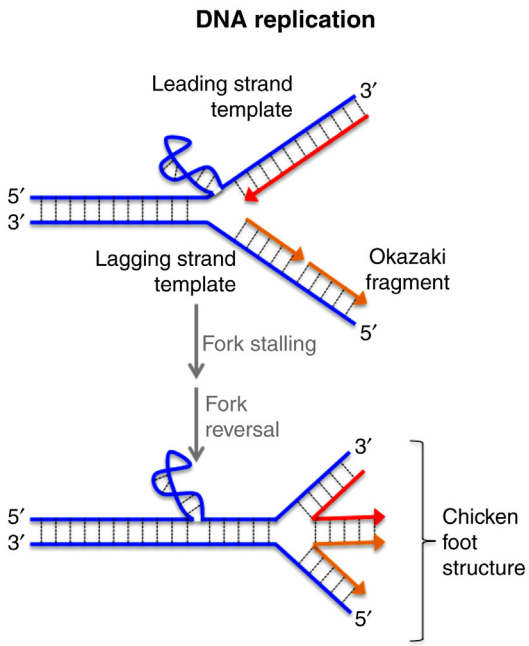
Glossary of terms

Base excision repair (BER) Repair mechanism that removes lesions that do not distort the DNA helix, for example, those caused by oxidative damage. Contains two subpathways, short patch BER and long patch BER.

"Chicken foot" structure AKA reversed fork. Fourstranded structure resembling a chicken's foot formed when replication forks stall, for example, at a structure that blocks DNA polymerase, as illustrated. Fork collapse and regression results in extrusion of the two newly replicated strands. Their annealing forms the middle toe of "chicken-foot."

Embryoid body Three-dimensional aggregate of pluripotent stem cells.

5′ Flap Single-stranded DNA produced when the 5′ end of a downstream Okazaki fragment, nick or gap is displaced by DNA polymerase.



Global genome nucleotide excision repair (GG-NER) A subpathway of NER that repairs bulky DNA lesions in both transcribed and untranscribed regions (see NER).

G-quadruplex (G4-DNA; tetraplex) An exceedingly stable DNA structure consisting of square planar tetrads of guanines held together by Hoogsteen bonds. Can involve 1, 2, or 4 DNA strands.

Hairpin Structure involving base pairing between two different regions of a single DNA molecule. The constituent base pairs can be canonical Watson–Crick base pairs or non-Watson–Crick base pairs.

Heterochromatin Highly compacted chromatin region that is transcriptionally inactive.

Hoogsteen bond An alternate type of hydrogen bond that can form between bases to stabilize various non-Watson-Crick base pairs, triplets or tetrads.

Hypomorphic mutation A mutation that impairs but does not abolish gene function.

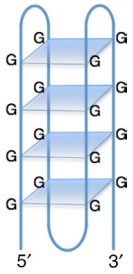
Leading and lagging strands Replication proceeds continuously on the leading strand and discontinuously on the lagging strand via the production of a series of Okazaki fragments, as illustrated earlier.

Microsatellite instability (MSI) Genome-wide variability in the number of repeats in different short tandem repeat arrays (microsatellites). Commonly seen in cells with impaired MMR.

Mismatch repair (MMR) Repairs DNA mismatches or small insertions or deletions (INDELs).

Nucleotide excision repair (NER) Repairs a variety of bulky DNA lesions. Proceeds by one of two subpathways, GG-NER or TCR, that differ in how they recognize DNA damage but that converge for the downstream repair steps.

Okazaki fragments Short DNA fragments, ~200 nucleotides in length, produced during lagging strand synthesis as illustrated earlier.



G4-DNA

Okazaki initiation zone (OIZ) Region of lagging strand where synthesis of the Okazaki fragment begins. This region is frequently single-stranded and thus prone to the formation of intrastrand structures.

Origin of replication (ORI) Sequence where DNA replication is initiated.

Reversed fork see chicken-foot

Transcription coupled repair (TCR) A form of NER active in transcribed genes where it removes transcription-blocking lesions (see NER).

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CHAPTER

6

Modeling Fragile X Syndrome Using Human Pluripotent Stem Cells

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The clinical and molecular complexity of fragile X syndrome (FXS), the most prevalent form of inherited intellectual disability, calls for a suitable model system that can capture the various facets of the disease (Santoro, Bray, & Warren, 2012). The pathogenesis of FXS involves genetic, epigenetic, and cellular alterations, which lead to a complex and often severe neuropsychiatric phenotype and offer challenges to the search for an effective treatment. The identification of the trinucleotide repeat expansion mutation in the *FMR1* gene, which is found in almost all affected individuals, paved the way for the development of animal- and human-based models, which have been used to address the fundamental questions regarding the pathophysiology of this disorder (Verkerk et al., 1991).

The cognitive and behavioral impairments observed in FXS patients originate from the complete or partial absence of fragile X mental retardation protein (FMRP), a key regulator of neural function encoded by the gene *FMR1* (Santoro et al., 2012). The loss of *FMR1* expression is typically mediated by the expansion of a repetitive CGG sequence in the 5′UTR of the gene, which leads to the epigenetic inactivation of the promoter region. Full mutation FXS alleles, which contain more than 200 repeats, are characterized by extensive DNA methylation of the repeat region and the surrounding CpG island, together with the acquirement of repressive histone modifications and the loss of active histone marks (Coffee, Zhang, Warren, &

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Reines, 1999; Coffee, Zhang, Ceman, Warren, & Reines, 2002; Kumari & Usdin, 2010; Pieretti et al., 1991).

Much of our current knowledge on the profound impact of FMRP deficiency on higher brain functions is derived from studies on animal models. Loss of function mutations in *Fmr1* were introduced in several species, including the mouse (Bakker et al., 1994), rat (Hamilton et al., 2014), zebra fish (den Broeder et al., 2009; Tucker, Richards, & Lardelli, 2006), and fruit fly (Wan, Dockendorff, & Jongens, 2000; Zhang et al., 2001). *FMR1* is highly conserved across species (Verkerk et al., 1991), and FMRP-deficient models manifest some of the phenotypes associated with the human disorder. The *Fmr1*-mutated mouse, for instance, displays impaired memory and learning abilities (D'Hooge et al., 1997; Van Dam et al., 2000; Dobkin et al., 2000; Kooy et al., 1996), behavioral alterations (Mineur, Sluyter, De Wit, Oostra, & Crusio, 2002; Nielsen, Derber, McClellan, & Crnic, 2002; de Vrij et al., 2008), and susceptibility to epileptic seizures (Musumeci et al., 2000, 2007), which are all common in affected FXS patients. Analysis of brain sections of FXS mice revealed unusually long, thin, and tortuous dendritic spines of increased density, consistent with histological findings in brain autopsies of FXS patients (Nimchinsky, Oberlander, & Svoboda, 2001).

These animal models provided an invaluable experimental tool for studying the cellular roles of FMRP, and offered new insights regarding the effects of its absence on neuronal architecture, complex behavior and cognition (Busquets-Garcia, Maldonado, & Ozaita, 2014). The excessive protein synthesis in several brain regions of FMRP-deficient mice, for example, highlighted the role of FMRP as a translational repressor and suggested that the disruption of translational homeostasis, together with the lack of stimulus induced protein synthesis, plays an important role in the pathophysiology of the disease (Dölen et al., 2007; Qin, 2005; Udagawa et al., 2013). These models also demonstrated the interaction of FMRP with several key neural signaling pathways, the most highly acknowledged of which are the metabotropic glutamate receptor (mGluR)–mediated long-term depression pathway (Bear, Bear Huber, & Warren, 2004; Dölen et al., 2007) and the GABA signaling pathway (Curia, Papouin, Seguela, & Avoli, 2009; D'Hulst et al., 2006; Gantois et al., 2006). These findings suggested new therapeutic strategies to tackle the molecular defects underlying the manifestations of FXS.

However, in spite of their immense value in studying the consequences of FMRP deficiency in vivo, FXS animal models have a number of limitations. To date, there is no animal model for CGG expansion–mediated *Fmr1* silencing, which is responsible for the majority of cases in humans. The knockin mouse model carrying an expanded CGG tract failed to recapitulate the loss of *Fmr1* expression or the aberrant hypermethylation associated with the repeat expansion in humans (Brouwer et al., 2007). The inability to silence the expanded *Fmr1* locus prevented the use of animal models to study the epigenetic mechanisms responsible for the inactive state.

In addition, interspecies dissimilarities in neuronal function call for the validation of evidence derived from animal studies in human-based models. The recent failure of therapies based on mouse models to correct disease-related phenotypes in FXS patients suggested that the reliance on *Fmr1* knockout mice as a single model may limit the translation of preclinical data to human patients (Bailey et al., 2016; Berry-Kravis et al., 2016; Scharf, Jaeschke, Wettstein, & Lindemann, 2015).

HUMAN-BASED MODELS FOR FXS

Different in vitro systems have been developed to study FXS in cultured human cells. As neural tissue is particularly inaccessible, several studies utilized peripheral blood mononuclear cells (PBMCs), fibroblasts, and immortalized lymphoblastoid cell lines derived from patients with FXS. These models enabled the characterization of the genetic and epigenetic landscape of the *FMR1* locus in FXS somatic cells, but could not be utilized to study the disease phenotype in the target tissue (Brasa et al., 2016; Coffee et al., 1999, 2002; Kumari & Usdin, 2010; Pieretti et al., 1991; Pietrobono, 2004). Nevertheless, molecular defects identified in patient-derived primary cell cultures can serve as potential biomarkers for assessing the efficacy of therapeutic interventions in clinical trials. Higher rates of basal protein synthesis (Gross & Bassell, 2012; Kumari et al., 2014), together with alterations in several FMRP targets, including extracellular signal–regulated kinase (ERK) 1/2 (Weng, Weiler, Sumis, Berry-Kravis, & Greenough, 2008), phosphoinositide-3-kinase (PI3K) (Gross & Bassell, 2012), matrix metalloproteinase-9 (MMP9) (Dziembowska et al., 2013), and others (Hoeffer et al., 2012; Kumari et al., 2014; Westmark et al., 2011), were detected in patient-derived cells, and proposed as potential outcome measures for studies in humans.

Another approach to the modeling of FXS is the use of various CGG repeat constructs to compare the influence of different CGG repeat lengths on the regulation of gene expression independently of its natural genomic context. However, transgenes containing expanded CGG tract were unable to accurately reproduce the epigenetic inactivation of the repeats, thus curtailing the capability of these models to elucidate the pathophysiology of the disease (Sandberg & Schalling, 1997; Sølvsten & Nielsen, 2011).

The cognitive and behavioral manifestations of FXS have motivated the search for a cellular platform that can capture the neuronal defects associated with the disease. Several studies have relied on primary neural cell lines derived directly from postmortem fetal (Bhattacharyya et al., 2008; Castrén et al., 2005) or adult (Schwartz et al., 2005) tissue. Generation of human neural stem cell lines from an 18-week-old FXS fetus revealed morphological and functional defects associated with neural differentiation (Castrén et al., 2005). FMRP-deficient neurospheres generated larger numbers of Tuj-1 positive cells that had shorter radial processes and a smaller cell body volume. The mutant neural progenitors gave rise to reduced numbers of GFAP-positive cells, an observation that was attributed to increased apoptotic cell death. Electrophysiological examinations also revealed differences between the affected cells and their normal counterparts associated with alterations in Ca^{2+} signaling. However, examination of a 14-week-old FXS fetus by another group (Bhattacharyya et al., 2008) revealed unaltered neurogenesis and no morphological defects in the newly born FXS neurons. Instead, the authors reported changes in the expression of genes associated with several signal transduction pathways (Bhattacharyya et al., 2008; McMillan, Kamps, Lake, Svendsen, & Bhattacharyya, 2012). These inconsistencies in findings can be attributed to differences in the specific developmental stage of the tissue samples, or to the different derivation and culturing methods applied.

Therefore, although postmortem neural cultures provide a glimpse into the neural phenotype of FXS patients, the restricted access to primary neural tissue and the variation of the phenotype as a function of the culture method and the specific developmental stage make

it difficult to generalize the results or to draw conclusions about the fundamental cellular alterations associated with the disorder. Furthermore, the use of embryonic samples precludes the analysis of the association between the neural phenotype and its corresponding cognitive manifestations, which vary greatly between affected individuals (Gallagher & Hallahan, 2012). Finally, primary cell cultures can only be used to study the disease phenotype in a narrow developmental window and thus ignore the dynamic characteristics of FXS throughout the course of neurodevelopment.

MODELING FXS IN HUMAN PLURIPOTENT STEM CELLS

The introduction of human pluripotent stem cells (PSCs) opened a new avenue for the study of FXS. Human PSCs have the ability to self-renew and to differentiate into the various cell types comprising the human body (De Los Angeles et al., 2015), thus providing an inexhaustible source of neuronal cells carrying the genetic background associated with the disease (Avior, Sagi, & Benvenisty, 2016). The ability of PSCs to model the early stages of embryonic development is particularly important for the study of a neurodevelopmental disorder, such as FXS.

Different methodologies for the generation of pluripotent cell lines have been utilized for modeling FXS (Fig. 6.1). Preimplantation genetic diagnosis (PGD) makes it possible to identify human embryos with specific genetic abnormalities, and provides a source of embryonic stem cells (ESCs) that carry specific mutations or chromosomal aberrations in their natural genetic background. FXS was one of the first disorders modeled using PGD-derived embryos (Eiges et al., 2007; Turetsky et al., 2008; Verlinsky et al., 2005).

The discovery that mature cells can be reprogrammed back to the pluripotent state by the delivery of exogenous transcription factors provided another strategy for the derivation of disease-specific PSCs (Avior et al., 2016; Takahashi & Yamanaka, 2006). The utilization of human induced pluripotent stem cells (iPSCs) avoids the ethical issues raised by the use of human embryos, and allows the generation of PSC lines from affected individuals with a known clinical phenotype. The generation of FXS patient-derived iPSCs revealed a fundamental distinction between the FXS-ESC and the FXS-iPSC models, and constituted an invaluable tool for the study of this disorder (Urbach, Bar-Nur, Daley, & Benvenisty, 2010).

Recently, another key step in the modeling of FXS has led to the successful genetic correction of full mutation FXS-PSC lines using CRISPR/Cas9 technology. Edited FXS-ESCs and iPSCs were generated by the targeted deletion of the CGG repeat tract in the *FMR1* gene. The deletion of the expanded sequence reactivated the silenced *FMR1* promoter in FXS-iPSCs, and restored the expression of FMRP to normal levels (Park et al., 2015). Genetically edited patient-derived cells form the ideal isogenic control for investigating the disease phenotype, and can serve in the development of targeted therapy.

Various groups have utilized ESC and iPSC models to address the unsolved questions regarding the pathophysiology of FXS. PSC models have been used in different research fields dealing with this disorder, such as the analysis of the epigenetic silencing of *FMR1*, the

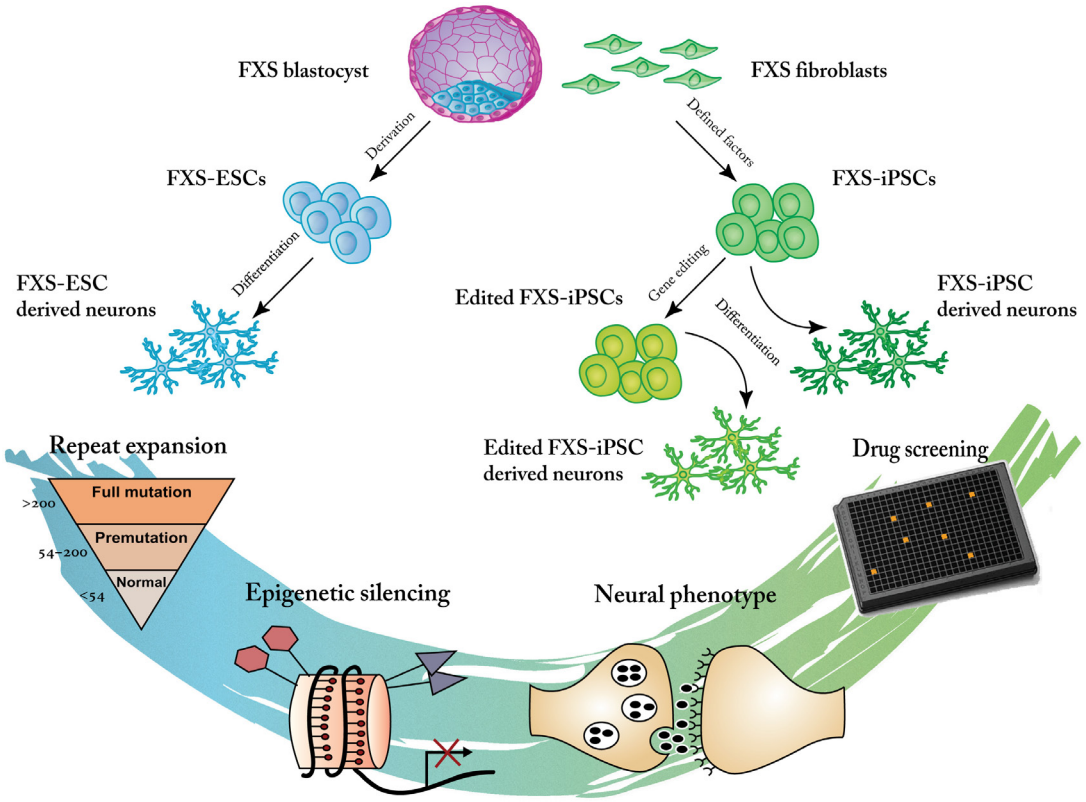

FIGURE 6.1 Current utilization of human fragile X syndrome pluripotent stem cells (FXS-PSCs) models in the study of FXS. Different methodologies have been employed to derive FXS-PSCs. FXS embryonic stem cells (FXS-ESCs) are produced from affected blastocysts harboring the full *FMR1* mutation, identified by preimplantation genetic diagnosis (PGD). FXS induced pluripotent stem cells (FXS-iPSCs) are obtained from patient-derived somatic cells, which are reprogrammed into PSCs by a defined set of factors. FXS-PSCs can be differentiated into human FXS neurons, thus enabling the analysis of FXS-related manifestations in disease-relevant cell types. The CRISPR/Cas9 gene-editing technique has been utilized to eliminate the repeat region, and produce isogenic edited FXS-iPSCs, in which *FMR1* expression is restored both in the undifferentiated state and upon neural differentiation. The differences between the available models are suggestive of their differential use in different fields of research. As FXS-iPSCs harbor a silenced *FMR1* allele, they are a preferable platform for characterizing the effects of FMRP loss in neural cells, and for screening compounds that could rescue disease-associated defects. As FXS-ESCs show significant levels of FMRP expression, which diminishes upon prolonged neural differentiation, they are a suitable model for studying the temporal nature of CGG-mediated epigenetic silencing. Repeat instability in FXS-ESCs makes them a suitable model for analyzing the mechanisms of CGG repeat expansion.

pathological instability of expanded CGG tracts, and the molecular mechanisms underlying disease-related phenotypes (Fig. 6.1). Understanding the fundamental differences between the available models is crucial for studying many facets of the disease.

In this chapter, we describe the utilization of these different cellular models of FXS in disease research, and summarize the new discoveries made by the use of FXS-PSCs.

HUMAN ESCs AS A DEVELOPMENTAL MODEL FOR FXS

In 2007, Eiges et al. (2007) reported the establishment of a human ESC line harboring a full CGG expansion, and used it to characterize the early events in the transcriptional inactivation of *FMR1*. Surprisingly, although a full mutation was present, FXS-ESCs showed significant expression levels of *FMR1* mRNA that correlated with typical features of euchromatin in the gene promoter. Spontaneous differentiation of the FXS-ESC line by the generation of teratomas in immunodeficient mice was accompanied by a decline in the expression of FMRP and by the acquirement of epigenetic marks associated with inactive chromatin. This silencing in *FMR1* transcription occurred despite low levels of methylation of the *FMR1* gene.

The detection of *FMR1* expression in the undifferentiated state and its decrease upon differentiation suggested the existence of a multistep developmentally regulated process for *FMR1* inactivation. The downregulation of *FMR1* mRNA prior to DNA hypermethylation of the promoter suggested that hypermethylation might be a late event in the silencing process, which occurs following the transcriptional silencing of the gene and the acquirement of repressive histone modifications. These findings were in line with a previous work based on the transfer of human expanded *FMR1* locus into mouse embryonic carcinoma (mEC) cells using microcell fusion (Wöhrle, Salat, Hameister, Vogel, & Steinbach, 2001). The introduction of hypermethylated full mutation alleles to the murine embryonic environment caused demethylation and destabilization of the repeats, suggesting that the expanded *FMR1* locus might be actively transcribed in the early stages of embryogenesis. The examination of chorionic villi samples from the affected embryos also supported this notion, and revealed active FMRP expression in extraembryonic tissue up to 10.5–12.5 weeks of gestation (Willemsen, Bontekoe, Severijnen, & Oostra, 2002).

More recently, two additional FXS-ESC lines were used to further characterize the temporal sequence of events in *FMR1* inactivation, which suggested a novel role for *FMR1* transcription in the silencing process (Colak et al., 2014). In this study *FMR1* expression was monitored over 60 days of in vitro neural differentiation. *FMR1* mRNA was gradually downregulated until it was completely absent, 51 days after the initiation of the differentiation process. By this time, the *FMR1* promoter switched to a repressive state characterized by increased levels of the repressive mark H3K9me2, and decreased levels of the active mark H3K4me2. The authors suggested that the formation of RNA:DNA duplex between the CGG repeat portion of the *FMR1* transcript and its DNA template promoted the differentiation-induced epigenetic silencing. Knocking down *FMR1* mRNA by a specific shRNA prevented the loss of active histone marks and the acquirement of repressive histone marks in the *FMR1* promoter region.

Interestingly, a small molecule that prevented the linearization of the hairpin structure of the CGG repeat mRNA was able to block the epigenetic silencing of *FMR1*. However, this small molecule was unable to reactivate the *FMR1* promoter after the inactivation occurred, thus suggesting independent mechanisms for the induction and the maintenance of the repressive epigenetic state. These findings provided additional evidence for the developmentally regulated fashion of *FMR1* inactivation.

Recently, the characterization of eight additional full mutation FXS-ESCs was reported, thus revealing another facet of epigenetic silencing in FXS (Avitzour et al., 2014). Apparently, partial levels of DNA methylation may already exist in the undifferentiated state (24%–65% in six out of nine ESC lines). The methylation levels of the FXS-ESC clones in this study

remained stable over time and were correlated with the *FMR1* transcription level and the distribution of repressive chromatin marks. These results suggest that the hypermethylation of a full mutation is irreversible and might also occur before or during blastocyst formation. CGG repeats that are not hypermethylated during this time frame, undergo developmentally regulated inactivation triggered by differentiation. To overcome the inherent heterogeneity in the cell culture, the authors applied bisulfite single colony sequencing, which revealed the complete dichotomy of hypermethylated and hypomethylated full mutation alleles. The findings indicated that the partially methylated FXS-ESC lines are a mixture of *FMR1* hyper- and hypomethylated cells. In addition, the authors reported the isolation of female FXS-ESC lines, which can be used to study X-inactivation patterns in affected female patients (Avitzour et al., 2014).

iPSCs IN MODELING FRAGILE X SYNDROME

The generation of iPSC lines from fibroblasts of affected FXS patients revealed a fundamental distinction between ESC and iPSC models of FXS (Urbach et al., 2010). While FXS-ESC lines show significant expression levels of FMRP, the *FMR1* locus in FXS-iPSCs is completely silent, and exhibits the repressive epigenetic modifications associated with the somatic state (Fig. 6.2). Characterization of additional iPSC lines (Doers et al., 2014; Sheridan et al., 2011) supported the notion that the inactive *FMR1* locus is resistant to the reprogramming process, and showed that once established, *FMR1* hypermethylation is irreversible and clonally maintained.

The differences between FXS-ESCs and FXS-iPSCs must be taken into account when choosing the most suitable model for each specific research question. As FXS-ESCs recapitulate the early embryonic stages of the FXS pathology, they are an appropriate tool for studying the developmental aspects of the disease, and primarily the temporal sequence of events leading up to the inactivation of *FMR1*. FXS-ESCs are a preferable model to study additional early processes in the natural history of FXS, as the instability of the expanded CGG repeat tract during early embryonic development and gametogenesis. However, as complete *FMR1* silencing in FXS-ESCs can be achieved only after about 50 days of neural differentiation, it is very challenging to utilize them to study the consequences of the absence of *FMR1* in neural cells. Therefore, the complete inactivation of the gene in FXS-iPSCs makes them a better model for uncovering the neuronal defects associated with the disease, and the optimal cellular platform for targeted drug discovery. As the intellectual impairment in FXS patients is highly variable (Gallagher & Hallahan, 2012), the generation of iPSCs from clinically defined individuals can link their clinical profile to the cellular and molecular alterations found in vitro, a connection that cannot be made using embryos after PGD analysis.

Furthermore, the presence of a completely inactive *FMR1* locus in FXS-iPSCs can teach us about the role of different epigenetic modifications in the maintenance of the silenced state. The treatment of FXS-iPSCs with the DNA methyltransferase inhibitor, 5-azacytidine, was shown to reactivate *FMR1* gene expression in FXS undifferentiated iPSCs and iPSC-derived neurons by demethylating the *FMR1* promoter and partially inducing histone modifications associated with active transcription (Bar-Nur, Caspi, & Benvenisty, 2012). This study was supported by earlier reports in FXS-lymphoblastoid cell lines (Chiurazzi, Pomponi, Willemsen,

	Normal somatic cells	FXS somatic cells	FXS-ESCs	FXS-iPSCs	Edited FXS-iPSCs	DNMTi-treated FXS-iPSCs
FMR1 expression	Expressed	Not expressed	Mostly expressed	Not expressed	Expressed	Partially expressed
DNA methylation	Unmethylated	Hypermethylated	Mostly unmethylated	Hypermethylated	Unmethylated	Partially unmethylated
Chromatin status	Open	Closed	Mostly open	Closed	Open	Partially open
H3 acetylation	High	Low	Mostly high	Low	High	High
H3K4 methylation	High	Low	Mostly high	Low	High	High
H3K9 methylation	Low	High	Mostly low	High	Low	High
CGG repeat length	Stable	Stable	Unstable	Not defined	No repeats	Not defined

FIGURE 6.2 **Characteristics of the *FMR1* gene in different PSC models of FXS.** *DNMTi*, DNA methyltransferase inhibitors.

Oostra, & Neri, 1998; Coffee et al., 1999, 2002; Pietrobono et al., 2002) and highlighted the role of DNA methylation as a maintenance mechanism that locks the *FMR1* promoter in the inactive state. Additionally, the ability to restore FMRP expression in FXS patient cells provided a new therapeutic strategy targeting the transcriptional silencing of *FMR1*.

Although it has been suggested that a silenced allele cannot be demethylated during the standard reprogramming process, there are still uncertainties regarding the effect of reprogramming on the epigenetic status of unmethylated full mutation alleles. On one hand, it was reported that the reprogramming of fibroblasts with full mutation and intermediate methylation levels (77%) results in some iPSC clones carrying unmethylated and active *FMR1* genes (Avitzour et al., 2014). On the other hand, it has also been shown that reprogramming of fibroblasts from a normal male carrying an unmethylated full *FMR1* mutation resulted in hypermethylated FXS-iPSC clones (de Esch et al., 2014). The ability of unmethylated full mutation alleles to undergo hypermethylation upon reprogramming might suggest that in normal individuals with a FXS mutation, the escape from *FMR1* silencing is caused by an epigenetic alteration rather than a unique genetic background.

Finally, recent advances in genome editing technologies have enabled the generation of genetically rescued human mutated cell lines (Park et al., 2015). It was reported that a targeted deletion of the CGG repeat tract in FXS-iPSCs was able to generate edited FXS-iPSC lines that

express normal levels of FMRP. The system was based on Cas9, an RNA-guided endonuclease that can be targeted to specific genomic locations by a short guide RNA. The authors used single-guide RNA that targeted Cas9 to an upstream sequence to the CGG repeats, so as to induce site-specific DNA double-strand breaks. The generations of double-strand breaks can cause insertion or deletion mutations via the activation of the error prone nonhomologous end joining repair pathway (NHEJ). The authors were able to isolate FXS-iPSC clones with deletions of the entire CGG repeat sequence. Interestingly, deletion of the CGG repeat tract caused the reactivation of *FMR1* expression to levels similar to normal cells. The edited iPSCs showed extensive DNA demethylation in the *FMR1* promoter, and acquired a transcriptionally active chromatin state characterized by H3 acetylation, H3K4 methylation, and H3K9 demethylation (Fig. 6.2). The extensive demethylation of the *FMR1* promoter following the removal of the repeats pointed to the existence of constant regulation of the promoter's epigenetic status based on the repeat length.

NEURAL DIFFERENTIATION OF FXS-PSCs

The transcriptional silencing of *FMR1* leads to impairments in neuronal function. Elucidating the primary and secondary changes in neuronal circuitry that stem from the absence of FMRP is critical for understanding the pathology of FXS and devising new strategies for treatment. Although animal models are the major tools for the examination of FXS manifestations in vivo, the physiological differences between affected animal and human cells may hinder the application of preclinical experiments on human patients. Interspecies differences can partially explain why candidate drugs developed in animal studies fail to achieve the same effect in human clinical trials (Berry-Kravis et al., 2016; Scharf et al., 2015).

Therefore, the ability of PSCs to differentiate into various cell types provides a tremendously valuable tool for assessing the outcomes of *FMR1* inactivation in disease-relevant human cells. The characterization of disease-associated phenotypes in affected cells can unveil the molecular defects underlying FXS manifestations, and can also serve as a discovery tool for novel candidate drugs. PSCs represent an early state in embryonic development, and their neural derivatives retain an immature cellular identity that resembles fetal rather than adult cells. However, as FXS is a developmental disorder with an early onset, the utilization of neurons differentiated from FXS-PSCs may still serve as a useful tool for the dissection of the molecular mechanisms affected in the disorder.

Analysis of neural phenotypes can be carried out in either FXS-ESCs or FXS-iPSCs. *FMR1* silencing in FXS-ESCs occurs late during their differentiation, which presents a challenge when attempting to characterize FXS-related phenotypes in these cells. Interestingly, analysis of directed neural differentiation of FXS-ESCs suggested that the mutated cell lines differed from normal cells even in the early stages of neural induction, before complete inactivation of *FMR1* occurred (Telias, Segal, & Ben-Yosef, 2013). Unlike normal ESCs, FXS-ESCs failed to demonstrate an increase in *FMR1* transcription upon neural differentiation, and displayed reduced and delayed development of neural rosettes and aberrant expression patterns of key neural genes, such as *SOX1*, *NOTCH1*, and *PAX6*. Further studies connected FMRP to the regulation of additional members in the SOX superfamily, and revealed abnormal overexpression of *SOX2* and downregulation of *SOX9* in FXS-NPCs that might lead to defective neural

development (Telias, Mayshar, Amit, & Ben-Yosef, 2015). Whereas in mice a *FMR1* deficiency led to elevated levels of glycogen synthase kinase 3β (GSK3β) in brain samples (Portis et al., 2012), neural progenitor cells derived from FXS-ESCs showed reduced levels of *GSK3β*, thus highlighting the need to validate molecular phenotypes that differ between mouse brains and human neural cells.

Morphologically, FXS-derived neurons that completely lacked FMRP by day 60 of in vitro neural differentiation had significantly smaller somata and shorter neurites than their wild-type counterparts. Electrophysiological examination of FXS-ESC–derived neurons revealed poor synaptogenesis and a reduced response to glutamate (Telias, Kuznitsov-Yanovsky, Segal, & Ben-Yosef, 2015). Current clamp techniques uncovered the incapacity of FXS neurons to fire trains of action potentials, and detected a significant decrease in spike duration and amplitude. These findings were linked to an intrinsic reduction in the ability to activate Na^+ and K^+ currents, thus preserving the FXS neurons in an electrically immature state. FXS neurons had fewer releasable synaptic vesicles and reduced spontaneous and active synaptic activity. Interestingly, the coculture of FXS neurons with normal rat neurons significantly increased the synaptic activity of the affected cells, which might hint at additional mechanistic links between FMRP expression mosaicism and a milder cognitive phenotype (Cohen et al., 1996).

The complete silencing of *FMR1* in FXS-iPSCs makes them a powerful tool for identifying the role of FMRP in neural signaling pathways. Several studies (Doers et al., 2014; Sheridan et al., 2011) have examined the morphological defects associated with the disease, thus supporting the notion that FXS involves defects in initial neurite outgrowth, as the FXS-iPSC–derived neurons also had fewer and shorter processes than their normal counterparts.

Another approach in characterizing FXS-associated phenotypes has focused on the differences between FXS and normal neurons at the molecular level (Halevy, Czech, & Benvenisty, 2015). Global transcriptomic analysis of FXS-iPSC–derived neurons revealed aberrant expression patterns of axon guidance and neural differentiation genes, which were linked to a known master regulator of neurogenesis, the transcriptional repressor *REST*. The levels of *REST*were elevated in FXS neurons, suggesting that the FMRP deficiency led to its disinhibition. It was demonstrated that the repression of *REST* by FMRP is mediated by the miRNA pathway, thus showing that the brain-enriched miRNA hsa-mir-382, which targets the mRNA of *REST*, is downregulated in the affected cells. The introduction of mimic-mir-382 inhibited the excessive expression of *REST* in the affected cells and restored the levels of its axon guidance target genes (Halevy et al., 2015). Thus, a novel role for FMRP in the maturation of miRNA, mediating the inhibition of *REST*and the subsequent activation of axon guidance pathways was presented.

A fundamental challenge to the utilization of PSCs to study human diseases is the inherent variation between different cell lines that can mask disease-related phenotypes and interfere with the comparison between normal and affected cells. One of the most powerful tools to overcome the variation arising from the different genetic backgrounds of PSC lines is the phenotypic rescue of affected cell lines by correction of the disease-causing mutation. Genetic correction of FXS-affected cells (Park et al., 2015), together with the utilization of methylation or repeat mosaicism found in some FXS patients, can enable the derivation of isogenic cell lines that differ solely in the expression of *FMR1*. Such cell lines can be utilized to validate the connection of FMRP loss to specific pathological features, ensuring the generalizability of the results.

Another challenging aspect of studying PSC-derived FXS neurons is the detection and interpretation of cell type–specific in vitro phenotypes, which are often subtle and have uncertain functional significance. Moreover, neural populations derived by different differentiation protocols might differ in their neural cell identity and in their pathological features. The development of validated and robust differentiation protocols, together with new tools to assess neuronal integrity, will contribute to the analysis of FXS-associated phenotypes, and will allow the characterization of the role of FMRP in distinct neuronal subpopulations or in other cell types affected by the disorder. As FMRP is primarily involved in translational control, proteomic approaches to identify protein level alterations in human FXS neurons could be beneficial in identifying the phenotypes of FXS at the cellular level. In vivo grafting of FXS-NPCs into rodent brains would facilitate the analysis of mature circuit integrated FXS-neurons, and might enable in vivo candidate drug testing. Thus, PSC-derived neurons are highly promising candidates for uncovering novel pathological mechanisms associated with FXS.

PSC MODELING OF CGG REPEAT INSTABILITY

The expansion of repetitive DNA sequences is the cause of more than 30 human inherited diseases (López Castel, Cleary, & Pearson, 2010). In affected pedigrees, a single-repeat tract becomes expanded and unstable, and can not only change during transgenerational transmission, but also within the same individual. Transgenerational expansion is associated with anticipation, which implies that the disease phenotype becomes more severe and appears at earlier ages across generations (López Castel et al., 2010).

In FXS-related disorders, the expansion of the CGG repeats to different lengths is correlated with different phenotypes. While expansion to more than 200 repeats is associated with the clinical presentation of FXS, carriers of premutation alleles (55–200 repeats) are at risk of developing fragile X–associated primary ovarian insufficiency (FXPOI) and fragile X–associated tremor/ataxia syndrome (FXTAS) (Gallagher & Hallahan, 2012). Meiotic instability of CGG repeats is responsible for repeat length differences between parents and children, with expansion to the full CGG mutation taking place during maternal transmission from a premutation carrier. Mitotic repeat instability is reflected in repeat length heterogeneity within affected individuals, which might influence the clinical phenotype and the response to treatment (Pretto et al., 2014). Interestingly, several studies have suggested that CGG repeats instability is predominant in early stages of embryonic development (Devys et al., 1992; Wöhrle, Hennig, Vogel, & Steinbach, 1993).

Understanding the mutational process causing repeat expansion is important for deciphering the pathogenesis of FXS-associated disorders and might give rise to strategies that identify alleles at risk or modulate the repeat expansion size. Human PSCs can serve as a suitable model for repeat instability, as they recapitulate the specific stages in which this phenomenon occurs.

Different factors influence CGG repeat instability. The propensity of tandem repeat tracts to form unusual DNA structures is thought to be a key intermediate of repeat instability that interferes with a wide range of DNA metabolic processes, such as DNA replication, repair, and recombination (López Castel et al., 2010; Usdin, House, & Freudenreich, 2015). AGG

interruptions, which might destabilize the secondary structure formed by the uninterrupted CGG sequence, were claimed to result in a more stable CGG repeat size because maternal alleles with no AGGs are more prone to undergoing expansion to full mutation (Jarem, Huckaby, & Delaney, 2010; Latham, Coppinger, Hadd, & Nolin, 2014; Nolin et al., 2015; Yrigollen et al., 2014). Other studies suggested a role for *cis*-elements proximal to the repeats in the determination of expansion frequency, in that some haplotypes are more likely to undergo repeat expansion than others, even with the same repeat length (Nolin et al., 2011). Additionally, the association between hypermethylation of *FMR1* and the mitotic stability of the repeats hint at the role of epigenetic silencing of *FMR1* in stabilizing the length of the repetitive sequence. The introduction of a mitotically stable *FMR1* locus into undifferentiated murine embryonal carcinoma cells, but not into somatic murine cells, was associated with both demethylation and destabilization of the repeats, thus further enhancing the connection between DNA methylation and the stabilization of expanded CGG repeats (Wöhrle et al., 2001).

Recently, alterations in the replication program of the expanded CGG tract were suggested to be a potential mechanism for repeat instability during the early embryonic stages (Gerhardt et al., 2014a). In this study, a single-molecule analysis of replicated DNA (SMARD) was used to monitor the replication program in FXS-ESCs. Whereas in nonaffected cells the repeats were replicated approximately equally in either the 3′ to 5′ or 5′ to 3′ direction, in FXS-ESCs the replication fork predominantly progressed from 3′ to 5′, with the CCG strand serving as the template for the lagging strand. These changes in the sequence serving as the lagging-strand template might favor the formation of stable DNA secondary structures in CGG repeat–containing Okazaki fragments, and therefore predispose the newly synthesized strand to expansion during mitotic division. The aberrant replication profile in the affected cells was connected to the absence of a replication initiation site approximately 50-kb upstream to the CGG repeats. Surprisingly, upon differentiation, the replication profile of the FXS lines became similar to nonaffected cells, consistent with the switch from repeat instability to repeat stability.

Based on these findings, it was proposed that in normal cells, the homeostasis of the CGG repeat length is maintained by a balance between contractions and expansions. When the secondary structure–prone CGG strand is in the nascent lagging strand, it occasionally undergoes expansions. When the same strand serves as the nascent leading strand, it is prone to undergoing contraction. Thus, developmentally regulated inactivation of the replication origin upstream to *FMR1* in FXS-ESCs might lead to a predisposition for repeat expansion.

In a further study, the authors connected a single nucleotide polymorphism (SNP) variant that cosegregates with the haplogroup at the highest risk for repeat expansion to the missing replication origin upstream to *FMR1* (Ennis, Murray, Brightwell, Morton, & Jacobs, 2007; Gerhardt et al., 2014b). In FXS-ESC lines that lacked the upstream replication origin, the SNP variant C replaced the T that was found in normal and premutation cell lines. The authors suggested that the substitution of T for C leads to repeat instability due to the inactivation of the replication origin upstream to the repeats.

However, there are still many open questions regarding the mechanism of repeat expansion. As premutation human ESC lines did not contain the specific nucleotide substitution at the replication initiation site and showed a normal replication profile of repeats, it is unclear how the mechanism of expansion from a normal range of CGG repeats to the premutation range occurs. The involvement of additional factors could contribute to the repeat expansion in FXS patients who do not carry the specific nucleotide substitution.

THE USE OF FXS-PSCs FOR TARGETED DRUG DISCOVERY

To date, the treatment of FXS patients aims to alleviate individual symptoms, without targeting the underlying molecular defects. Enormous efforts have been invested in translating new knowledge about the neuropathology of FXS into therapeutic interventions. The behavioral and neurobiological similarities between FXS and other autism spectrum disorders have raised the hope that the development of therapeutic interventions for FXS might also provide a gateway for the treatment of other neurodevelopmental disorders.

The main strategy for drug development for FXS is based on the targeting of the dysregulated signaling pathways downstream to FMRP (Darnell & Klann, 2013; Hagerman, Des-Portes, Gasparini, Jacquemont, & Gomez-Mancilla, 2014; Richter, Bassell, & Klann, 2015). Analysis of the molecular defects in the FXS mouse model guided the design of genetic and pharmacological rescue experiments in which the targeting of different FMRP-regulated proteins was able to achieve reversal of the associated neural phenotypes (Gross, Hoffmann, Bassell, & Berry-Kravis, 2015). Many targets of FMRP, together with various proteins involved in translational homeostasis, have been suggested as therapeutic targets for FXS, including mGluR1/5, GSK3β, MMP-9, ERK 1/2, PI3K, $GABA_A$, and others, as reviewed by Gross et al. (2015). However, the recent failure of the selective mGluR5 inhibitors, Basimglurant and Mavoglurant, to show clinical improvement in FXS patients raises a question about the ability of null mutation mice to predict the therapeutic potential of novel candidate drugs, and highlights the need for identification of human-specific outcomes of the FMRP deficiency (Bailey et al., 2016; Berry-Kravis et al., 2016; Scharf et al., 2015).

Neural populations derived from affected human PSCs offer a novel platform for the in vitro testing of therapeutic interventions. Their use in large-scale drug screening has immense promise for identifying compounds that could tackle the neuronal functional deficiencies associated with the disease. This would require the development of quantifiable, reproducible, and efficient in vitro assays for the evaluation of phenotypic defects in FXS neurons. Such assays could be based on alterations in cellular morphology, functional or metabolic processes, cellular death, and the response to specific cellular stressors. The utility of such a system ranges from the identification of disease biomarkers, a better understanding of the disease pathogenesis, to the ability to develop automated screens to detect compounds able to rescue the associated phenotypes.

The discovery that a full mutation allele can be expressed under specific circumstances led to another therapeutic strategy for FXS, targeting the pathological core of the disease; namely, the transcriptional silencing of *FMR1* (Tabolacci & Chiurazzi, 2013). As the expanded region lies within the 5′UTR of the gene, the open reading frame of *FMR1* is not disrupted, and enables the production of functional FMRP in the undifferentiated state or in rare high-functioning individuals carrying an unmethylated full mutation (Avitzour et al., 2014; Eiges et al., 2007; de Esch et al., 2014; Smeets et al., 1995). Restoration of *FMR1* expression in adult neural stem cells and their progeny has been able to correct hippocampus-dependent learning impairments in FMRP-deficient mice, suggesting that *FMR1* reactivation might ameliorate cognitive deficits even throughout the adult life (Guo et al., 2011).

As mentioned earlier, the loss of *FMR1* expression is accompanied by the acquirement of DNA hypermethylation and by repressive histone modifications indicative of heterochromatin. The ability of the FDA-approved DNA demethylating agent, 5-azacytidine, to reactivate

FMRP expression in FXS-iPSCs–derived neurons opened up possibilities of pharmacologically restoring FMRP expression (Bar-Nur et al., 2012). 5-Azacytidine administration in pharmacological concentrations was shown to robustly reactivate *FMR1* expression, triggering DNA demethylation of the *FMR1* promoter together with the elevation of H3 acetylation and H3K4 methylation, which are active markers indicative of euchromatin (Bar-Nur et al., 2012). Although the effect of 5-azacytidine increased in a concentration-dependent manner, the expression levels were nevertheless lower than observed in normal cells, and the amounts of repressive H3K9 methylation were not affected by the treatment. The clinical application of the tested compounds is restricted due to significant side effects and chemical instability, and the consequences of long-term treatment are unknown (Gnyszka, Jastrzebski, & Flis, 2013). Identification of new compounds that can reactivate the silenced gene in a less toxic or a more specific manner may suggest transcriptional reactivation as a feasible strategy for the treatment of FXS. As full mutation iPSCs contain a completely inactivated *FMR1* locus, they are the most suitable cellular platform for FMRP expression–based screening. To date, two large-scale compound screens have been conducted to detect molecules that can reactivate the silenced *FMR1* locus. The first study developed a cellular platform based on FXS-iPSC–derived neural progenitors, and used it to test 50,000 compounds, including agents with a known mode of action, randomly selected compounds, and molecules covering a broad chemical and biological space (Kaufmann et al., 2015). FMRP expression was monitored using a high-content imaging assay based on immunostaining. Although the screening was able to identify a small set of compounds that induced FMRP expression, the level of expression was lower than the level achieved using 5-aza-2′-deoxycytidine, and the toxicity was higher.

Another screen utilized a TR-FRET–based assay to quantify the levels of FMRP expression (Kumari et al., 2015). The assay was based on the use of two anti-FMRP antibodies, one of which was labeled by a donor fluorophore (europium cryptate) and the other with an acceptor (d2). Upon the binding of the two antibodies to FMRP, the close proximity between them enabled energy transmission from the donor to the acceptor, enhancing its fluorescence and eliminating background signals. Although the authors identified several compounds that reactivated *FMR1* expression to some extent, the effect was still modest and no detectable FMRP levels appeared in western blot analysis.

Even though these studies had limited success in finding new efficient agents that counteract the epigenetic silencing of the expanded *FMR1* locus, they illustrate the feasibility of FXS-iPSC–derived neural cells to serve as a platform for high-throughput screening to detect new candidate drugs that will be able to efficiently restore *FMR1* expression.

CONCLUSIONS

Although a large amount of information has been obtained on the pathophysiology of FXS, many key questions for the development of targeted treatment remain unanswered. The challenges associated with the study of FXS include complex and divergent neuropsychiatric phenotypes, the dysregulation of a wide range of molecular pathways, genetic and epigenetic variations, gender differences, and the lack of a suitable animal model. These challenges preclude reliance on a single model in the investigation of the disease, and require the use of an array of complementary disease models focusing on the various alterations associated with FXS.

Human PSCs offer a tremendously useful model system for FXS by integrating the different facets of the disease and providing a source for expandable, disease-relevant cellular populations. The development of both iPSCs and ESC models for FXS present complementary tools that can be differentially used according to the nature of the specific research question. Over the last few years FXS-PSCs have been used to investigate different aspects of the disease, such as the neural defects caused by the absence of FMRP, the epigenetic silencing of full mutation alleles, the expansion mechanism of the CGG repeats, and others. These studies have confirmed the value of using FXS-PSCs in the study of FXS, and highlight their great potential in different research fields.

The effectiveness of PSC modeling of FXS can be enhanced by the use of isogenic genetically corrected cell lines, and by the implementation of efficient and robust differentiation protocols. The derivation of FXS-iPSCs from clinically characterized individuals could target the uncertainties regarding the genotype–phenotype correlation in FXS, and shed light on the reasons for the clinical variability between affected individuals. Moreover, the characterization of scalable, reproducible cellular phenotypes in FXS neurons can be utilized for drug screening and the development of new disease biomarkers.

Thus, the use of pluripotent cellular models has great potential to further elucidate the molecular abnormalities associated with FXS, and to aid in the development of novel therapeutic interventions to alleviate the difficulties experienced by affected individuals.

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CHAPTER

7

Animal Models of Fragile X Syndrome

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INTRODUCTION

The identification of the *FMR1* gene, involved in fragile X syndrome (FXS), was a first step on the road to the understanding of the cellular and molecular basis of FXS. In silico analyses have revealed important functional domains of the protein encoded for by *FMR1*, named fragile X mental retardation protein (FMRP) (Bagni & Oostra, 2013). To gain more insight into the pathological, physiological and molecular processes of FXS the toolbox of the biomedical researcher is well-equipped. Reverse genetics strategies greatly facilitated the study of gene function and human genetic disease, including advanced methods of in silico biology, studies of tissues from patients with FXS, cellular models (Chapter 6) and animal models as described in this chapter. Tissues available from patients with FXS include white blood cells, cultured skin fibroblasts, induced pluripotent stem cells (IPSC), and postmortem brain tissue. Immortalized human cells have the ability to proliferate indefinitely and thus can be a source of a permanent growing cell line to perform in vitro studies

All these resources and wide range of methodologies have been invaluable for investigating *FMR1* gene function and modeling FXS. However, no tissue allows conclusive in vivo studies on the molecular pathogenesis of FXS and the gap between *FMR1* genome sequence and mechanistic understanding in gene function has been illuminated and sometimes even bridged by studying different model organisms. Unfortunately no natural occurring animal model of FXS have been described thus genetically modified animal models were generated during the last 25 years. The wide variety of model organisms of FXS allows *FMR1* gene function studies within the context of a whole organism, including vertebrate and nonvertebrate

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models. Nonmammalian (vertebrate) model organisms will be typically used in early biomedical research and should deliver fast answers to the function of a gene. *FMR1* gene function has been studied in the fruit fly *Drosophila melanogaster* and the zebra fish *Danio rerio*. In particular, *FMR1* gene function in early development can be easily assessed in these nonmammalian models. In addition, their central nervous system is relatively simple in comparison with that of mammalian species and often known in detail. However useful, vertebrate models are taxonomic more equivalent to humans and as such have been widely used in modelling human disease. Since the discovery of the expanded CGG > 200 tract in the *FMR1* gene in 1991, particularly the laboratory mouse has been used in modelling FXS (Bakker et al., 1994; Verkerk et al., 1991). Interestingly, very recently, a fragile X knockout rat has been generated using Zinc Finger Nuclease (ZFN) technology.

This chapter will provide an overview of the different animal models generated to understand *FMR1* gene function and to mimic FXS. The knowledge gathered by these animal models has paved the way for preclinical research focused on targeted therapeutic interventions for FXS.

RODENT MODELS OF FRAGILE X SYNDROME

Although rats and mice separated in evolution between 12 and 25 million years ago (Gibbs et al., 2004) (for comparison humans and chimpanzees separated approximately 6 million years ago), these two mammalian species are often used interchangeably in neuroscience research despite differences in their physiology and original ecological niches. As the first mammalian species to be domesticated for scientific purposes, laboratory rats (*Rattus norvegicus*) have been the subject of physiological studies since the early twentieth century. As such, rats were the favored mammalian model and have provided a robust experimental foundation for many areas of biomedical research. The advent of genetically modified mice (but not rats) in the 1980s led to the production of thousands of transgenic lines and has shifted the popularity of rodent models to mice (*Mus musculus*) over the past 30 years. Recent advances including the mapping of the rat genome (Gibbs et al., 2004) and development of new genetic techniques that enable genetic modification of this species (Cui et al., 2011; W. Li et al., 2014; Tesson et al., 2011) have begun to level the playing field for rats in this regard as well. Together both rodent models enable cross-mammalian comparison of neurological disease from core cellular pathophysiology to circuit and behavioral endophenotypes.

Taken together, the major benefits of rodents are that they:

- have many similarities to humans in terms of anatomy, physiology, and genetics (almost every gene in the human genome has a counterpart in rodents)
- naturally develop diseases affecting humans
- exhibit extensive genomic conservation with humans, making them particularly useful for the study of human diseases
- are amenable to genetic engineering
- enable rigorous control of genetic background and environmental factors
- are small in size, relative cheap to house, and easy to breed
- have short generation times, usually around 10 weeks
- have short lifespans (one rodent year equals about 30 human years)

Despite these common attributes and their similar appearance, rats and mice both bring unique attributes to the lab bench. Rats benefit from:

- a long history of extensively developed behavioral paradigms
- a rich repertoire of social behaviors
- closer correspondence to human metabolism/physiology,
- larger size facilitates multiple approaches (e.g., MRI, monitoring physiology, or when sample size is limiting)

In contrast, mice benefit from:

- provide a larger choice of transgenic tools
- benefit approaches requiring smaller organ size (e.g., stereotaxic targeting of individuals organs or brain regions),
- require less physical space to maintain and experiment on.

Similar to humans, mouse and rat *Fmr1* gene are encoded by 17 exons located on the X-chromosome. The *Fmr1* gene is highly conserved among these species, and the rodent homologs of *Fmr1* shows 95% sequence identity at the nucleic level, whereas Fmrp shows 97% identity in amino acid sequence compared with human FMRP (Ashley et al., 1993). Like in humans, rodent FMRP is approximately 76 KDa and is expressed widely throughout the brain. It's expression level is dynamically regulated over postnatal development with an apparent decrease with age (Bakker et al., 2000; De Diego Otero et al., 2000; Irwin et al., 2000; Khandjian et al., 1995; Till et al., 2015).

Although sporadically intragenic loss of function mutations have been identified in fragile X individuals (Chapters 1 and 2), the predominant cause of FXS in humans is the expansion and hypermethylation of a trinucleotide (CGGn) tract in the 5′ untranslated region of the *FMR1* gene that blocks transcription at the locus (Verkerk et al., 1991). Among random individuals this repeat length varies in size between 5 and 55 CGG repeats. In rodents, the length of the CGG repeat tract in rats is 4 units in size and is strain-dependent in mice, ranging between 9 and 11 units. Given a lack of naturally occurring models of FXS in model organisms and the transgenic technologies initially available, mice were the first species of choice to begin to model this genetic disorder.

MOUSE MODELS OF FRAGILE X SYNDROME

A series of mouse models has been developed for the fragile X syndrome and this chapter begins with an overview of the different models and their respective genetic backgrounds (Table 7.1).

Knockout mouse: A first model was generated by inactivating the *Fmr1* mouse gene. Briefly, the wild-type murine *Fmr1* gene was replaced with a nonfunctional *Fmr1* gene by homologous recombination of a targeting vector containing a neomycin resistance cassette in exon 5 in embryonic stem (ES) cells (Bakker et al., 1994). The E14TG2a ES cells were obtained from the 129P2/OlsHsd strain and transfection took place in Rotterdam. The resulting chimeras were backcrossed with C57BL6/J in Rotterdam and in parallel in Antwerp. Formally these two mutant colonies should be designated as B6.129P2-*Fmr1*^{tm1Cgr}/Cgr and B6.129P2-*Fmr1*^{tm1Cgr}/Ant, respectively. The Rotterdam colony was transferred to the laboratory of

TABLE 7.1 Fragile X Mouse Models

Mouse model type	Description	Stem cell	Transgene	Background	Official name	Jackson code	Available at fragile X mutant mouse facility[a]
Knockout	*Fmr1* knockout	129/OlaHsd		C57BL/6	B6.129P2-*Fmr1*^{tm1Cgr}	3025	Yes
	Fmr1 knockout	129/OlaHsd		FVB	FVB;129P2-*Fmr1*^{tm1Cgr}	2700	No
	Fmr1 knockout	129/OlaHsd		FVB	FVB.129P2-*Fmr1*^{tm1Cgr}	3024	No
	Fmr1 knockout	129/OlaHsd		Sighted FVB	FVB.129P2- Pde6b^{+}Tyr$^{c\text{-}ch}$ *Fmr1*^{tm1Cgr}	4624	No
	Sighted FVB	129/OlaHsd		Sighted FVB	FVB.129P2- Pde6b^{+}Tyr$^{c\text{-}ch}$	4828[b]	No
Conditional knockout	*Fmr1* conditional restoration	129/OlaHsd			*Fmr1* cON		Yes
	Fmr1 conditional knockout	129/OlaHsd			*Fmr1* CKO		Yes
	Fmr1 constitutionally inactivated	129/OlaHsd			*Fmr1* KO2		Yes
Point mutation		129/SvJ		C57BL/6	B6.129-*Fmr1*$^{tm1(I304N)Rbd}$	10504	
				FVB	FVB.129-*Fmr1*$^{tm1(I304N)Rbd}$	8909	
YAC rescue	Transgenic YAC rescue		YapRV.2 (modified from YAC 209G4)	C57BL/6	TG298		Yes
	CGG90YAC		YapRV-EX (modified from YAC 209G4)	FVB	TG296		Yes
Repeat expansion	$CGG_{dut}KI$	129/OlaHsd		C57BL/6	B6.129P2-*Fmr1*^{tm2Cgr}	24102	Yes
	$CGG_{nih}KI$	129S6/SvEv		C57BL/6			

[a]*The mutant mouse facility is a FRAXA sponsored repository at Baylor college (Dr. David Nelson). For details, see http://www.fraxa.org/funded-research/nelson/*
[b]*4828 is the control strain for 4624.*

Dr. Steve Warren at Emory University, who donated this version of the colony to the Jackson laboratories, where these are available as B6.129P2-*Fmr1*^{tm1Cgr}/J stock no 3025. Additionally, the chimeras were backcrossed to FVB/N mice at Erasmus University. This colony was donated to the Jackson laboratory where it is available as FVB.129P2-*Fmr1*^{tm1Cgr}/J stock no 3024. A not yet fully backcrossed version FVB;129P2-*Fmr1*^{tm1Cgr}/J is also still available as stock 2700. While the FVB strain reproduces vigorously, a major drawback is that it suffers from a severe visual impairment due to two unrelated genetic defects. First, FVB/N is an albino strain as a consequence of a dysfunctional tyrosinase locus. The unpigmented eyes of albino animals (Tyr$^{c/c}$) result in impaired vision. Second, it is homozygous for the retinal degeneration mutation (Pde6b$^{rd1/rd1}$), resulting in postnatal degeneration of the rods in the eye in the first month after birth. To be able to use the fragile X mice in an FVB/N background in test that are dependent on intact vision, the FVB;129P2-*Fmr1*^{tm1Cgr} strain was backcrossed to the FVB/N strain in Antwerp, while in this case selecting against albinism and the retinal degeneration mutation in the homozygous state (Errijgers, Fransen, D'Hooge, De Deyn, & Kooy, 2008). The resulting FVB.129P2- Pde6b^{+}Tyr$^{c\text{-}ch}$ *Fmr1*^{tm1Cgr} /Ant strain that contains the knockout mutation in a sighted FVB background was transferred to Dr. William Greenough at the University of Illinois, who transferred the stock to the Jackson laboratory, where it is available as stock 4624 under the name FVB.129P2- Pde6b^{+}Tyrc-ch *Fmr1*^{tm1Cgr} /J. The corresponding sighted FVB stain without the *Fmr1* mutation is available as stock 4848 and can be used as a control for this specific mutant (Table 7.1).

The knockout model in the various genetic backgrounds is the most widely used fragile X animal mouse model so far. It is generally accepted to be a valid model for the FXS. Like patients, *Fmr1* knockout (KO) mice do not produce Fmrp. However, the *Fmr1* promoter remains intact and results in residual transcription of detectable abnormal *Fmr1* RNA of about 27% of wild-type level (Bakker et al., 1994; Braat et al., 2015; Mientjes et al., 2006; Yan, Asafo-Adjei, Arnold, Brown, & Bauchwitz, 2004). It shows many features compatible with the clinical hallmarks of patients, including macroorchidism (Fig. 7.1), mild facial dysmorphism, neuroanatomical and functional brain abnormalities, impaired cognitive functioning and deficits in specific behavioral assessments and increased susceptibility to seizures, all discussed in greater detail later.

A difference in test results dependent on the genetic background was sometimes observed (Berman et al., 2014; Errijgers et al., 2008; Paradee et al., 1999; Pietropaolo, Guilleminot, Martin, D'Amato, & Crusio, 2011). To assess the importance of the effects of genetic background on behaviors in a systematic way, Spencer et al. (2011) crossed male wild-type mice of five different genetic backgrounds (A/J, DBA/2J, FVB/NJ, 129S1/SvImJ and CD-1) to female *Fmr1* heterozygous mice on a pure C57BL/6J background. The resulting F1-hybirds were examined in an extensive behavioral test battery. The test results clearly indicate that multiple behavioral responses, including autistic-like traits are dependent on genetic background (Kooy, 2003).

Conditional knockout mouse: In order to be able to study the effect of the lack of Fmrp expression in specific tissues or distinct cell types or at various time points during development, a conditional knockout mouse was generated (Mientjes et al., 2006). A targeting construct was designed containing a neomycin resistance cassette flanked by cre-lox sites in intron 1 and an additional cre-lox site in the *Fmr1* promotor region (Fig. 7.2). Transfection of the targeting construct in E14 ES cells resulted in an *Fmr1* conditional restoration (cON) model: despite the presence of an intact *Fmr1* gene, the presence of a neomycin cassette in the antisense

FIGURE 7.1 ***Fmr1* knockout mice develop progressive macroorchidism with time** (Bakker et al., 1994; Kooy et al., 1996; Slegtenhorst-Eegdeman et al., 1998). Fmrp is high expressed in Sertoli cells during very early development of the testis in neonatal mice and it has been proposed that increased Sertoli cell proliferation during testis development is the cause of macroorchidism in *Fmr1* knockout mice (Bakker et al., 2000; Slegtenhorst-Eegdeman et al., 1998).

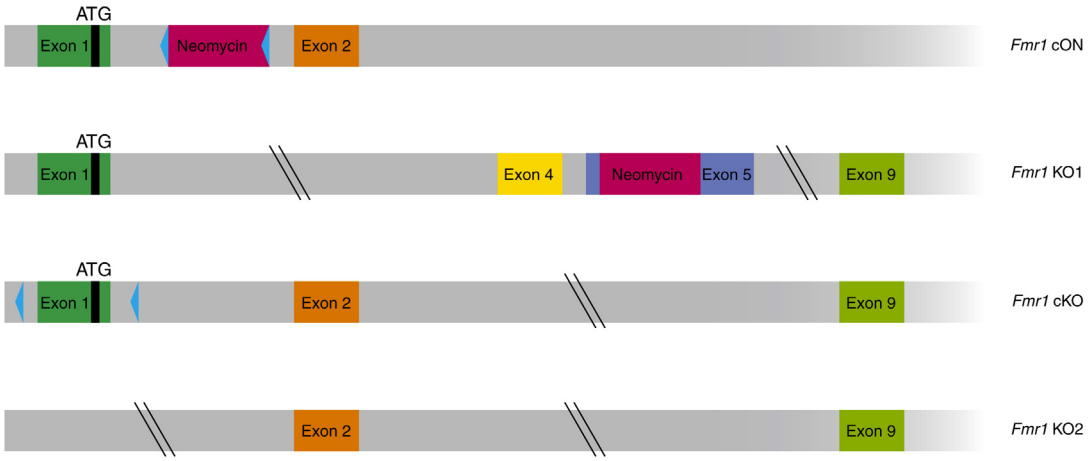

FIGURE 7.2 **Schematic presentation of the different mouse models for FXS.**

orientation in intron 1 reduces the expression of Fmrp in brain tissue to less than 10%–20% of wild-type levels. Subsequent in vitro excision of the neomycin cassette resulted in a conditional fragile X knockout mouse model due to the presence of two cre-lox sites flanking exon 1 of the fully intact *Fmr1* gene. This mouse is called an *Fmr1* CKO mutant. Constitutively inactivated *Fmr1* KO2 lines, lacking exon 1, as well as the neomycin cassette were obtained

through crossing the cON mice with CAG-cre expressing mice in an FVB/N background. All mutants were subsequently backcrossed for many generations to C57BL/6J mice.

The KO2 model has been far less characterized in comparison with the previously mentioned knockout model. It generates neither *Fmr1* mRNA nor Fmrp. From the relatively limited number of studies reported using this model, no differences between both types of *Fmr1* mutants have been observed. In this chapter we will specifically indicate if a study has been performed with *Fmr1* KO (2).

The I304N point mutation model: Recently a mouse model for the I304N mutation was reported (Zang et al., 2009). This *Fmr1*^{I304N} model recapitulates one of the rare missense mutations that has been reported in a patient with FXS with an extremely severe phenotype with profound intellectual disability and excessive macroorchidism (De Boulle et al., 1993). It was generated by injecting an I304N containing, self-excising cre-lox ACNF cassette into 129/SvJ derived ES cells. The resulting chimeras were backcrossed for many generations with C57BL/6J (B6.129-*Fmr1*$^{tm1(I304N)Rbd}$), as well as with FVB/N mice (FVB.129-*Fmr1*$^{tm1(I304N)Rbd}$). The mutation in both backgrounds were deposited in the Jackson laboratories as stock 10,504 and 8,909, respectively.

The isoleucine to asparagine mutation is located in the second RNA binding KH domain of the protein and was demonstrated to hamper RNA and ribosome binding. However, protein expression levels were also reduced in an age-dependent way, with the lowest expression recorded at 2 weeks of age during a critical period for synapse development. In contrast to expectations, the *Fmr1* I304N mice are not more severely affected than *Fmr1* KO mice but phenocopy many of their deficits as assessed by behavioral testing, degree of macroorchidism and electrophysiological long-term depression (LTD) recordings. At the moment it is unclear whether the difference in the severity of the symptoms between this mouse model and the human patient is a consequence of the reduced expression of the protein in the animal model or of the presence of a second, apparently unrelated mutation in the *PHKA2* gene associated with liver glycogenosis in the patient (De Boulle et al., 1993).

The rescue model: YAC transgenic mice were generated by transgenesis of a slightly modified version of YAC 209G4 containing the human *FMR1* gene including a CGG repeat of approximately 20 repeat units (Heitz et al., 1991) in C57BL/6 mice (A. M. Peier et al., 2000). The resulting line TG298 was extensively characterized and showed overexpression of the *FMR1* mRNA and protein. In knockout mice carrying the YAC transgene, macroorchidism was absent, indicating a functional rescue by the human protein. Further indications for a functional rescue came from a more thorough evaluation of the *Fmr1* KO phenotype, that showed opposing behavioral responses of the YAC transgenic line TG298 in a knockout compared with knockout mice (A. M. Peier et al., 2000; Spencer, Graham, Yuva-Paylor, Nelson, & Paylor, 2008). Additional lines carrying the same human *FMR1* gene, but in this case manipulated to contain an expanded repeat of 92 CGG repeats, derived from a premutation male with the configuration (CGG)9AGG(CGG)9AGG(CGG)72 in an FVB background were subsequently generated (A. Peier & Nelson, 2002). Multiple lines of these CGG90YAC transgenic mice show moderate intergenerational stability, but methylation of the repeat has not been observed even if the repeat length exceeds the methylation-size threshold in man by far.

The repeat expansion model: In order to study the repeat expansions in a mouse model, knock-in models containing an expanded CGG repeat in the *FMR1* gene were generated. Additional advantage of these animals was that these were predicted to generate FMRP during

early embryonic development before methylation takes place, like patients but unlike the knockout models that have a constitutional inactivation of the gene. Two independent mouse models were generated by two different groups (reviewed by Berman et al., 2014). One model was generated in the Rotterdam and commonly referred to as the so-called Dutch mouse (CGG dut KI). This mouse model was generated by replacing the native murine CGG_8-repeat of the endogenous *Fmr1* gene with a CGG_{98} repeat derived from a human patient, including the flanking sequences by homologous recombination in E14 embryonic stem cells (Bontekoe et al., 2001). The line was inbred in both the C57BL/6 and the FVB background. These CGG KI mice show moderate instability of repeat length upon paternal and maternal transmission. Both expansions and contractions of typically fewer than 10 repeats are observed. A similar knockin mouse was developed at the National Institutes of Health with an initial CGG118 tract (Entezam et al., 2007). The CGG nih KI mice were generated using in vitro generated repeats flanked by Sfi I compatible sites, that were targeted into exon 1 of the mouse gene modified to contain two adjacent but incompatible Sfi I sites. This allowed the synthetic CGG repeat track to be inserted into the mouse *Fmr1* gene locus in the correct orientation with minimal changes to the mouse flanking sequence. As a result of this strategy, the CGG nih mouse retains the translational TAA stop codon just upstream of the CGG118 repeat that is present in the endogenous murine gene but not in the human gene. The NIH mouse was generated in 129S6/SvEv-derived TC1 and inbred in C57BL/6. Both the CGG dut mice and the CGG nih mice show elevated *Fmr1* mRNA levels, decreased FMRP levels, moderate intergenerational expansions, no methylation (even when repeat numbers were >300) and ubiquitin-positive intranuclear inclusions (Brouwer et al., 2008; Entezam et al., 2007; Willemsen et al., 2003).

THE PHENOTYPIC SPECTRUM OF THE KNOCKOUT MOUSE

Neuroanatomical Phenotype in *Fmr1* Knockout Mouse

The first observations of dendritic spines in brain tissue originate from 1891 by Santiago Ramon y Cajal using Golgi's staining method. Dendritic spines, small membranous protrusions along neuronal dendrites, are sites that usually receive excitatory input from axons although sometimes both inhibitory and excitatory connections are made onto the same spine head as well. The dendrites of a single neuron can contain hundreds to thousands of spines and express receptors and signaling molecules that are essential for synaptic strength. Spines are highly dynamic structures and their morphology can change very rapidly upon different types of stimulation, a process termed synaptic plasticity (Yang, Pan, & Gan, 2009). Spine abnormalities are considered a hallmark of patients with intellectual disability (Purpura, 1974). Therefore, spine abnormalities have been intensively studied in the knockout mouse model (Fig. 7.3). Some studies found a higher dendritic spine density in cortex or hippocampus of *Fmr1* KO adult mice (Galvez, Smith, & Greenough, 2005; McKinney, Grossman, Elisseou, & Greenough, 2005), while others found spine density to be decreased (Braun & Segal, 2000), while others found no differences in spine characteristics between both genotypes (Grossman, Elisseou, McKinney, & Greenough, 2006; Irwin et al., 2002). Overall though, most of the studies agree on immature dendritic spine phenotype either in the cortex or the hippocampus of *Fmr1* KO mice as compared with the wild-type littermates (Levenga & Willemsen, 2012; Pop et al., 2014;

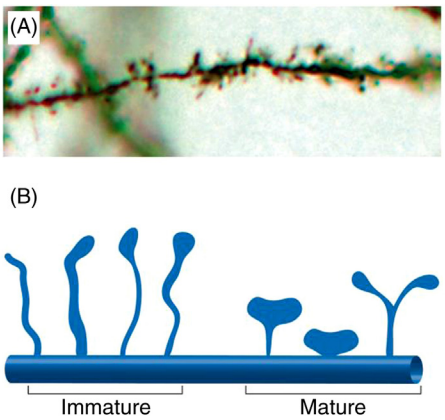

FIGURE 7.3 **Morphology of dendritic spines.** Photomicrograph (A) shows a detail of the dendrite from a murine *Fmr1* KO hippocampal neuron stained with Golgi impregnation staining method. The Golgi technique selectively impregnates single neurons with silver chromate and allows visualization of dendrites and axons. Photomicrograph (B) shows graphical representation of dendritic spine morphologies defined as mature or immature.

Portera-Cailliau, 2011). The same immature dendritic spine phenotype was found in primary hippocampal neuron cultures (Braun & Segal, 2000; De Vrij et al., 2008), as well as in dissociated neuronal cultures, but only in a C57BL/6 background (Levenga & Willemsen, 2012).

The interstrain differences sometimes lead to different results for the same aspect studied, and therefore, it is important to choose the correct strain for each study (seemingly identical strains, but distributed by different suppliers or even by different branches of the same supplier have been demonstrated to have subtle, but sometimes relevant genomic differences). Additional factors that may contribute to the differences in findings include the brain region studied, the age of the mice and the methodology used. Recently, superresolution imaging using stimulated emission depletion (STED) microscopy showed only subtle differences in spine composition that are age and brain region dependent (Wijetunge, Angibaud, Frick, Kind, & Nagerl, 2014).

Long-Term Potentiation and Long-Term Depression

Learning and memory involves long-term potentiation (LTP) and long-term depression (LTD) which are considered to be the major cellular mechanisms underlying learning and memory (Kandel, 2001). LTP is best described as a strengthening of the connection between the presynaptic and the postsynaptic compartment of a neuron for a longer period of time. LTP coincides with the appearance of more AMPA receptors at the postsynaptic membrane, increasing the sensitivity to signals from the presynaptic neurons and thus strengthening the connection (Matsuzaki, Honkura, Ellis-Davies, & Kasai, 2004). LTD is the antithesis of LTP and is seen as the weakening of the synapse, reflected by a reduced number of ion receptors at the postsynaptic membrane (for review, see Malenka & Bear, 2004). Thus, the gain or loss of AMPA receptors at the postsynaptic membrane is related to the strength of the synapse which is reflected in the morphology of the spines.

A proper balance between LTP and LTD underlies learning and memory formation. As patients with FXS suffer from intellectual disability, learning and memory among other processes

are defective. Therefore it was postulated that FMRP might be involved in the LTP/LTD mechanisms, and its absence disturbs the balance and the normal function of the synapse of the patient. Since the generation of the *Fmr1* KO mouse, researchers have tried to investigate the differences in LTP or LTD in this model to understand the underlying molecular mechanisms of intellectual disability in FXS patients. Electrophysiological studies in *Fmr1* KO mice and wild-type mice showed that FMRP contributes to postsynaptic LTP induction in the anterior cingulate cortex (ACC) (Chen et al., 2014), whereas the role of FMRP in hippocampal and cortical LTP is still controversial (Godfraind et al., 1996; Hu et al., 2008; J. Li, Pelletier, Perez Velazquez, & Carlen, 2002; Paradee et al., 1999; Shang et al., 2009). Recently, age-dependent deficits were demonstrated in the prefrontal cortex of 12-month old mice, whereas younger mice didn't show reduction of synaptic plasticity (Martin, Lassalle, Brown, & Manzoni, 2016). Impaired LTP may explain some of the behavioral deficits observed in *Fmr1* KO mice.

Different types of LTD can be distinguished, such as NMDA receptor metabotropic glutamate receptor (mGluR) or endocannabinoids dependent LTD. An important type of LTD is induced by activation of group 1 metabotropic glutamate receptors (mGluR) (Huber, Roder, & Bear, 2001). This form of LTD is dependent on local protein synthesis at the synapse and also results in a reduced number of AMPA receptors in the postsynaptic membrane (Schuman, Dynes, & Steward, 2006). This type of LTD is not observed if mRNA translation is inhibited. Activation of group 1 mGluRs, either with paired-pulse low-frequency synaptic stimulation (PP-LFS) or with the selective agonist (S)-3,5-dihydroxyphenylglycine (DHPG), results in a persistent decrease in synaptic strength that is mechanistically distinct from classical NMDA receptor (NMDAR)-dependent LTD. A model (mGluR theory) was proposed in which FMRP normally functions as a repressor of translation of specific mRNAs at the synapse (see Chapter 9). Indeed, *Fmr1* KO mice show enhanced hippocampal mGluR-LTD (reviewed in Sidorov, Auerbach, & Bear, 2013). Enhancement in cerebellar mGluR-LTD has been reported as well (Koekkoek et al., 2003).

Behavioral Phenotype in Fmr1 Knockout Mouse

Since the generation of the *Fmr1* KO mouse in 1994 an extensive suite of different behavioral tests have been performed by many research groups all over the world. These behavioral tests have revealed dissimilarities in their outcome measurements, and in some cases opposite results that in part can be attributed to variations in genetic background. In addition, many reported *Fmr1* KO behavioral phenotypes are seemingly opposite to the most prominent clinical features in patients with FXS. Interestingly, it has been suggested that this phenomenon reflects the phenotypic variability in humans (Dobkin et al., 2000). In this section, a condensed summary of 25 years of behavioral studies is provided. Based on relevant behavioral deficits in patients with FXS the battery of test has been divided in specific subgroups, including seizures and hypersensitivity, cognitive functioning, attention and hyperactivity, social and emotional functioning, and anxiety.

Seizures and Hypersensitivity

In approximately 20% of FXS males spontaneous epileptic seizures are observed, however, seizures are infrequent and often partial (Hagerman & Stafstrom, 2009). Since *Fmr1* KO

mice have never been reported to display spontaneous epileptic seizures, acoustic stimulation (typically with a 125 decibel, high-intensity siren) was applied to provoke audiogenic seizures. The vulnerability to audiogenic seizures was greater on *Fmr1* KO mice compared with wild-type littermates and depended on background and age, with the youngest mice on an FVB background showing the highest susceptibility (Kooy, 2003; Musumeci et al., 2000). It has been proposed that increased cortical excitability may underlie audiogenic seizure vulnerability or alternatively deficiencies in LTP (Kooy, 2003).

Patients with FXS display hyperarousal and increased sensitivity to sensory stimuli. Enhanced responses to auditory tones have been demonstrated in electrophysiological recordings in the auditory cortex of *Fmr1* KO mice (Rotschafer & Razak, 2014). At the behavioral levels opposite results have been reported. Enhanced prepulse inhibition (PPI) and reduced startle versus impaired PPI and increased startle responses were described (reviewed in Kazdoba, Leach, Silverman, & Crawley, 2014). These divergent findings in mice can in part be explained by the different genetic backgrounds and testing protocols in use by each laboratory.

Cognitive Functioning

A vast majority of male patients with FXS display intellectual disability (ID) which can range from mild to severe. The behavioral test battery to study learning and memory deficits in mice is broad and may include the Morris Water Maze, the Barnes Maze and E- and cross-shaped mazes, passive avoidance, contextual fear conditioning, novel object recognition and radial arm maze. These tests rely on different brain regions including hippocampus, amygdala, and prefrontal cortex. Overall the results show mild deficits in spatial learning and working memory in *Fmr1* KO mice when compared with wild-type littermates (reviewed in Kazdoba et al., 2014). Again, some of the test results are poorly reproducilble across laboratories, suggesting the testing conditions strongly influence the results. Currently, a reliable and reproducible test that can be used to study the effect of drugs on cognition in the FXS model is lacking and a reliable and robust behavioral task is desperately warranted.

Attention and Hyperactivity

Patients with FXS are hyperactive and display attentional deficits and impulse control (Chapter 1). *Fmr1* KO mice show contradictory results in attention tasks (nose-poke responses), however, consistent behavioral findings were reported for impaired inhibitory control, disrupted performance in olfactory distracters and increased locomotor activity in the open field paradigm (Bakker et al., 1994; Ding, Sethna, & Wang, 2014; Krueger, Osterweil, Chen, Tye, & Bear, 2011; A. M. Peier et al., 2000; Sidorov et al., 2014).

Social and Emotional Functioning

One of the core features of autism and therefore also FXS is abnormal social behavior, but unlike in ASD where individuals show social withdrawal, patients with FXS, especially young children, seek more social contact and show interest in communication

(S. S. Hall, Lightbody, Hirt, Rezvani, & Reiss, 2010; Tranfaglia, 2011). In *Fmr1* KO mice, social behavior was studied by several different methods which led to an abnormal phenotype observed by some groups ranging from increased social preference in *Fmr1* KO mice (Gantois et al., 2013; Spencer, Alekseyenko, Serysheva, Yuva-Paylor, & Paylor, 2005), through no difference (McNaughton et al., 2008; Pietropaolo et al., 2011), to even a decrease in social interest (Mineur, Sluyter, de, Oostra, & Crusio, 2002). Recently, a novel reliable and robust behavioral assay (Automated Tube Test, ATT) to characterize the social dominance behavior of adult male *Fmr1* KO mice was developed and validated. FXS mice in the ATT showed a highly dominant behavior over wild-type littermates (de Esch et al., 2015). Of interest, the social behavioral phenotype of the *Fmr1* KO mice heavily depended on previous social experiences.

Anxiety

Anxiety is a core feature in the vast majority of patients with FXS. Anxiety-related behavioral tests include preference for dark spaces in the elevated plus-maze, open quadrants in the zero-maze, and avoidance of the center of the open field. *Fmr1* KO mice displayed increased anxiety-related responses in the exploratory tests but a lowered anxiety was reported if measured in the open field paradigm (reviewed in Kazdoba et al., 2014).

Twenty five years of preclinical research has led to the identification of most of the involved pathways underlying the pathophysiology of FXS (see Chapters 8–16). Encouraged by these discoveries, an increasing number of pharmaceutical companies are interested in developing and testing candidate drugs to improve the condition of FXS patients. More and more research groups are testing these drugs on different levels for behavioral, molecular, and morphological rescue of the abnormalities in *Fmr1* KO mice. But while many FXS phenotypes were improved in preclinical studies with drugs targeting these pathways in the *Fmr1* KO mouse model, attempts to translate these animal-model success stories into treatment of patients in clinical trials have been disappointing up till now (see Chapters 19–20). Complicating factors, particularly in animal studies, include the use of the mutation in different mouse inbred strains, variability in functional studies between laboratories, a publication bias toward positive results and an a lack of reliable and objective primary outcome measures in mice. While most studies focused on one single impaired pathway at a time, the key solution for finding a cure for FXS might be to target multiple pathways and proteins simultaneously. In the years to come, animal studies aimed at a multitarget approach, as well as a search for reliable outcome measures, including sensitive biomarkers and relevant robust functional phenotypes, are more likely to provide us with new preclinical data in the quest for targeted therapy for FXS (Zeidler, Hukema, & Willemsen, 2015).

RAT MODELS OF FRAGILE X SYNDROME

The first *Fmr1* KO rat model has been created on the outbred Sprague Dawley background using a zinc-finger-nuclease (ZFN) methodology to target deletion within *Fmr1* exon 8 (Hamilton et al., 2014). Important as a research tool in their own right, the development of rat models of FXS also enable comparisons of phenotypes to be made between

mammalian species, thereby increasing confidence that phenotypes and treatments may translate to the human condition. Initial studies in *Fmr1* KO rats reveal that several cellular and physiological phenotypes associated with the loss of Fmrp are conserved across mammalian species. Like FXS individuals and their murine counterparts, *Fmr1* KO rats do not produce Fmrp, exhibit macroorchidism, and have subtle alterations in the density of dendritic spines (Hamilton et al., 2014; Till et al., 2015). Consistent with findings in *Fmr1* KO mice, loss of Fmrp in rats also results in elevated basal protein synthesis, as well as enhanced expression and protein synthesis independence of group 1 mGluR-LTD in CA1 of the hippocampus (Till et al., 2015).

Sensory processing and sensory integration problems are common features of FXS. Studies of the development of sensory-evoked activity in the visual cortex indicate early network changes in *Fmr1* KO rats. Despite exhibiting a normal developmental trajectory of visual responses, *Fmr1* KO rats show hypoexcitability of light evoked responses in the visual cortex at developmental ages corresponding to late gestation in humans, as well as mild hyperexcitability at later ages (Berzhanskaya, Phillips, Shen, & Colonnese, 2016). Moreover, spontaneous resting state activity that arises at ages equivalent to human birth and infancy lack modulation in *Fmr1* KO rats. This persistent activated state in *Fmr1* KO rats appears to result from failure to develop normal synchronization of firing in deep layers of the visual cortex (Berzhanskaya, Phillips, Gorin et al., 2016). These findings are consistent with studies in *Fmr1* KO mice that suggest that the loss of FMRP biases cortical states toward activation at times of low arousal (Gibson, Bartley, Hays, & Huber, 2008; Hays, Huber, & Gibson, 2011). Sensory processing is also affected in adult *Fmr1* KO rats; fMRI in awake rats reveals that loss of Fmrp alters activation in the mesolimbic/habenular circuit that underlies reward processing (Kenkel et al., 2016).

While key cellular phenotypes are conserved and network alterations are consistent between *Fmr1* KO mice and rats, they appear to manifest in predominantly distinct behavioral dysfunction. For example, in contrast to previous reports in mice, loss of Fmrp in rats does not affect locomotor activity in an open field (Hamilton et al., 2014). This difference alone confers an advantage to rats over mice since hyperactivity can confound performance analysis in behavioral paradigms that rely on locomotion. Also in contrast to findings in *Fmr1* KO mice (Baker et al., 2010; D'Hooge et al., 1997; Paradee et al., 1999; Van Dam et al., 2000), flexible spatial learning is intact in *Fmr1* KO rats as measured by reversal learning in the spatial reference memory and delayed matching to place versions of the Morris water maze (Till et al., 2015). Although still in the early stages of exploration, the findings that cellular phenotypes converge between mammalian species while behavioral phenotypes do not, raise important questions regarding the importance of animal models showing face validity with the human condition.

Compared to mice, rats show increased flexibility in response to novel situations and extensive social interactions. Indeed, loss of Fmrp in rats leads to a deficit in an associative recognition memory task that requires the hippocampus to bind multiple associations to form a memory; in contrast, versions of this task that do not require an intact hippocampus are unaffected in *Fmr1* KO rats (Till et al., 2015). Deficits in perseverative chewing and juvenile play have also been reported in *Fmr1* KO rats (Hamilton et al., 2014). Use of these paradigms to assess social and cognitive behaviors through development, provides the opportunity to examine intervention strategies across the lifespan.

ZEBRA FISH MODELS OF FRAGILE X SYNDROME

Zebrafish, *Danio rerio*, is a very convenient animal model to study embryonic development in a vertebrate system. Within 24 h, all organs are developed and in 90 days the zebrafish will mature. Moreover, the embryos develop outside the mother and are transparent, allowing direct observation of their embryonic development. The zebra fish genome encodes for all three human orthologues of FMRP, FXR1P, and FXR2P (Engels et al., 2004; Tucker, Richards, & Lardelli, 2004; van't Padje et al., 2005). To study the function of FMRP during development, one of the approaches which can be used is the morpholino antisense oligonucleotide knockdown technique. Injecting an antisense oligonucleotide morpholino in fertilized eggs will result in a transient knock down of the target gene, that is, *fmr1*. Using this approach, a FXS phenotype has been observed including abnormal axonal branching and neuronal guidance and defasciculation defects (Tucker, Richards, & Lardelli, 2006). It is now commonly assumed that the phenotype of this morpholino injected *fmr1* knockdown fish model potentially resulted from experimental artefacts, a recently underscored phenomenon for this technology (Schulte-Merker & Stainier, 2014). In line therewith, a genetic *fmr1* KO zebrafish model developed with ENU-mutation screening, shows no obvious phenotype at young age (den Broeder et al., 2009). In contrast, adult *fmr1* KO zebra fish did show behavioral and synaptic abnormalities (Kim et al., 2014; Ng, Yang, & Lu, 2013; Shamay-Ramot et al., 2015). In particular, the vertebrate zebra fish model holds great potential benefits for high-throughput drug screening.

Drosophila Models of Fragile X Syndrome

Many features of fruit fly make it an attractive model to study basic biology. With a rapid life cycle (10 days at 25°C), fruit fly has four distinct developmental stages: embryo, larva, pupa, and adult. It is estimated that 100,000 neurons are present in fly brain. These neurons form complex circuits and neuropil that mediate multifarious and complicated behavior, such as flight navigation, aggression, grooming, feeding, learning and memory, sleep, and circadian rhythms. *Drosophila* has been a powerful model system due to the ease of manipulating the expression and function of its genes. Genetic tools developed in fly provide quick and easy ways to generate human disease models by mutation, expression, inactivation or misexpression of orthologous genes in fly (Dietzl et al., 2007; Rubin & Spradling, 1982). Furthermore, *Drosophila* has emerged as a premiere model system for the study of human diseases due to the realization that flies and humans share many structurally and functionally related gene families (Bonini & Fortini, 2003). Development of diseases models in the fly will allow genetic approaches to be applied to address specific hypotheses concerning disease progression and to test candidate modifier genes or therapeutic drug compounds. Most importantly, fly models will also allow the identification of novel modifiers through unbiased genetic screens.

FMRP Protein is Well-Conserved Between *Drosophila* and Mammals

FMRP, along with its autosomal paralogs, the Fragile-X-Related Proteins FXR1P and FXR2P, compose a well-conserved, small family of RNA-binding proteins (fragile

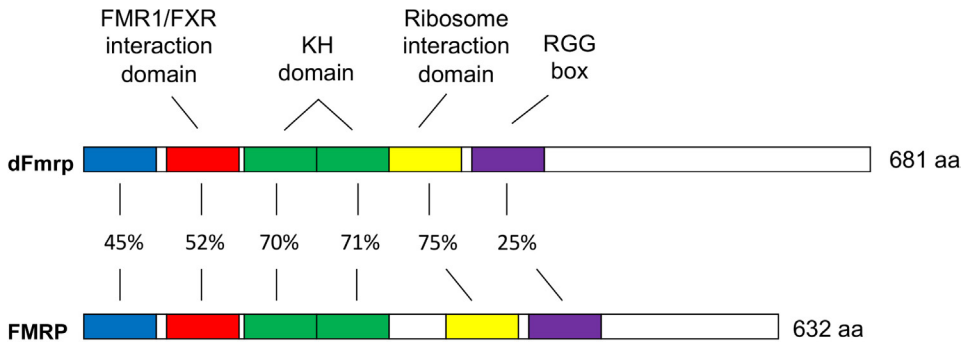

FIGURE 7.4 **Conservation of *Drosophila* FMR1 protein (dFmrp) and mammalian FMRP.** dFmr1 encodes a protein with 681 amino acids that shares a high degree of conservation with mammalian FMRP. The two KH domains are about 70% identical between dFmrp and FMRP. The RGG box, another RNA-binding motif found in FMRP is also present in dFmrp. The high degree of sequence conservation suggests that dFmrp is a functional homolog of FMRP in *Drosophila*.

X-related gene family) that share over 60% amino acid identity (Siomi et al., 1995; Y. Zhang et al., 1995). Unlike their mammalian counterparts, the fly genome harbors a single *FMR1* gene homolog, also referred to as *dFmr1* or *dfxr* (*dFmr1* here, as per Flybase annotation). Sequence comparisons show a high level of similarity between the functional domains of fly and mammalian Fmrp, with overall 56% similarity and 35% identity (Gao, 2002; Zhang et al., 2001; Fig. 7.4). dFmrp is comprised of two KH domains, ribosomal- and self-association domains, an RGG box, as well as a nuclear localization signal (NLS) and nuclear export signal (NES).

Not only is dFmrp highly homologous to mammalian Fmrp, but also it exhibits similar homopolymer RNA binding properties. In vitro translated dFmrp can strongly bind to poly(G), weakly to poly(U) but not to poly(A)/(C) (Wan, Dockendorff, Jongens, & Dreyfuss, 2000). Mutations in each of the KH domains abolish homopolymer binding, consistent with a functional role for these motifs as suggested from human genetics studies (De Boulle et al., 1993). More recently, dFmrp in analogy with human FMRP was unexpectedly found associated with chromatin in nucleus, suggesting a new role of Fmrp in the DNA damage response (Alpatov et al., 2014; W. X. Zhang et al., 2014).

Immunohistochemical data show that dFmrp is ubiquitously expressed during the early stages of embryogenesis, with strong expression in the mesoderm, the brain lobes and ventral ganglia developing at later stages (Dockendorff et al., 2002; Wan et al., 2000; Zhang et al., 2001). Other tissues where dFmrp has been detected are the developing imaginal discs, testes, ovaries and the ring gland (Zarnescu et al., 2005; Zhang et al., 2001). Just like its mammalian counterpart(s), dFmrp is enriched in neurons and with low or absent levels in glia.

FXS Fly Model Displays the Defects in Behavior, Synaptogenesis and Spermatogenesis

To characterize the physiological functions of *dFmr1*, several loss-of-function mutations ranging in strength from weak hypomorphs to nulls have been isolated in the *dFmr1* locus

(Dockendorff et al., 2002; Inoue et al., 2002; Lee et al., 2003; Morales et al., 2002; Zhang et al., 2001). *DFmr1* is not essential for viability, although some variability has been reported in the numbers of adult homozygotes (Dockendorff et al., 2002; Morales et al., 2002). Homozygous mutant adults display abnormalities in behavior, synaptogenesis and spermatogenesis, some of which may be viewed as resembling the phenotypes observed in FXS patients.

Behavioral Defects

Examination of locomotor activity in adult flies lacking *dFmr1* revealed a statistically significant arrhythmic behavior (Dockendorff et al., 2002; Morales et al., 2002). Overall, *dFmr1* mutants exhibit erratic activity patterns with brief periods of high activity. To determine whether this is a result of an alteration in the circadian clock or simply due to reduced locomotor activity, the overall activity of *dFmr1* null flies kept in the dark for a total of 9 days was measured and no significant difference was found compared to controls (Dockendorff et al., 2002). This suggests that the arhythmicity observed is not due to defects in motor function and locomotion ability, but rather in the circadian clock. Interestingly, just like mutants lacking normal circadian function, *dFmr1* nulls can be driven to display normal rhythms and even anticipate lights turning on and off when trained in light: dark cycles (Dockendorff et al., 2002; Inoue et al., 2002). This suggests that the molecular clock in itself is intact, and the defects observed may be due to downstream effectors of the clock. To address this possibility, the expression of known molecular components of the circadian clock, such as timeless and period, were examined and found no significant changes in *dFmr1* mutants cycles (Dockendorff et al., 2002; Inoue et al., 2002; Morales et al., 2002). However, using a reporter construct (CRE-Luciferase) to monitor the downstream activity of the molecular clock, it was found that the amplitude of oscillations was reduced, suggesting that *dFmr1* affects at least this aspect of the clock output (Dockendorff et al., 2002). Indeed FXS patients have shorter sleep duration, greater variation in sleep duration and sleep timing problems, which might be related to the disturbance of circadian rhythms.

Courting *Drosophila* males perform a characteristic sequence of behaviors: orienting toward and following the female, tapping her with his forelegs, vibrating one wing, licking her genitalia, and attempting to copulate (J. C. Hall, 1994). The percentage of time that the male spends performing any of these behaviors toward a target during a defined period of time is referred to as the courtship index (CI) (Siegel & Hall, 1979). Learning and memory can be examined in *Drosophila* by utilizing conditioned courtship behavior. In conditioned courtship, a male fly learns to modify his courtship behavior after experience with an unreceptive female (Hall, 1994). This is a complex associative learning paradigm that potentially involves multiple sensory inputs (Hall, 1994). Previous studies have found that the *dFmr1* mutants display reduced levels of naive courtship activity relative to wild type and fail to display any detectable memory (immediate and short-term) of courtship conditioning while the learning during training remains unchanged (Chang et al., 2008; McBride et al., 2005). In addition, it also has been shown that dFmrp is acutely required for the formation of long-term memory using an olfactory learning and memory assay (Bolduc, Bell, Cox, Broadie, & Tully, 2008). These works together indicate an important role of dFmrp in learning and memory in *Drosophila*.

Synaptogenesis Defects

Using *Drosophila* larval neuromuscular junction (NMJ, an ionotropic type of synapse), it was shown that the loss of *dFmr1* resulted in an increased number of synaptic boutons and overelaboration of synaptic terminals, similar to the dendritic overgrowth phenotype reported in the *Fmr1* knock-out mouse, as well as in FXS patients (Hinton, Brown, Wisniewski, & Rudelli, 1991; Nimchinsky, Oberlander, & Svoboda, 2001; Zhang et al., 2001). As expected, overexpression of *dFmr1* results in underelaborated synaptic terminals with enlarged synaptic boutons (Zhang et al., 2001). Using tissue specific drivers to overexpress the protein either pre- or postsynaptically, it was found that *dFmr1* functions on both sides of the synapse, but is predominantly presynaptic (Zhang et al., 2001). Electrophysiological studies revealed that evoked synaptic neurotransmission is significantly increased at the NMJ in *dFmr1* mutants, suggesting that the average synaptic efficacy is upregulated in these mutants (Zhang et al., 2001). In addition, miniature excitatory junctional currents (mEJC) had a mildly increased frequency in nulls flies compared to controls and also showed a significant increase in frequency when *dFmr1* was over expressed on the pre- but not postsynaptic side (Zhang et al., 2001). This result was surprising in that both loss- and gain-of-function conditions resulted in increased efficacy of synaptic transmission, suggesting that the physiology of the synapse is highly sensitive to the level of dFmrp. A similar effect was observed with electroretinograms (ERGs) recorded at the histaminergic photoreceptor synapse, although in this case the transmission efficacy was decreased by modulating the level of dFmrp (Morales et al., 2002). These results are similar to the observation that in mouse the level of Fmrp is critical and overexpression of Fmrp could overcorrect the behavioral phenotypes affected in *Fmr1* KO mice (Peier et al., 2000).

Just like its mammalian counterpart, dFmrp plays a role in dendrite morphogenesis. A detailed developmental analysis of multiple dendritic (MD) neurons in *dFmr1* mutants showed that dFmrp is a negative regulator of neurite extension (Lee et al., 2003). In contrast, overexpression of dFmr1 allows the extension of the major dendritic branches, but blocks the formation of higher order structures, thus reduces the overall dendritic complexity (Lee et al., 2003, p. 2952). Other studies focused on the dorsal cluster neurons (DC), which control eclosion and the lateral (LNv) neurons, which control circadian rhythms (Dockendorff et al., 2002; Morales et al., 2002). In the absence of *dFmr1*, both of these neuronal types exhibit overextended neurites, with overbranching, as well as axonal pathfinding defects. Interestingly, overexpression of *dFmr1* in both wild type and mutant backgrounds results in failure of axonal extension, once again suggesting that dosage is critical for normal functions (Morales et al., 2002). Recently, the mushroom body (MB), a highly plastic brain region that is essential for many forms of learning and memory, was also investigated (Michel, Kraft, & Restifo, 2004; Pan, Zhang, Woodruff, & Broadie, 2004). Phenotypic analyses showed that, in the absence of *dFmr1*, MB neurons display a more complex architecture, including overgrowth, overbranching, and abnormal synapse formation (Michel et al., 2004; Pan et al., 2004). Taken together, these data showed that *dFmr1* is a potent negative regulator of neuronal architecture and synaptic differentiation in the fly nervous system.

Spermatogenesis Abnormality

Although *dFmr1* mutants are viable and lack obvious morphological abnormalities, they cannot be maintained as a stock using standard fly husbandry (Zhang et al., 2004). A detailed

analysis of *dFmr1* expression during spermatogenesis showed that the protein is upregulated in the late and larger spermatocytes (first four stages of spermatogenesis) compared to the more mature, elongated spermatids (last two stages of spermatogenesis) (Zhang et al., 2004). Consistent with this expression pattern, an age-dependent enlargement (100% penetrant in newly enclosed, but insignificant in 3 days old males) in the middle region of the testes was observed. This enlargement is not due to an overproliferation of spermatids, but rather due to the accumulation of misarranged spermatid bundles. Moreover, at the next developmental stage, coiled spermatid bundles appear to be degenerating in *dFmr1* mutant testes and thus very few individual spermatozoa are present in the mutant seminal vesicles (Zhang et al., 2004). The studies using electron microscopy showed that the basis of this degenerative phenotype is the loss of the central pair of microtubules without effects on the overall integrity of the axoneme (Zhang et al., 2004).

Utilities of Fly Model Toward Understanding the Molecular Basis of FXS

Development of *Drosophila* models for FXS has provided new avenues to understand the molecular pathogenesis of this disease. The power of fly genetics has enabled the field to identify and dissect biological pathways regulated by FMRP. For example, using two different fly models, we demonstrated that the microRNA pathway is critical for FMRP function in neural development and synaptogenesis, which provided the first link between microRNAs and human genetic diseases (Jin et al., 2007). Also the study of *dFmr1* revealed that dFmrp biochemically and genetically interacted with the adenosine-to-inosine RNA-editing enzyme dADAR, which unexpectedly linked dFmrp to the RNA-editing pathway in maintaining the proper NMJ synaptic architecture (Bhogal et al., 2011). More recently, a role for dFmrp was reported in the DNA damage response (DDR), which revealed an unexpected nuclear role of FMRP in DDR and uncovered a feed-forward mechanism by which *dFmr1* and early DDR induced by replication stress reciprocally regulate each other, thereby synergistically triggering activity of the DDR signaling cascade (Zhang et al., 2014). All these examples reflected the power of fly genetics and how much we could learn by studying the fly model of FXS.

In addition to the typical use of *Drosophila* (i.e., screening for novel genes and their mutations), the fruit fly is becoming the model of choice when a combination of gene alteration, pharmacological and functional assays of a phenotype is needed. Such a combined approach is particularly valuable in studies of complex systems, such as the CNS. It was discovered a few years ago that one of the phenotypes in *Fmr1* KO mice is the enhanced metabotropic glutamate receptor (mGluR) activity (Chapter 9). Consistent with the findings in mice, the enhanced mGluR activity was also observed in *dFmr1* mutant as well (McBride et al., 2005). More importantly, it was demonstrated that administration of various mGluR antagonists rescues the behavioral phenotypes previously reported in the fly (McBride et al., 2005), which provided the initial proof of the concept that mGluR antagonists could ameliorate some of the cognitive and behavioral deficits in human patients, and led to a series of human clinical trials.

Furthermore, *Drosophila* could also be directly used to identify small molecules that could modify the phenotypes associated with human diseases. A small molecule screening against the fruit fly model of FXS was one of such examples (Chang et al., 2008). The discovery that *dFmr1*-deficient *Drosophila* will die when they are reared on food containing increased levels

of glutamate, is consistent with the theory that loss of fragile X mental retardation protein (FMRP) disrupts the regulation of glutamate signaling. Two thousand compounds were screened against this lethal phenotype, and nine compounds were identified for their abilities to rescue the lethality, including three that implicate the GABAergic inhibitory pathway, which suggested the potential of GABA agonists for the therapeutic intervention of FXS.

CONCLUDING REMARKS

As stated earlier, a series of animal models have been developed for the FXS and these have significantly contributed to our molecular understanding of the consequences of the absence of Fmrp. The toolbox of animal models is likely to grow exponentially due to the development of novel genomic-editing technologies, most notably CRISPR/Cas9. These technologies enable a much more easily manipulation of the genome of each vertebrate or invertebrate with great precision. It is in this light of interest to mention the initiative to generate and distribute CRISPR/Cas9 rat models of autism, including FXS. Currently this rat *Fmr1* KO model is available to any qualified researcher, with minimal cost and restrictions. In summary, animal models are likely to become an even more important research tool for the FXS in the years to come.

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CHAPTER

8

RNA and Protein Targets of FMRP

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INTRODUCTION

Fragile X syndrome (FXS) is typically caused by the silencing of the *FMR1* gene, resulting in a lack of the gene product, fragile X mental retardation protein (FMRP). Elucidating the functions of this protein is critical to understanding the molecular pathology of the disorder. Nearly 25 years of research on FMRP has revealed many different functions, particularly those performed in the postsynaptic compartments of neurons. While FMRP has been found to participate in a variety of molecular processes, from mRNA transport (Bassell & Warren, 2008; Ohashi et al., 2002; De Diego Otero et al., 2002), cytoskeleton remodeling (De Rubeis et al., 2013), and even the DNA damage response in the nucleus (Alpatov et al., 2014), the most well-characterized function of FMRP is that of translational repression of a subset of mRNAs at the synapse. FMRP likely binds several hundred different mRNAs at the ribosome, preventing their local synaptic translation until required, following a specific neuronal stimulus. Upon the appropriate neuronal cues, such as signaling through the metabotropic glutamate receptor (mGluR) pathway, FMRP dissociates from these bound mRNAs, relieving the repression and allowing a burst of local synaptic translation to occur in response to neuronal stimuli. Activity-dependent protein synthesis at the synapse, which is disrupted in the absence of FMRP, is a key event in the modulation of synaptic plasticity, a process hypothesized to be the basis of memory and learning (Huber, Gallagher, Warren, & Bear, 2002; Weiler et al., 1997; Bear, Huber, & Warren, 2004). In addition to binding and repressing mRNA translation, FMRP has been shown to interact with other proteins in the brain (Pasciuto & Bagni, 2014), which appears to be completely distinct and separate from the protein's RNA-binding functions (Alpatov et al., 2014; Myrick et al., 2015a). These varied and differentiated binding properties of FMRP are facilitated by multiple conserved functional domains that have been identified throughout the protein.

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The ability of FMRP to bind specific mRNA targets is mediated by three KH domains, as well as an arginine glycine–rich domain (RGG-box), each of which is conserved and has been characterized as an RNA-binding domain within FMRP, as well as other RNA-binding proteins (RBPs). The KH2 domain of FMRP has been studied extensively, due in part to the discovery of a missense mutation in the domain that renders FMRP nonfunctional, and was the underlying cause of a severe form of FXS (DeBoulle et al., 1993). The KH1 domain, as well as the recently described KH0 domain (Myrick, Hasimoto, Cheng, & Warren, 2015b), has not been as highly studied with regard to its functionality. The RGG-box domain has been reported to have a strong affinity for G-rich and structured RNA sequences, namely G-quadruplexes (Ramos, Hollingworth, & Pastore, 2003). In addition to RNA-binding domains, FMRP also contains two Agenet (also known as Tudor) domains that mediate interactions with other proteins, such as the recently characterized association of FMRP with methylated histones (Alpatov et al., 2014). Overall, FMRP is a multidomain, multifunctional protein that binds and modulates the activity of its mRNA and protein ligands.

As FMRP has been estimated to associate selectively with approximately 4% of all neuronal mRNAs (Ashley, Wilkinson, Reines, & Warren, 1993; Brown et al., 2001), identification of these mRNA partners has been a major research priority in the field. Knowledge of FMRP targets would not only help elucidate the molecular pathways that are affected by the absence of FMRP, which would help further characterize and define the role of FMRP in synaptic function, learning, and memory, but will also help identify possible therapeutic approaches. In addition to this, putative FMRP target genes appear to have a striking overlap with genes associated with autism spectrum disorders (ASDs) and schizophrenia (Folsom, Thuras, & Fatemi, 2015; Fernandez, Rajan, & Bagni, 2013; Tang et al., 2015; Fromer et al., 2014; Purcell et al., 2014; Iossifov et al., 2012; Darnell et al., 2011). This correlation has helped fuel the search for, and confirmation of, genuine FMRP targets. Given the extremely useful information that can be gained through identification of FMRP-binding partners, this chapter focuses on the mRNA and protein molecules that interact with FMRP, the experimental approaches that have been used to define these ligands, and the factors that specify FMRPs binding.

APPROACHES TO DEFINING THE RNAs/PROTEINS ASSOCIATED WITH FMRP

FMRP was characterized as an RBP soon after the discovery of the *FMR1* gene in 1991 (Siomi, Siomi, Nussbaum, & Dreyfuss, 1993). Given this functional knowledge and the importance of FMRP for proper synaptic development and functioning, a major research endeavor has been, and will continue to be, the identification of the molecular binding partners of FMRP. Identification of FMRP ligands has helped to define pathways affected by the absence of the protein and will aid in directing future research priorities. Numerous approaches have been employed to try and establish a catalog of FMRP binding targets, including methods at the molecular and cellular levels, as well as computational studies of the target datasets generated in the laboratory. Each approach has unique advantages and drawbacks, and each adds novel information and supports the ultimate goal of identifying genuine FMRP targets.

Molecular Approaches

The first high-throughput, large-scale study attempting to identify specific mRNA transcripts interacting with FMRP used a coimmunoprecipitation technique followed by microarray interrogation (RIP-Chip; Brown et al., 2001). This study identified 432 mRNAs in brain that were associated with FMRP, more than half of which showed abnormal translational profiles in FXS patient cells. This work also established a clear correlation between the absence of FMRP and aberrant translational control of a subset of brain mRNAs, which had been hypothesized (Jin & Warren, 2000), but unproven until that point. This seminal study provided the first robust list of mRNAs that are targeted by FMRP and also display an abnormal translational profile. Although endogenous FMRP from mouse brain tissue was assayed to determine mRNA targets in these experiments, this in vitro approach may capture interactions that are not physiologically relevant due to the decompartmentalization of FMRP and mRNAs during sample processing. In addition, while neurons are the main point of interest regarding FMRP function, whole brain tissue used in these studies is comprised of many cell types, which may identify interactions that are valid, but are of less interest. Thus, some of the mRNAs characterized via this approach may include targets that are not present under physiological conditions and likely identifies targets of FMRP in multiple cell types, including but not limited to neurons.

To combat the shortcomings of a whole brain, in vitro approach, a novel technique called antibody-positioned RNA amplification (APRA) was developed to identify mRNAs associated with FMRP in vivo using hippocampal neurons isolated from mouse brain (Miyashiro et al., 2003). By covalently linking a degenerate oligonucleotide sequence to an FMRP antibody, Miyashiro et al. (2003) applied this antibody to primary neuronal cell cultures. The antibody locates FMRP and as a result positions the oligonucleotide near mRNAs that are at a very short distance to the FMRP molecule. A subsequent in situ transcription reaction enriched sequences that are within the reach of the oligonucleotide, which suggests a direct association between FMRP and the transcribed RNA. The mRNAs transcribed through this process were then used to interrogate cDNA microarrays containing a subset of known neural genes to identify FMRP interactors. This led to the identification of ~1000 genes that were presumed to be associated with FMRP via the APRA method. A small subset of these (83) were selected for in vitro experimentation to confirm the interaction. Approximately 60% of this subset was validated as bound to FMRP in vitro, which if true within the larger dataset, suggests that ~600 genes are targets of FMRP in vivo. The APRA technique has the advantage of observing interactions in the specific cells of interest; in this case, hippocampal neurons. The APRA technique likely identifies genuine in vivo interactions, though the assay is performed in cultured cells, which requires some tissue processing and cell maturation ex vivo before experimentation. Additionally, the novelty of this approach is arguably its biggest drawback; the method remains largely untested and has not been used for the identification of targets for any other RBPs in the literature.

The inability to reliably identify in vivo RNA:protein interactions on a large scale in the tissue of interest hindered the identification of targets for all types of RBPs, including FMRP. The advent of a technique called crosslinking immunoprecipitation (CLIP) made it possible to "freeze" in vivo RNA:protein interactions in tissue, which allows for stringent purification without disruption of the associations (Ule et al., 2003). Furthermore, after

the crosslinking process, the unbound RNA is enzymatically digested, while RNA directly bound to a protein is protected from the enzyme activity, allowing the determination of the location of binding within the transcript. The laboratory where CLIP was developed used the technique in conjunction with high-throughput sequencing (HITS-CLIP) to isolate and identify mRNAs associated with FMRP in mouse brain (Darnell et al., 2011). HITS-CLIP was applied to mouse brain tissue slices from wildtype (WT) and *Fmr1* knockout (KO) models and two different FMRP antibodies were used to isolate the FMRP:mRNA crosslinked complexes. Applying rigorous controls and filtering methods resulted in a set of 842 transcripts that were associated with ribosome-bound FMRP in mouse brain. Many of the genes identified as FMRP targets overlap significantly with other gene sets of interest. For example, 32% of the 842 FMRP targets were found to be members of the postsynaptic proteome. Additionally, 62% of the mGluR5 receptor complex genes and 34% of the NMDAR receptor complex genes were FMRP targets. The FMRP target set defined by Darnell et al. (2011) also significantly overlapped with a number of genes associated with ASD, providing a molecular link to support the observed clinical overlap between the two conditions. In addition to identifying genes bound by FMRP, this study revealed a potential mechanism by which FMRP regulates translation. By mapping the HITS-CLIP data to the mouse genome, it was revealed that most FMRP binding occurs in the coding sequence (CDS) of its mRNA targets (66% of FMRP CLIP tags were in the CDS). Surprisingly, within the CDS of individual genes or all FMRP target genes collectively, there was no apparent pattern as to the position that FMRP was bound; the CLIP tags were dispersed in a seemingly random fashion throughout the CDS. Additionally, there was no identifiable consensus sequence or structural motif in this dataset that might serve as a molecular docking station for FMRP and the data didn't support any previously postulated target motifs (which will be discussed later in this chapter). Based on these findings, the authors postulated and presented several different experimental data points showing that FMRP is bound to transcripts on which ribosomes are stalled, and FMRP was essential for this stalling. A disadvantage of this approach is similar to that of the Brown et al. (2001) approach; tissue slices of the whole brain were used, which may identify FMRP:mRNA interactions that take place in cell types other than neurons. However, this landmark study provided the first in vivo catalog of FMRP-binding partners and revealed that FMRP seems to be somewhat arbitrarily bound across the CDS regions of its targets and may regulate translation of these bound mRNAs by ribosome stalling.

A variant of CLIP called photoactivatable ribonucleoside–enhanced crosslinking immunoprecipitation (PAR-CLIP) was developed to improve upon the relatively low efficiency of RNA:protein crosslinking in standard CLIP (Hafner et al., 2010). The method requires the incorporation of a nucleoside analog [such as 4-thiouridine (4SU)] in cultured cells and uses 365-nm UV to crosslink rather than the 254-nm UV in the original CLIP and HITS-CLIP procedures. The combination of these two modifications improves RNA recovery by over 100-fold. The PAR-CLIP method was applied to HEK293 cells, a human kidney cell line, that had been engineered to stably express FMRP, and high-throughput sequencing was used to identify bound transcripts (Ascano et al., 2012). The researchers performed PAR-CLIP on isoform 1 and isoform 7 of FMRP, as well as the FXR1P and FXR2P paralogs, and the well-studied I304N FMRP mutant. In agreement with the HITS-CLIP data, Ascano et al. (2012) found that a majority of FMRP is bound to the

coding region of targets. In stark contrast, however, the PAR-CLIP technique isolated and identified more than 6000 transcripts associated with FMRP. RIP-Chip assays were performed to support the PAR-CLIP data and rank the FMRP targets by enrichment status. Using this method, the authors found more than 900 target genes that were identified by PAR-CLIP and at least twofold enriched by RIP-Chip. While an overlap analysis between this dataset and others was not performed, it did include many targets that overlap with ASD-associated genes, similar to previous studies. The researchers were able to identify two enriched sequence motifs within the sequence data, which will be discussed in detail later in this chapter. In addition, a useful consequence of the PAR-CLIP crosslinking process at 365 nm with incorporated 4SU is the mutation of uridine to cytosine at locations where the RNA is in direct contact with a protein. Subsequent bioinformatic analyses of mRNAs isolated by PAR-CLIP enabled the identification of FMRP:protein contact points with nucleotide-level resolution, which provided a novel interaction map at a very high resolution for the associations that were captured. The major drawback of this study was the use of human kidney cell lines as the tissue of choice. Although it was a human-derived cell line, as opposed to the mouse brain tissue or cultured neurons used in the other studies, the transcripts expressed in these kidney cells represents only about two-thirds of the transcripts expressed in neural tissue, equivalent to thousands of genes that are absent from the model system (Suhl, Chopra, Anderson, Bassell, & Warren, 2014). Each transcript expressed in the brain, but not the cell line, represents a potential interaction that was not analyzed by the PAR-CLIP method. Additionally, FMRP may interact with different transcripts in different cell types.

In each of the reports discussed here, several hundred to several thousand mRNA targets were found to be associated with FMRP. In contrast to these findings, a very recent CLIP study in cultured neurons found that FMRP was significantly associated with just a handful of transcripts, where only seven mRNAs were enriched twofold or more when comparing WT neurons to *Fmr1* KO neurons (Tabet et al., 2016). One transcript, diacylglycerol kinase kappa (*Dgkκ*), was enriched by FMRP CLIP approximately eightfold more than the next most highly enriched gene. *Dgkκ* is a member of the DGK protein family, which act as master regulators of the diacylglycerol and phosphatidic acid signaling pathways, and are intertwined with the mGluR and translational control pathways. Interestingly, additional experiments by Tabet et al. (2016) showed that FMRP positively regulates the translation of *Dgkκ*, in contrast to its typical function as a negative regulator of translation. Genetically silencing *Dgkκ* in cultured neurons and in live mouse models supported these findings, as electrophysiological experiments showed altered synaptic plasticity and FXS-like phenotypes were observed in mouse behavioral studies. Additionally, the silencing of *Dgkκ* led to long, immature dendritic spines in hippocampal tissue; a hallmark morphological feature in FXS mouse models and humans. These data suggested a novel mechanism of neuron dysfunction in the absence of FMRP whereby *Dgkκ* levels are reduced, causing a disruption in neuronal gene regulation. These findings are very intriguing and run counter to several of the axioms in the field of FMRP research, such as the canonical role of FMRP as a translational suppressor. Surprisingly, none of the previous studies of FMRP targets identified *Dgkκ* as an interactor, which may be due to a variety of reasons (see Tabet et al., 2016 for hypotheses). A main focus of follow-up research will likely revolve around the role of FMRP as a translational activator of *Dgkκ*, the location of the FMRP:*Dgkκ* interaction, and the nature of the interaction (i.e., what motif within *Dgkκ* is

being targeted by FMRP). Overall, this study presented potentially field-shifting data on how the loss of FMRP affects the translation of neuronal genes and provides a novel mechanism of dysfunction for further investigation.

Cell Biology and Proteomic Approaches

Since FMRP is an RBP, its function is tightly linked to the translation of the mRNA cargoes that it binds. As a result, significant efforts have been made to identify, measure, and characterize the proteins whose translation is regulated by FMRP. Work along these lines began first with establishing that FMRP levels impacted protein synthesis rates. This question has been approached in several different model systems ranging from cell culture to rodent models. A variety of approaches have been applied to this question, including radioactive metabolic labeling to more modern approaches using light and heavy isotopes containing amino acids for in vivo incorporation of a label into newly synthesized proteins for mass spectrometry (MS)–based proteomics.

One of first reports linking FMRP expression to cell biological effects on translation came from studies in *Xenopus laevis* oocyte preparations in which the authors demonstrated that microinjection of FMRP mRNA suppressed the translation of several mRNA transcripts that were known to be bound by homodimerized FMRP protein (Laggerbauer, Ostareck, Keidel, Ostareck-Lederer, & Fischer, 2001). In another early study, Li et al. (2001) showed that FMRP suppressed the translation of specific transcripts rather than acting as a global suppressor of translation. Using the cell-free experimental preparation rabbit reticulocyte lysate (RRL), they showed that FMRP suppressed the translation of the parathyroid hormone transcript, which binds FMRP, but not a beta-globin transcript, which does not. They confirmed the specificity of FMRP to these observations by showing that the removal of the FMRP-binding site abolished the inhibitory effect of FMRP activity (Li et al., 2001). Additional evidence for selective regulation of FMRP-containing granules was shown by experiments in which RNA-containing granules form under FMRP-deficient conditions, and subsequent formation of granules were enhanced in response to mGluR-induced protein synthesis (Aschrafi, Cunningham, Edelman, & Vanderklish, 2005). This pointed to a role for FMRP in translational control mechanisms under specific conditions of neuronal stimulation or development.

A role for FMRP in translational control was also shown in a study by Sharma et al. (2010) using the *Fmr1* KO mouse model. They showed that FMRP loss in KO mice resulted in upregulation of the mechanistic (or mammalian) target of rapamycin (mTOR) pathway in the hippocampus. Additionally, they showed enhanced levels of interaction between the cap-dependent initiation factors, eIF4G and eIF4E in *Fmr1* KO mice (Sharma et al., 2010). Some of these findings were later confirmed from western blot analyses performed from postmortem brain tissues obtained from FXS patients (Hoeffer et al., 2012). Another study did not identify changes in mTOR signaling in FMRP KO mice but instead showed that extracellular regulated kinase (ERK) signaling was altered (Osterweil, Krueger, Reinhold, & Bear, 2010). While it is not clear why these groups observed different signaling effects, it is evident that the signal transduction pathways affected by FMRP loss can impact translational control. As FMRP is likely to bind and interact with a subset of mRNAs only, this supports the notion that

FMRP modulates the translation regulation of specific transcripts under compartment- and stimuli-specific conditions. Thus, identifying these proteins, as well as regulatory pathways controlling their FMRP-dependent expression would be of great benefit to the understanding of FXS and assessing the role of FMRP on protein synthesis.

One of the first studies to examine global translation in an FXS model measured cerebral protein synthesis (CPS) using radioactive labels in *Fmr1* KO mice at early age time points. While they showed that CPS declined as their experimental mice aged overall, they found evidence for brain regional–specific enhancement of CPS in *Fmr1*-null mice. These in vivo experiments provided support for the notion that FMRP is a suppressor of translation in brain (Qin, Kang, Burlin, Jiang, & Smith, 2005). Additional studies using metabolic radioactive labeling have confirmed enhanced protein synthesis rates in brain tissues obtained from FXS model mice (Dolen et al., 2007; Osterweil et al., 2010) and from FXS premutation knockin mice (Qin et al., 2014). Findings from FXS rodent models have recently been confirmed in tissues obtained from humans. Kumari et al. (2014) used H3 leucine incorporation to measure protein synthesis in human fibroblasts isolated from FXS patients. They found elevated protein synthesis and upregulation of the mTOR signaling pathway also (Kumari et al., 2014).

The aforementioned studies confirmed global elevated protein synthesis rates in the absence of FMRP, but did not address the central question of which specific proteins are affected either directly by FMRP removal or as a consequence of aberrant regulation due to FMRP loss. One of the first studies to address this question was performed using MS to identify proteins perturbed by FMRP removal using stable isotope labeling with amino acids in cell culture (SILAC). SILAC is a nonradioactive labeling method that is based on MS and detects differences in protein abundance among samples using differences in identical peptides based on their mass differences. Two populations of cells can be labeled with amino acids, with one population containing normal isotopes, while a second population is labeled with amino acids containing heavy isotopes (i.e., 13C or 15N). Using SILAC, Liao, Park, Xu, Vanderklish, and Yates (2008) detected 132 proteins with altered expression comparing *Fmr1* KO and WT cortical neurons. Importantly, these proteins included ones with direct relationships to synaptic function and transmission, epilepsy, and autism (Liao et al., 2008). Corroborating evidence was obtained from another study, in which isobaric tags for relative and absolute quantification (iTRAQ) labeling was used to identify protein changes in the hippocampi of *Fmr1* KO mice. Quantification using iTRAQ revealed ~20 proteins with differential expression between *Fmr1* KO and WT tissues. While the number of protein alterations found was not as extensive as the earlier SILAC study, they identified proteins involved in cell differentiation, mitochondrial function, and synaptic vesicle release (Klemmer et al., 2011). As several key presynaptic proteins were identified in this study including synaptophysin and synapsin1, they further tested the idea whether presynaptic function was altered in *Fmr1* KO. In agreement with their iTRAQ data, they found impaired paired pulse facilitation (PPF) on Schaffer collateral inputs onto CA1 hippocampal pyramidal cells in *Fmr1* KO mice as compared to WT controls. Another interesting finding was the presence of increased eukaryotic elongation factor 2 (eEF2), a potential link to enhanced protein synthesis levels observed in numerous FXS model systems. Finally, data from in vivo SILAC labeling of heterozygote *Drosophila dfmr1*$^{+/-}$ (partial

loss of *dfmr1* function) also identified some cytoskeletal interacting proteins altered under conditions of reduced *dfmr1* activity (Xu et al., 2012).

Matic, Eninger, Bardoni, Davidovic, and Macek (2014) exploited SILAC in mouse embryonic fibroblasts (MEFs) from *Fmr1*-null and WT mice to examine proteins altered under FMRP-deficiency conditions. Using SILAC they performed analyses in fibroblastic cell lines to characterize protein expression and phosphoregulation in FMRP-null conditions. Through this approach, the researchers identified expression changes in 511 proteins. Interestingly, they found roughly equal numbers of proteins that showed decreased expression compared to those that were increased. Importantly, they also detected significant and extensive alteration of phosphorylation of peptides in *Fmr1* KO fibroblasts compared to controls, with 683 peptides showing altered phosphorylation (including both up- and down-regulated phosphorylation). Proteins whose expression or phosphoregulation increased in FMRP-KO tissues included cell cycle control, p53, vasopressin, ribosomal subunits, and regulatory proteins. Reduced expression or phosphoregulation was detected in pathways affecting synaptic plasticity (long-term potentiation, long-term depression, gap junctions, and extracellular matrix interaction), axon guidance, peroxisome proliferator–activated receptor (PPAR) pathway, and lysosomal degradation. Using the Search Tool for Retrieval of Interacting Genes (STRING) database to probe for known protein–protein interactions, the group also identified several clusters of interaction in *FMR1*$^{-}$ cells. As might be expected from the proteomics data, not only cell cycle and ribosomal function were identified, but also peptides belonging to the ubiquitin/proteasome system. This may point to increases in protein turnover under conditions of FMRP loss either as compensation for increased protein synthesis or as a direct result of translational dysregulation resulting from the loss of FMRP activity. They also observed phosphoregulation and expression changes in several other cellular signaling pathways. They confirmed altered activity in the MEK/ERK pathway in the absence of FMRP, with decreased phosphorylation of ERK1/2. Although they did not observe significant exaggerated mTOR activity (as assayed by phosphoregulation) in FMRP-null MEFs, they exhibited differential expression of several proteins in the mTOR pathway, such as Rictor, PTEN, and S6. Novel pathways were also revealed with dysregulated expression of Wnt and p53 signaling cascades, as well as pathways previously shown to be involved in ASDs, but not with FXS.

Finally, a recent study using stable isotope labeling in mice (SILAM) measured protein expression in differences in brains of *Fmr1* KO and WT mice across age (Tang et al., 2015). Importantly, they determined that developmental time points played a key role on the effects of FMRP loss on protein expression with younger-aged mice showing greater numbers and levels of dysregulated protein expression. In concordance with other studies, they identified several proteins related to synaptic function, transmission, and structure as altered in the *Fmr1* KO brain. Confirming and extending mechanistic studies in the *Fmr1* KO mouse, they characterized changes in an array of scaffolding and actin cytoskeleton–interacting proteins. Especially interesting was the fact that these scaffolding molecules were known to interact within the postsynaptic compartment to regulate the localization and surface expression of receptors and included proteins such as Homer1, Shank-family proteins, and MAGUK family members. Proteomic approaches are critical to further elucidating the molecular mechanism perturbed in FXS because while determining the identity of mRNA species interacting with FMRP will reveal potential translational targets of

FMRP, identification of perturbed proteins will reveal both direct and indirect effects of the loss of FMRP activity.

Computational Approaches

Several of the approaches described aimed to characterize mRNA ligands of FMRP and produced a set of genes that were putative FMRP interactors. However, the level of concordance between datasets had not been thoroughly assessed to determine the relative precision of each technique. While each method identified some number of genuine FMRP targets, the datasets are all likely to be only a subset of the true catalog of FMRP:mRNA interactions due to a number of factors, including assay limitations and stochastic experimental variation. A group of consensus target genes (i.e., those identified in all studies) would provide a powerful list of the most reproducible FMRP:mRNA interactions and a higher level of confidence in their authenticity. Using computational methods, the four largest studies regarding FMRP mRNA targets were analyzed to identify the genes most consistently associated with FMRP (Suhl et al., 2014). Each individual dataset analyzed, of which there were five in total (Brown et al., 2001; Darnell et al., 2011; Miyashiro et al., 2003; Ascano et al., 2012, which included both PAR-CLIP and RIP-Chip assays), was compared for FMRP target concordance in a pairwise manner. The Miyashiro et al. (2003) dataset, which utilized the APRA approach discussed earlier, did not show a high degree of overlap with any of the other datasets, whereas all other comparisons showed significant overlap in putative FMRP target genes; the Brown et al. (2001) and Darnell et al. (2011) datasets revealed a particularly high level of overlap. The genes common to the Brown et al. (2001), Darnell et al. (2011), and Ascano et al. (2012) RIP-Chip datasets were assessed to identify the most stringent set of FMRP targets found in all studies [note that Ascano et al. (2012) RIP-Chip includes genes that were identified by PAR-CLIP *and* showed at least a twofold enrichment by RIP-Chip analysis]. This multidataset overlap revealed 53 genes that were identified as FMRP targets in all datasets. This consensus list contains several genes previously identified as FMRP targets (e.g., MTOR and TSC2); several genes not well established as FMRP targets, but of potential interest based on function (e.g., PI4KA); and those known to be associated with ASD, for example CHD8 (Bernier et al., 2014). As with previous studies of FMRP targets, there was a highly significant level of overlap with this consensus set and genes associated with ASD. One weakness of analyzing the concordance of multiple studies in this way is that if just one dataset lacks a target gene, it is excluded as a top candidate despite potentially strong evidence in multiple other studies. To address this, the authors took advantage of the fact that three of the datasets included enrichment information (i.e., how much of a certain target gene was recovered relative to other genes). By using a rank aggregation method, a target list of the top 40 most highly enriched genes from Brown et al. (2001), Darnell et al. (2011), and Ascano et al. (2012) RIP-Chip datasets that are involved in the development of FXS, autism, or intellectual disability was generated (Table 8.1). This approach allowed targets that may be missing in one dataset, but highly enriched in the other two datasets, to be included in the list of top candidates, and identified FMRP target genes that are associated with neurodevelopmental disorders and intellectual disability. Overall, this study represents the first thorough analysis of multiple FMRP target datasets and has helped define a collection of genes that have been consistently identified by different techniques.

TABLE 8.1 List of Highly Enriched FMRP Target Genes Associated with Neurodevelopmental Disorders.

Rank	Gene	Rank	Gene
1	TSC2	21	ATP2B2
2	MTOR	22	ARHGEF7
3	NAV1	23	COBL
4	CREBBP	24	ALS2
5	EHMT1	25	MAPK8IP1
6	TRIO	26	PRPF8
7	DST	27	TRAPPC10
8	ANKRD11	28	CUX1
9	CYFIP2	29	TSHZ1
10	ITPR1	30	CIC
11	SMARCA4	31	FOXK2
12	SKI	32	HERC1
13	ANK3	33	MYT1L
14	CHD8	34	PI4KA
15	HERC2	35	BAI2
16	BCR	36	GPRIN1
17	BSN	37	MAST4
18	SPTAN1	38	LPHN1
19	SCAP	39	JAK1
20	HCFC1	40	FASN

FMRP-BINDING DETERMINANTS

Knowledge of the genes that are translationally regulated by FMRP is extremely useful in determining the molecular pathways involved in FXS and other related neurodevelopmental disorders. However, a finer resolution of the binding kinetics of FMRP and its ligands would provide precise information about the nature of the interactions and help to better define the true compendium of FMRP targets. Information about the sequence or structural recognition motifs bound by FMRP would also provide insight into the structure and function of the variety of binding domains within FMRP. As such, significant effort has been put forth to identify the binding elements that FMRP recognizes within target genes.

Structural RNA Motifs Targeted by FMRP

One of the first recognized and most well-studied binding motifs of FMRP is an RNA secondary structure called a G-quadruplex or G-quartet (Darnell et al., 2001; Schaeffer

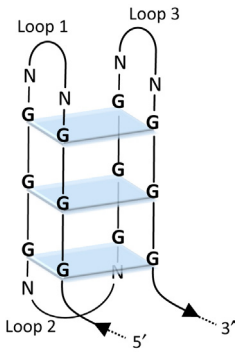

FIGURE 8.1 **An example of a G-quadruplex structure, a fragile X mental retardation protein (FMRP)–binding motif.**

et al., 2001). These structures are formed by several sets of tandem guanine (G) nucleotides in a linear sequence that fold to create stacked, planar tetrads of G nucleotides, with intervening loop sequences of variable length (see Fig. 8.1 for an example of a G-quadruplex structure).

FMRP binds to this structure via its RGG domain (Darnell et al., 2001; Ramos et al., 2003; Vasilyev et al., 2015). The consensus G-quadruplex sequence bound by FMRP determined by Darnell et al. (2001) was four DWGG motifs with zero to two interspersed nucleotides as loop sequence between them (where D = any nucleotide except C; W = U or A). Interestingly, mutational analysis suggested that it's not simply the G-quadruplex structure that binds FMRP, but a specific sequence component of the interaction is required as well (Darnell et al., 2001). More recent studies of FMRPs interaction with the G-quadruplex have solved the structure of the RGG domain with a bound G-quadruplex RNA, which revealed new insights into the conformation of the RGG domain while bound to a G-quadruplex and confirmed the requirement of specific sequences/nucleotides (Vasilyev et al., 2015). These studies implicated both sequence and structural elements in FMRPs recognition of target mRNAs via the RGG domain.

The I304N mutation is located within the KH2 domain of FMRP, which is one of three KH domains within the protein. This mutation, which is the cause of severe FXS in a patient, was found to abrogate the RNA-binding and ribosome-association properties essential to FMRP function (Siomi, Choi, Siomi, Nussbaum, & Dreyfuss, 1994; Feng et al., 1997a). Thus, there was a strong interest in identifying the target mRNAs or recognition motifs of this domain. Through in vitro RNA selection experiments, a loop–loop pseudoknot structure, termed the FMRP kissing complex, was identified as a recognition element of FMRP (Darnell et al., 2005). The high-affinity selection process, which utilized full-length FMRP, as well as an isolated KH1 and KH2 domain fragment, selected two sequences that comprised ~20% of all recovered RNA sequences after several rounds of an in vitro selection, indicating they were the most tightly bound sequences. Interestingly, these two FMRP-associated candidate RNAs shared significant homology at the 5′ and 3′ ends, suggesting a potential consensus sequence for FMRP binding. Through a series of experiments, it was revealed that the structural context was crucial for FMRP association to the targets and each of the sequences had the capacity to

form the kissing complex structure. As the KH2 domain is essential for FMRP functioning, the determination of a KH2 domain–binding element was very promising. However, to date there have been no FMRP targets identified that contain a similar sequence or predicted kissing complex structure (Anderson, Chopra, Suhl, Warren, & Bassell, 2016).

Sequence Motifs Targeted by FMRP

Other studies have focused on identifying linear sequence motifs that act as recognition sequences of FMRP. Two independent studies reported that FMRP-bound mRNAs with U-rich motifs; one study used a cDNA in vitro selection approach (Chen, Y, Seto, Liu, & Toth, 2003), while the other study used a yeast three-hybrid system (Dolzhanskaya, Sung, Conti, Currie, & Denman, 2003). More recent approaches include PAR-CLIP (Ascano et al., 2012) and a technique called RNACompete, which assessed the binding sequences of over 200 RBPs in vitro, including FMRP (Ray et al., 2013). Using the PAR-CLIP method in a human kidney cell line identified ACUK and WGGA (where K = T or G; W = A or T) as sequences bound by FMRP, whereas the RNACompete technique found GACR as the core binding motif (where R = G or A) of both human FMRP and *Drosophila* dFmrp. Each of these studies identified conflicting recognition sequences, leaving the true recognition sequence, if any, uncertain and bringing into question the role of RNA sequence in FMRP binding.

To help determine if any of the proposed sequences are authentic binding sites for FMRP, two unbiased bioinformatic analyses were performed (Suhl et al., 2014; Anderson et al., 2016). These studies reanalyzed the available raw data that accompanied several FMRP target datasets, namely Ascano et al. (2012), Brown et al. (2001), Darnell et al. (2011), and Miyashiro et al. (2003), to determine if any of the proposed motifs were significantly enriched among the most reliable FMRP targets and CLIP sequences.

Suhl et al. (2014) examined each dataset, including the high-confidence consensus datasets generated from the shared FMRP target genes identified in the Ascano et al. (2012), Brown et al. (2001), and Darnell et al. (2011) studies for the proposed binding sequences ACUK, WGGA, and GACR. The average number of each motif per kilobase in the FMRP target genes was compared to the average number of each motif per kilobase in all other non-FMRP target genes. These comparisons revealed no enrichment of the ACUK motif in FMRP targets, suggesting it likely does not have a major influence on FMRP binding. The GACR motif was highly enriched in nearly every FMRP target set analyzed, including all consensus gene sets, and indicates that GACR is a likely contributor to FMRP target recognition. The WGGA sequence was modestly enriched in some of the datasets, but not all of them, leaving the validity of the motif as a recognition sequence in question. However, the similarity between the WGGA motif and the DWGG motif previously identified as a G quadruplex–forming motif targeted by the RGG box domain of FMRP (Darnell et al., 2001) spurred deeper investigation into the binding of FMRP to the WGGA sequence. Instead of focusing on the total number of WGGA sequences present in FMRP targets, the authors analyzed the distribution of WGGA sequences in the target genes, postulating that clustered WGGA motifs may be able to form G-quadruplexes and serve as a structural recognition motif for FMRP. This analysis showed there was a tremendous enrichment of closely clustered WGGA sequences in FMRP target genes compared to the rest of the genes in the genome. Of note, 53% of the shared target genes from the Brown et al. (2001), Darnell et al. (2011), and Ascano et al. (2012) RIP-Chip consensus

list had at least one WGGA cluster, compared to 15% in other genes in the genome. These results suggest that GACR is an FMRP-recognition sequence, and highly clustered WGGA motifs may form G-quadruplex structures recognized by FMRP.

The analysis by Anderson et al. (2016) built upon these results by focusing on the binding sites themselves, rather than the gene level study performed by Suhl et al. (2014). To do this, two FMRP CLIP datasets (Ascano et al., 2012; Darnell et al., 2011) were compared to identify sites where both studies observed FMRP binding. By identifying a set of consensus CLIP tags between these studies, a high-resolution assessment of FMRP-binding determinants was performed. First, an unbiased motif discovery analysis of the shared FMRP CLIP tags was performed to determine motifs that were enriched within these short sequences. The most enriched motif found in this analysis was TGGA, which is one of the possibilities of WGGA. Two other highly enriched motifs, AGGA and TGGT, were among the top 10 most highly enriched sequences. In fact, there were 12 motifs containing GG out of the 26 total motifs identified, supporting WGGA, and more broadly G dinucleotides, as a binding sequence of FMRP. Other enriched motifs within the shared binding sites included GAC and a novel motif TAY (where Y = C or T). Of note, ACUK and U-rich motifs were not identified as highly enriched through this analysis. Investigation of potential structural motifs within the shared CLIP sites was also performed. The kissing complex structure was not significantly enriched in shared FMRP-binding site data compared to random gene sequences of the same length. When the data were searched for clustered WGGA sequences that may represent G quadruplex–forming motifs, there was no enrichment of these clusters in the shared binding sites. Reasoning that the G-quadruplex may not actually be bound by FMRP, but may act as a structural hindrance that stalls FMRP and aids in the repression of translation, the authors analyzed whether WGGA clusters formed just downstream of the binding sites and again found no enrichment. Despite highly clustered WGGA sequences in FMRP target genes, there was no detectable clustering at the binding site level, leaving the role of WGGA clusters in FMRP-binding site recognition unresolved. The shared CLIP tags were found to be distributed throughout the CDS, confirming what each study had observed independently, and also identified a subset of sites in the 3'UTR of target genes that were identified by both studies. Through an unbiased motif search of shared CLIP tags, this study supported WGGA, and dinucleotide G's in general, and GAC as sequence elements involved in FMRP-binding site recognition and confirmed the finding that FMRP binds across the CDS of target genes.

Non-RNA FMRP Interactions

Binding a specific set of neuronal mRNAs and suppressing their translation at the synapse until the appropriate neuronal activity signals occur is the most well-studied function of FMRP. However, FMRP also has the ability to bind molecules other than mRNA and can influence a wide range of processes in a variety of cellular compartments, including transcription in the nucleus, ion channel regulation in the presynaptic space, and RNA interference in the cytoplasm. FMRP has also recently been shown to bind to chromatin and participate in the DNA-damage response. These functions of FMRP shed new light onto the pathology of the disorder and draw attention to new avenues of research for the study and treatment of FXS.

FMRP has been found to interact with a variety of proteins in the cytoplasm and the nucleus, both directly and indirectly, and carries out a number of different functions

[see SnapShot article by Pasciuto and Bagni (2014) for an overview]. Proteins, such as the FMRP paralogs: FXR1P and FXR2P, as well as the NUFIP and CYFIP proteins have been known to associate with FMRP (Schenck, Bardoni, Moro, Bagni, & Mandel, 2001; Bardoni et al., 2003; Bardoni, Schenck, & Mandel, 1999), though the functional significance of some of these interactions is still not well understood, particularly those that take place in the nucleus. FMRP has also been implicated in the RNA interference pathway through its interaction with the RNA-induced silencing complex (RISC) proteins (Ishizuka, Siomi, & Siomi, 2002; Jin et al., 2004), which is a potential mechanism by which FMRP regulates translation of its target mRNAs (Muddashetty et al., 2011; Kenny et al., 2014). However, the nature of the interaction of FMRP, the RISC machinery, and target mRNAs is not completely understood.

Novel and surprising functions of FMRP relating to its non-mRNA interactions have recently been discovered. Several studies have identified unique features of FMRP previously unknown that have compelled a shift in mindset regarding the typical mRNA transport and synaptic translational regulation functions of FMRP. One example of this is the discovery of FMRP's ability to regulate neurotransmitter release from the presynaptic side of a neuronal connection (Deng et al., 2013). This is achieved via an interaction between FMRP and the large-conductance calcium-activated potassium (BK) channels. The BK channels are a major influencer of action potential (AP) duration, which is directly associated with the amount of neurotransmitter released during information transmission from cell to cell. Using electrophysiological, biochemical, and genetic techniques, Deng et al. (2013) showed that FMRP specifically associates with the BK channels via the β4 protein subunit, and that loss of FMRP significantly increased AP duration in hippocampal tissue slices. This was found to be specifically due to the loss of the amino-terminus of FMRP, as addition of an FMRP fragment (amino acids 1–298) to the experimental system was able to restore normal AP duration. In addition, this increase in AP duration caused by the loss of FMRP was found to be independent of the translation regulation properties of the protein, suggesting that a different function of FMRP is required for normal AP duration. As FMRP was determined to associate with BK channels, and the typical translation regulation function of the protein was not the cause of the AP duration phenotype, the researchers performed experiments that showed FMRP to be critical for the BK channel's response to calcium ion signaling. These results revealed a previously unrecognized presynaptic function of FMRP that would prove to be a very important mechanistic finding for a patient with a novel missense mutation in *FMR1*. In a different study, a developmentally delayed male patient was referred for *FMR1* repeat expansion testing due to several clinical manifestations often observed in FXS patients, such as intellectual disability and developmental delay. While repeat expansion testing showed a normal number of repeats, sequencing of the patient's *FMR1* gene revealed a novel missense variant (c.G413A; p.R138Q) at a highly conserved nucleotide and amino acid (Collins et al., 2010). This variant falls within the recently described KH0 domain, and is near the amino-terminal Agenet domains, reported to be important for protein–protein and RNA–protein interactions (Ramos et al., 2006; Zalfa et al., 2005; Kenny & Ceman, 2016). Functional analysis of the variant revealed that the typical postsynaptic functions of FMRP, such as RNA binding and polyribosome association, remained intact, the pathogenicity of this variant uncertain (Myrick et al., 2015a). However, a *Drosophila* model of the R138Q variant showed a specific presynaptic phenotype, which initiated additional studies on the effect of the R138Q variant on BK channel binding and AP duration. The researchers found that the variant disrupted the association between FMRP and BK channels, specifically at the β4 subunit. Additionally, the

R138Q variant was unable to rescue the AP duration defect when perfused into cultured *Fmr1*-deficient mouse neurons, whereas WT FMRP could. Altogether, these findings suggest that the presynaptic activities of FMRP are independent of its typical postsynaptic functions and that the loss of FMRP functionality, specifically in the presynapse, can have deleterious effects on the neural health.

FMRP is known to be present in the nucleus at a low level (Feng et al., 1997b) and is found in nucleolar granules, which contain maturing ribosomal precursor particles (Willemsen et al., 1996). This suggests that FMRP is actively shuttled from the cytoplasm to the nucleus, a phenomenon that has been observed in several cell types, including hippocampal neurons (Bakker et al., 2000). Despite clear evidence that FMRP can localize to the nucleus, the nuclear functions of the protein are poorly understood. A recent study that demonstrated novel capabilities of FMRP showed that the protein binds chromatin in the nucleus and, through this interaction, participates in the cell's DNA-damage response (Alpatov et al., 2014). The researchers found that FMRP is able to bind chromatin and this interaction was critical for FMRP's activity in the response to DNA damage, specifically in response to replication stress. The interaction with chromatin was found to be mediated by FMRPs Agenet domains, which targeted histone H3 in a methylation-dependent manner. As the previously described R138Q variant is close to the Agenet domains, the ability of this mutant to bind chromatin and contribute to the DNA-damage response was investigated. Experiments showed that the R138Q FMRP was deficient in its ability to bind chromatin and unable to aid in the response to DNA damage, as evidenced by the higher number of incidents of incomplete DNA repair observed with R138Q FMRP present. Similar to the Deng et al. (2013) and Myrick et al. (2015a) studies, the ability of FMRP to bind chromatin or participate in the DNA-damage response was independent from its canonical translational regulation and mRNA transport functions. Additionally, these chromatin binding and damage response activities take place in the nucleus, which distinguishes important functions of FMRP where very little was previously known.

Together, each of these studies broadens the number of known FMRP functions outside of the well-studied mRNA-binding activities at the synapse. Given the dearth of data regarding nuclear and presynaptic activities of FMRP and the interactions between molecules other than mRNA, exciting discoveries regarding these functions within previously overlooked cellular compartments are on the horizon and will help to better define FMRP.

Conclusions

The binding partners, recognition motifs, and functional domains of FMRP are highly varied and complex. FMRP has long been categorized as an RBP, though the number of recognized functions performed and where these functions are executed is on the rise. While there are several promising FMRP recognition motifs and putative mRNA targets, an overall lack of consensus between the largest FMRP target datasets highlights the need for further investigation of these topics. As each of the studies used a different approach for defining these features, variability between the datasets is inherent and each likely captures only a subset of true mRNA interactors of FMRP. The computational approaches identify an even smaller subset than each of the independent studies, though they are more likely to be true FMRP targets because of their reproducibility across different techniques, laboratories, and time (see Table 8.2 for a summary of the approaches, findings, and advantages/drawbacks of each study).

TABLE 8.2 Approaches Used to Identify mRNA Targets of FMRP

Study; journal	Technique	Major findings	Technique advantages	Technique disadvantages
Brown et al. (2001); Cell	RIP-Chip	432 Gene targets of FMRP; first empirical evidence of translational misregulation in absence of FMRP	Performed using mouse whole brain tissue	Immunoprecipitation is an in vitro approach; lysate contains all cell types in brain
Miyashiro et al. (2003); Neuron	APRA	~1000 Gene targets of FMRP, 83 tested by confirmatory studies	In vivo technique performed in cell type of most interest (neurons)	Untested technique; although in vivo, performed in cell culture
Darnell et al. (2011); Cell	HITS-CLIP	842 Gene targets of FMRP; FMRP bound throughout CDS of gene targets; proposed ribosome stalling as mechanism of translational suppression by FMRP	In vivo technique on mouse brain tissue; crosslinking freezes RNA:protein interactions and allows stringent conditions to eliminate background interactions	In addition to assaying in neurons, other brain cell types are likely included in analyses
Ascano et al. (2012); Nature	PAR-CLIP and RIP-Chip	Over 6000 gene targets of FMRP, ~900 confirmed by RIP-Chip with greater than twofold enrichment; identification of two sequence motifs bound by FMRP (ACUK and WGGA)	In vivo technique with very strong crosslinking procedure; performed in human cells	Performed on vector-expressed FMRP in a kidney cell line
Ray et al. (2013); Nature	RNACompete	Identification of a sequence motif bound by human FMRP and *Drosophila* dFmrp (GACR)	Competitive nature of the assay ensures the capture of sequences with high affinity for FMRP	Used FMRP KH domain fragment; does not detect interactions that require secondary structure
Suhl et al. (2014); Human Molecular Genetics	Computational analysis; gene level	Creation of consensus gene targets of FMRP identified in several different studies; confirmed recognition motifs as enriched (WGGA and GACR) and refuted recognition motif as enriched (ACUK); postulated distribution of WGGA sequences is important to FMRP binding	Overlap of four datasets allows for high confidence in consensus gene targets of FMRP; the high confidence consensus lists provide consistent gene targets to test enrichment of putative recognition motifs	Likely excludes true gene targets of FMRP; did not perform motif discovery analysis
Anderson et al. (2016); Nucleic Acids Research	Computational analysis; binding site level	Creation of consensus CLIP sequences bound by FMRP in two different studies; confirmed recognition motifs as enriched in consensus CLIP sequences (GAC and GGA); identified novel recognition sequence (TAY); G-quadruplex motif not enriched in consensus CLIP sequences	Overlap of two CLIP datasets allows for higher confidence in FMRP-binding sites; the high-confidence consensus-binding sites provide consistently identified sites to test enrichment of putative recognition motifs	Only two CLIP studies to determine consensus; CLIP studies performed in vastly different cell types

APRA, Antibody-positioned RNA amplification; CDS, coding sequence; HITS-CLIP, high-throughput sequencing crosslinking immunoprecipitation; PAR-CLIP, photoactivatable ribonucleoside–enhanced crosslinking immunoprecipitation; RNA-Chip, RNA immunoprecipitation followed by microarray interrogation.

Two CLIP datasets have indicated that FMRP binding occurs largely in the CDS and appears to be equally distributed across the region, which is seemingly in conflict with a model where FMRP targets specific sequences or structural elements. This becomes especially perplexing if one considers how selective FMRP is in the mRNAs with which it associates. If a sequence/structural recognition element is so frequent that it occurs throughout the CDS of a gene, how can it also be infrequent enough such that it's found only in the small fraction of the neuronal transcriptome with which FMRP associates? In addition to addressing this question, future studies will need to determine whether each of the RNA-binding domains of FMRP work separately or in concert to specify targets and reveal more about the true nature of the FMRP:mRNA interactions. In addition, studies at the protein level are critically important for determining the actual effect of absent or nonfunctional FMRP, as an imbalance in neuronal protein concentration is likely to be the major consequence of FMRP loss or dysfunction.

These future goals will support the identification of bona fide gene targets of FMRP, which will help define the molecular pathways most affected by the loss of the protein and ultimately help direct research efforts toward the development of effective treatments for the disorder.

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CHAPTER

9

The mGluR Theory of Fragile X: From Mice to Men

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INTRODUCTION

Although it arises from a single gene mutation, the neurological and psychiatric symptoms associated with fragile X syndrome (FXS) are myriad. These symptoms include intellectual disability and autism, hyperactivity, hypersensitivity to sensory stimuli, and epilepsy (L. W. Wang, Berry-Kravis, & Hagerman, 2010). The neural bases for these disruptions are not fully understood, however alterations in multiple circuits have been identified in the FXS brain. For these reasons, prospects historically were considered to be dim for developing a disease-modifying treatment. The best one could hope for was to manage symptoms with polypharmacy using drugs developed for other medical indications.

The mGluR theory of fragile X offered an alternate view on the treatment of FXS. This theory posits that it is possible to treat multiple aspects of FXS by correcting the core biochemical pathophysiology—excessive or poorly regulated neuronal protein synthesis (Fig. 9.1A). With recognition that group 1 metabotropic glutamate receptors ($mGlu_{1/5}$) comprise a major system within the brain to regulate synaptic protein synthesis came the proposal that inhibiting these receptors with small molecule drugs could improve multiple psychiatric and neurological aspects of the disease. The idea was first presented publicly in 2001, and by the time it was published in 2004 (Bear, Huber, & Warren, 2004) research was already underway to test it in labs around the world. Since then, a remarkable accumulation of scientific evidence has supported the mGluR theory showing, time and again, that it is possible to alter the course of the disease in animal models even after appearance of symptoms (Bhakar, Dolen, & Bear, 2012; Dolen & Bear, 2008; Scharf, Jaeschke, Wettstein, & Lindemann, 2015). These exciting results

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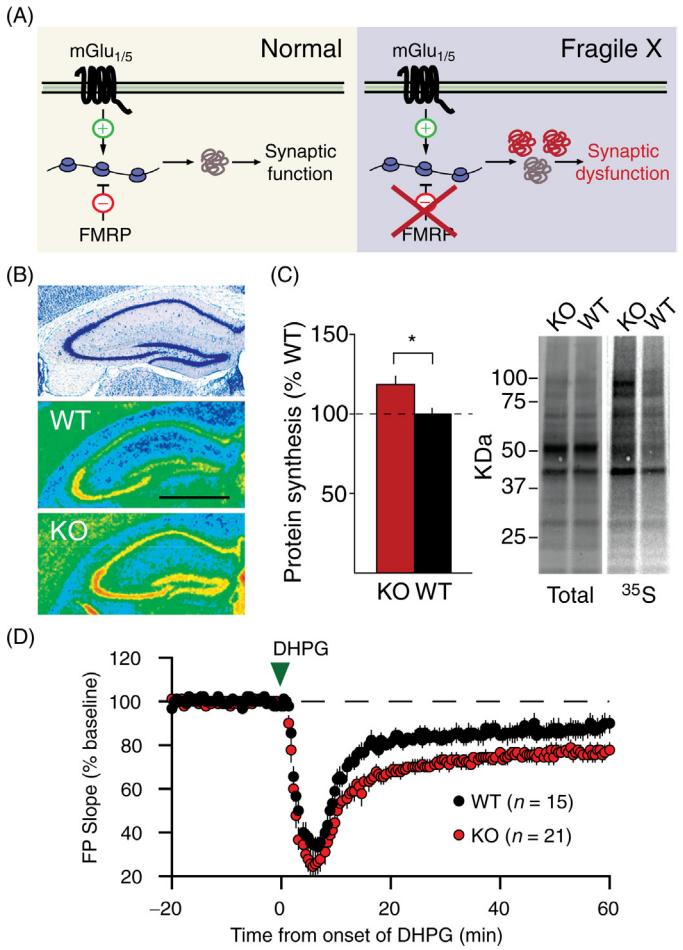

FIGURE 9.1 **Exaggerated protein synthesis and mGluR-LTD in the *Fmr1* KO mouse.** (A) The mGluR Theory of fragile X states that many pathological changes in FXS are due to excessive protein synthesis downstream of $mGlu_{1/5}$ activation, which occurs due to loss of FMRP. (B) The magnitude of mGluR-LTD, a form of synaptic plasticity that is dependent on de novo protein synthesis, is exaggerated in the *Fmr1* KO mouse compared with WT controls in CA1 of the hippocampus. (C) Nissl-stained coronal sections (top) and their corresponding pseudocolored autoradiograms (middle and bottom) show quantitative increases in translation rates throughout the hippocampus of 6-month old *Fmr1* KO mice in vivo (bottom) compared with WT controls (middle). (D) Metabolic labeling of slices prepared under conditions modeling slice electrophysiology experiments reveals rates of protein synthesis in the hippocampus are elevated in the *Fmr1* KO mouse compared to WT mice. *Source: Part B, Image from Huber, K. M., Gallagher, S. M., Warren, S. T., & Bear, M. F. (2002). Altered synaptic plasticity in a mouse model of fragile X mental retardation.* Proceedings of the National Academy of Sciences, *99(11), 7746–7750; Part C, Image from Qin, M., Kang, J., Burlin, T. V., Jiang, C., & Smith, C. B. (2005). Postadolescent changes in regional cerebral protein synthesis: an in vivo study in the FMR1 null mouse.* The Journal of Neuroscience, *25(20), 5087–5095; Part D, Image from Osterweil, E. K., Krueger, D. D., Reinhold, K., & Bear, M. F. (2010). Hypersensitivity to mGluR5 and ERK1/2 leads to excessive protein synthesis in the hippocampus of a mouse model of fragile X syndrome.* The Journal of Neuroscience, *30(46), 15616–15627.*

have led to the development of a number of novel treatment approaches that continue to be tested in clinical trials.

In this chapter we will briefly summarize what is known about the regulation of protein synthesis by FMRP and $mGlu_{1/5}$, and introduce the *Fmr1* KO mouse model that has been essential for FXS research. We will then discuss the mGluR theory, and summarize evidence for the validity of this strategy for treating FXS. Finally, we will address recent clinical findings and discuss some of the newer directions being pursued in FXS research as a consequence of the mGluR theory.

FMRP NEGATIVELY REGULATES TRANSLATION

The majority of FXS cases arise from transcriptional silencing of the *FMR1* gene, leading to loss of the brain-enriched mRNA binding protein FMRP (Ashley, Wilkinson, Reines, & Warren, 1993; Devys, Lutz, Rouyer, Bellocq, & Mandel, 1993; Hinds et al., 1993; Kremer et al., 1991; Verkerk et al., 1991). In the postnatal brain, FMRP is expressed in neurons and mature astrocytes where it localizes to multiple cellular compartments (Gholizadeh, Halder, & Hampson, 2015). Within neurons, FMRP is found in the soma, dendrites, and in individual postsynaptic dendritic spines (Feng et al., 1997b; Weiler et al., 1997). The structure of FMRP places it in the heterogeneous ribonucleoprotein family, which regulate the function of mRNAs (Siomi, Siomi, Nussbaum, & Dreyfuss, 1993). Three domains allow for interaction with mRNAs: (1) an N-terminal region, containing two Tudor domains that function as a nuclear localization signal (NLS); (2) a central region, which contains two RNA-binding K homology (KH) domains and a nuclear export signal (NES); and (3) a C-terminal region, which contains an RGG box RNA-binding domain (Bagni & Oostra, 2013; Darnell et al., 2005; Darnell, Warren, & Darnell, 2004). These multiple binding domains allow FMRP to interact with a broad range of mRNA targets (Sethna, Moon, & Wang, 2014).

Early studies pointed to a role for FMRP in the localization of mRNAs in the cell, and particularly in shuttling targets to and from the nucleus (Eberhart, Malter, Feng, & Warren, 1996). Subsequent experiments using cultured neurons showed that FMRP colocalizes with trafficking mRNPs in dendrites and dendritic spines, and this is regulated by synaptic activity (Antar, Afroz, Dictenberg, Carroll, & Bassell, 2004; De Diego Otero et al., 2002; Dictenberg, Swanger, Antar, Singer, & Bassell, 2008). However, other studies showed no significant changes in mRNA target expression or localization in the FXS mouse model (Steward, Bakker, Willems, & Oostra, 1998). It is possible that there are redundant molecular mechanisms that compensate for the absence of FMRP in the knockout mouse model of FXS.

There is now general consensus that FMRP plays an important role in the regulation of mRNA translation. In vitro studies showed the majority of FMRP in brain cosediments with translating polyribosomes, and this association exists in proximal dendrites (Ceman et al., 2003; Feng et al., 1997a; Khandjian et al., 2004; Tamanini et al., 1996). Other experiments revealed that mRNAs bound to FMRP were translationally suppressed (Laggerbauer, Ostareck, Keidel, Ostareck-Lederer, & Fischer, 2001; Li et al., 2001). The precise mechanism by which FMRP represses translation remains unclear, however the prevailing hypothesis suggests that it stalls the elongation step of protein synthesis. This hypothesis received strong support in 2011, when the Darnell group used high-throughput sequencing of RNAs isolated

by cross-linking immunoprecipitation (HITS-CLIP) to identify FMRP interactions with polyribosome bound mRNAs in the mouse brain. In addition to identifying 842 unique mRNA targets of FMRP, they demonstrated that FMRP is associated with transcripts on which ribosomes were stalled (Darnell et al., 2011). According to their model, which is now widely accepted, FMRP reversibly represses translation in a complex that consists of target mRNAs and stalled ribosomes. Subsequent work showed that FMRP represses elongation by direct binding to the ribosome (E. Chen, Sharma, Shi, Agrawal, & Joseph, 2014a). Thus, the loss of a "translational brake" leads to excess or inappropriate synthesis of synaptic proteins in FXS (Bhakar et al., 2012; Darnell et al., 2011). Other work suggests that FMRP also represses the initiation step of translation by recruiting cytoplasmic FMRP-interacting protein (CYFIP1), which consequently blocks the formation of the eIF4F complex thus preventing cap-dependent initiation of translation (Napoli et al., 2008).

The identified mRNA targets of FMRP encode a variety of proteins that are essential for synaptic function (Darnell et al., 2011). The ways in which these targets are dysregulated in FXS, and how this contributes to the disease pathology is an area of active investigation. However, it seems clear that the pathophysiology of FXS is directly linked to loss of mRNA binding by FMRP. Indeed, a rare I304N point mutation in the *FMR1* gene that results in the production of a mutant FMRP that cannot bind mRNA has been linked to a severe form of FXS (De Boulle et al., 1993). This discovery strongly suggests that understanding the mechanisms governing regulation of FMRP-mRNA interactions is key to understanding the pathogenesis of FXS.

ANIMAL MODELS OF FXS

Significant progress has been made over the past 30 years in understanding the roles that FMRP plays in cellular processes, as well as the functional and molecular consequences caused by the loss of FMRP in FXS. Integral to this progress in our understanding of the synaptic pathophysiology of FXS has been the use of animal models of FXS, most notably, the *Fmr1* KO mouse model. The *Fmr1* KO mouse, developed by the Dutch-Belgian Fragile X Consortium in 1994, remains the most widely studied animal model of FXS today and has greatly advanced our understanding of the pathophysiology of this complex disorder (Bakker et al., 1994). Initial investigation of the *Fmr1* KO mouse was directed at identifying pathogenic phenotypes, in particular, common features that the mouse model shares with humans afflicted with FXS. In the seminal paper characterizing the *Fmr1* KO mouse, it was revealed that male mice exhibit enlarged testes similar to human males with FXS, as well as hyperactivity and impaired cognitive function (Bakker et al., 1994). Further similarities have since been identified, including hyperarousal to sensory stimuli and network hyperexcitability (Gibson, Bartley, Hays, & Huber, 2008; Olmos-Serrano et al., 2010; Zhang & Alger, 2010); epileptiform activity and a resulting increased susceptibility to seizures (Chuang et al., 2005; Musumeci et al., 1999, 2000; Osterweil et al., 2013; Yan, Asafo-Adjei, Arnold, Brown, & Bauchwitz, 2004); deficits in social interaction (Liu & Smith, 2009); as well as learning and memory impairments (Brennan, Albeck, & Paylor, 2006; Dolen et al., 2007; Qin, Kang, & Smith, 2002; Zhao et al., 2005). *Fmr1* KO mice also exhibit abnormalities in dendritic spine structure, which are also observed in postmortem brain tissue from FXS patients (Comery et al., 1997; He

& Portera-Cailliau, 2013; Hinton, Brown, Wisniewski, & Rudelli, 1991; Irwin, Galvez, & Greenough, 2000; Wijetunge, Angibaud, Frick, Kind, & Nagerl, 2014).

Despite a plethora of features shared (to some degree) between the *Fmr1* KO mouse and humans with FXS, there are some phenotypes that seem to be opposing. Most notably, while patients with FXS most often exhibit severe generalized anxiety, *Fmr1* KO mice exhibit behaviors in the elevated plus maze and open field that are interpreted to reflect decreased anxiety (Chen et al., 2013; Li et al., 2001; Z. H. Liu, Chuang, & Smith, 2011; Yuskaitis et al., 2010). Interestingly, one compelling theory suggests that these behavioral paradigms, designed to be measures of anxiety in mice, may actually be measuring impulsivity or deficits in executive function (Liu & Smith, 2014). In 2000, a *Drosophila* model of FXS was generated upon the discovery of *dfmr1*, the invertebrate homolog of the *FMR1* gene (Wan, Dockendorff, Jongens, & Dreyfuss, 2000). More recently, a zebrafish model lacking *Fmr1* was generated and characterized, as well as two distinct rat models of FXS (den Broeder et al., 2009; Hamilton et al., 2014; Till et al., 2015). It is likely that investigation of the *Fmr1* KO rat models will offer added insight into our understanding of FXS that has not been possible with the mouse model, particularly in disease-relevant complex behaviors and network-level dysfunctions.

Although animal models provide a means for direct manipulation and probing of gene function, there are obvious limitations to the conclusions we can reasonably make about FXS in humans, particularly regarding cognition, complex behavior, and social interaction. Nonetheless, these animal models have not only led to the identification of common phenotypes between mice and humans lacking *Fmr1*, they have also generated a rich literature dissecting the role of FMRP and more generally, the synaptic pathophysiology of FXS.

DYSREGULATION OF SYNAPTIC PROTEIN SYNTHESIS IN THE *Fmr1* KO MOUSE

One of the hallmark phenotypes of the *Fmr1* KO mouse is the elevated rate of basal protein synthesis in the brain. This was first shown in the mouse hippocampus in vivo using autoradiography (Qin, Kang, Burlin, Jiang, & Smith, 2005) (Fig. 9.1B). In subsequent work, it was shown that this phenotype could be effectively modeled in acute brain slices using a metabolic labeling approach (Fig. 9.1C) (Dolen et al., 2007; Osterweil, Krueger, Reinhold, & Bear, 2010). Elevated rates of net translation in the *Fmr1* KO mouse hippocampus and cortex have now been observed using a number of different labeling techniques, by multiple independent research groups (Barnes et al., 2015; Bhattacharya et al., 2012; Dolen et al., 2007; Henderson et al., 2012; Muddashetty, Kelic, Gross, Xu, & Bassell, 2007; Osterweil et al., 2010). Even prior to the observation that cerebral protein synthesis was elevated in the *Fmr1* KO mouse, the functional consequences of the loss of FMRP at the synapse were being investigated. Based on evidence that FMRP, *Fmr1* mRNA and polyribosomes were all found at the base of dendritic spines, it was theorized that FMRP may provide an integral substrate for the structural changes necessary for protein-synthesis-dependent forms of synaptic plasticity including long-term potentiation (LTP) and long-term depression (LTD), the functional correlates of learning and memory (Huber, Kayser, & Bear, 2000; Kang & Schuman, 1996; Weiler & Greenough, 1999). Surprisingly, initial investigation of the *Fmr1* KO mouse revealed that LTP was normal in CA1 of the mouse hippocampus (Godfraind et al., 1996; Paradee et al., 1999).

Based on the observation that FMRP is synthesized upon activation of group I mGluRs with the agonist (S)-3,5-dihydroxyphenylglycine (DHPG), as well as the finding that local de novo protein synthesis was required for stable expression of LTD, it was hypothesized that mice lacking FMRP may have disrupted mGluR-LTD (Huber et al., 2000; Huber, Roder, & Bear, 2001; Snyder et al., 2001; Weiler & Greenough, 1999). Indeed, mGluR-LTD was found to be significantly altered in CA1 of the hippocampus of *Fmr1* KO mice. Surprisingly however, the magnitude of LTD was greatly enhanced rather than diminished (Fig. 9.1D) (Huber, Gallagher, Warren, & Bear, 2002). Furthermore, mGluR-LTD was no longer blocked by the protein synthesis inhibitor anisomycin in the *Fmr1* KO mouse (Nosyreva & Huber, 2006).

The fact that mGluR-LTD was maintained even in the absence of de novo protein synthesis suggested that "plasticity proteins" necessary for the induction and maintenance of LTD were already present in sufficient abundance in the *Fmr1* KO mouse. While the full list of mRNAs that are excessively translated in the *Fmr1* KO brain have yet to be identified, there are FMRP targets that have been shown to be translated in response to $mGlu_{1/5}$ activation at the synapse. These include the microtubule binding protein MAP1B (Lu et al., 2004), the immediate early gene Arc (Waung, Pfeiffer, Nosyreva, Ronesi, & Huber, 2008), the phosphatase STEP (Goebel-Goody et al., 2012), and the Alzheimer's protein APP (Westmark & Malter, 2007). The synaptic scaffolding protein PSD-95 and the plasticity protein CaMK2α have also been reported to be overtranslated at *Fmr1* KO synapses (Ifrim, Williams, & Bassell, 2015; Muddashetty et al., 2007; Osterweil et al., 2010; Zalfa et al., 2003).

THE mGluR THEORY OF FXS

Group I mGluRs are potent regulators of protein synthesis (Job & Eberwine, 2001; Todd, Mack, & Malter, 2003; Weiler & Greenough, 1993). This knowledge, along with the observation that mGluR-LTD in CA1 of the *Fmr1* KO mouse hippocampus is exaggerated and no longer dependent on protein synthesis, inspired the mGluR theory of FXS (Bear et al., 2004; Huber et al., 2002). This theory posits that synthesis of FMRP in response to $mGlu_{1/5}$ activation normally acts as a brake on mGluR-dependent protein synthesis. When FMRP is absent, the brake is lifted resulting in aberrant protein synthesis and myriad functional consequences that include network hyperexcitability, cognitive impairment, and augmented LTD (Bear et al., 2004) (Fig. 9.1A). Accordingly, inappropriate translation downstream of $mGlu_{1/5}$ signaling could explain many deficits shared between the *Fmr1* KO mouse and humans with FXS.

The important prediction of the mGluR theory was that negative modulation of $mGlu_{1/5}$ and its downstream signaling components could correct the underlying pathophysiology of FXS. Since the original publication of the theory in 2004, numerous observations have been made supporting the hypothesis that inhibition of $mGlu_5$ and associated signaling events is beneficial in alleviating aberrant phenotypes in FXS animal models. A number of targets have been investigated, including the $mGlu_5$ receptor itself, as well as downstream signaling pathways involved in translation control. These studies investigating the mGluR theory have had a substantial impact on the FXS community, not only by renewing hope of a targeted treatment, but also by leading to the identification of several novel therapeutic strategies.

CORRECTING FXS: TARGETING $mGlu_5$

In the crucial test of the mGluR theory of FXS, *Fmr1*$^{+/-}$ females were mated to *Grm5*$^{+/-}$ males, which lack a copy of the *Grm5* gene encoding $mGlu_5$ (Dolen et al., 2007). The rationale was that in male offspring lacking both the *Fmr1* gene and one copy of *Grm5*, reduction of $mGlu_5$ expression would downregulate $mGlu_5$ signaling. If the mGluR theory was correct, and if a 50% reduction in mGlu5 was sufficient, this genetic reduction strategy should ameliorate many of the disease phenotypes seen in the *Fmr1* KO mouse. Indeed, this genetic cross strategy resulted in a broad scale rescue of multiple neurological phenotypes in the *Fmr1* KO mouse, including excessive protein synthesis, exaggerated mGluR-LTD, susceptibility to audiogenic seizure (AGS), and cognitive deficits in a passive avoidance task (Dolen et al., 2007). While this study strongly supported the mGluR theory, one limitation was that $mGlu_5$ expression was reduced embryonically, persisting postnatally. The genetic reduction strategy did not allow an opportunity to evaluate whether postnatal, pharmacological downregulation of $mGlu_5$ signaling would show any therapeutic benefit.

To address this question, several studies tested the efficacy of acute administration of the $mGlu_5$ negative allosteric modulator (NAM) 2-Methyl-6-(phenylethynyl)pyridine (MPEP). These studies revealed that a multitude of cellular, electrophysiological, and behavioral phenotypes in the *Fmr1* KO mouse are resolved with acute inhibition of $mGlu_5$ (see Table 9.1 for a complete list of preclinical studies investigating $mGlu_5$ modulation in the *Fmr1* KO mouse) (Aschrafi, Cunningham, Edelman, & Vanderklish, 2005; Chuang et al., 2005; de Vrij et al., 2008; Hays, Huber, & Gibson, 2011; Koekkoek et al., 2005; Levenga et al., 2011; Meredith, de Jong, & Mansvelder, 2011; Min et al., 2009; Nakamoto et al., 2007; Osterweil et al., 2010; Su et al., 2011; Suvrathan, Hoeffer, Wong, Klann, & Chattarji, 2010; Thomas, Bui, Perkins, Yuva-Paylor, & Paylor, 2012; Vinueza Veloz et al., 2012; Yan, Rammal, Tranfaglia, & Bauchwitz, 2005). To examine the extent to which chronic postadolescent inhibition of $mGlu_5$ could correct FXS phenotypes another study used 2-chloro-4-((2,5-dimethyl-1-(4-(trifluoromethoxy) phenyl)-1H-imidazol-4-yl)ethynyl)pyridine (CTEP), a selective $mGlu_5$ NAM with a much longer duration of action than MPEP. As with MPEP, acute CTEP treatment corrected elevated protein synthesis and deficits in mGluR-LTD, and reduced the incidence of AGS. Most importantly, chronic treatment with CTEP for 30 days, initiated at postnatal day 30, rescued cognitive deficits, auditory hypersensitivity, aberrant spine density, as well as partial correction of macroorchidism (Michalon et al., 2012). Taken together these studies suggest that direct manipulation of $mGlu_5$, even in young adulthood, could be a useful therapy in humans with FXS.

CORRECTING FXS: TARGETING TRANSLATION CONTROL

In addition to directly targeting $mGlu_5$, manipulation of downstream signaling pathways has proven effective for treating FXS. Honing in on the specific signaling mechanism responsible for excessive protein synthesis in FXS could offer therapeutic advantages over global modulation of $mGlu_5$, with the obvious caveat that most signaling pathways are ubiquitous, and utilized throughout the body for functions unrelated to neuronal control of protein synthesis or fragile X.

TABLE 9.1 FX Phenotypes Corrected by $mGlu_5$ Manipulation

Fragile X phenotype (vs. WT)	$mGlu_5$ manipulation	References
Exaggerated mGluR-LTD	*Grm5*$^{+/-}$ cross CTEP	Dolen et al. (2007) Michalon et al. (2012)
Increased AMPAR internalization	MPEP	Nakamoto et al. (2007)
Impaired spontaneous EPSCs in juvenile hippocampus	MPEP	Meredith et al. (2011)
Increased protein synthesis	*Grm5*$^{+/-}$ cross MPEP CTEP	Dolen et al. (2007) Osterweil et al. (2010) Michalon et al. (2012)
Decreased number of mRNA granules in whole brain	MPEP	Aschrafi et al. (2005)
Increased glycogen synthase kinase-3 activity	MPEP	Min et al. (2009)
Increase beta amyloid	MPEP	Malter, Rayl, Westmarkl, & Westmarkl et al. (2010)
Increased ERK and mTOR signaling	CTEP	Michalon et al. (2012)
Increased dendritic spine/filopodia density	*Grm5*$^{+/-}$ cross Fenobam MPEP AFQ056 CTEP	Dolen et al. (2007) de Vrij et al. (2008) Su et al. (2011) Levenga et al. (2011) Michalon et al. (2012)
Altered visual cortical plasticity	*Grm5*$^{+/-}$ cross	Dolen et al. (2007)
Prolonged epileptiform discharges in hippocampus	MPEP	Chuang et al. (2005)
Increased persistent activity states in neocortex	MPEP, *Grm5*$^{+/-}$ cross	Hays et al. (2011)
Impaired presynaptic function in the amygdala	MPEP	Suvrathan et al. (2010)
Exaggerated inhibitory avoidance extinction	*Grm5*$^{+/-}$ cross	Dolen et al. (2007)
Associative motor-learning deficit	Fenobam	Vinueza Veloz et al. (2012)
Impaired eyelid conditioning	MPEP	Koekkoek et al. (2005)
Increased audiogenic seizure (AGS)	*Grm5*$^{+/-}$ cross MPEP CTEP	Dolen et al. (2007) Thomas et al. (2012), Yan et al. (2005) Michalon et al. (2012)
Defective prepulse inhibition of acoustic startle	MPEP AFQ056 CTEP	Levenga et al. (2011) Michalon et al. (2012)
Avoidance behavior deficits	Fenobam	Vinueza Veloz et al. (2012)
Decreased initial performance on rotorod	MPEP	Thomas et al. (2012)
Increased open-field activity	MPEP CTEP	Min et al. (2009), Yan et al. (2005)
Abnormal social interaction with unfamiliar mouse	*Grm5*$^{+/-}$ cross	Thomas et al. (2011)
Increased marble burying (repetitive behavior)	MPEP	Thomas et al. (2012)
Macroorchidism	CTEP (partial rescue)	Michalon et al. (2012)
Pubertal increase in body weight	*Grm5*$^{+/-}$ cross	Dolen et al. (2007)

AMPAR, α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; CTEP, 2-chloro-4-((2,5-dimethyl-1-(4-(trifluoromethoxy) phenyl)-1H-imidazol-4-yl)ethynyl)pyridine; EPSC, excitatory postsynaptic currents; FX, fragile X; mGluR; LTD, long-term depression; mGluR, metabotropic glutamate receptor; MPEP, 2-methyl-6-(phenylethynyl)-pyridine; mRNA, messenger RNA; WT, wild type.
MPEP, Fenobam, CTEP, AFQ056 = $mGlu_5$ negative allosteric modulators.

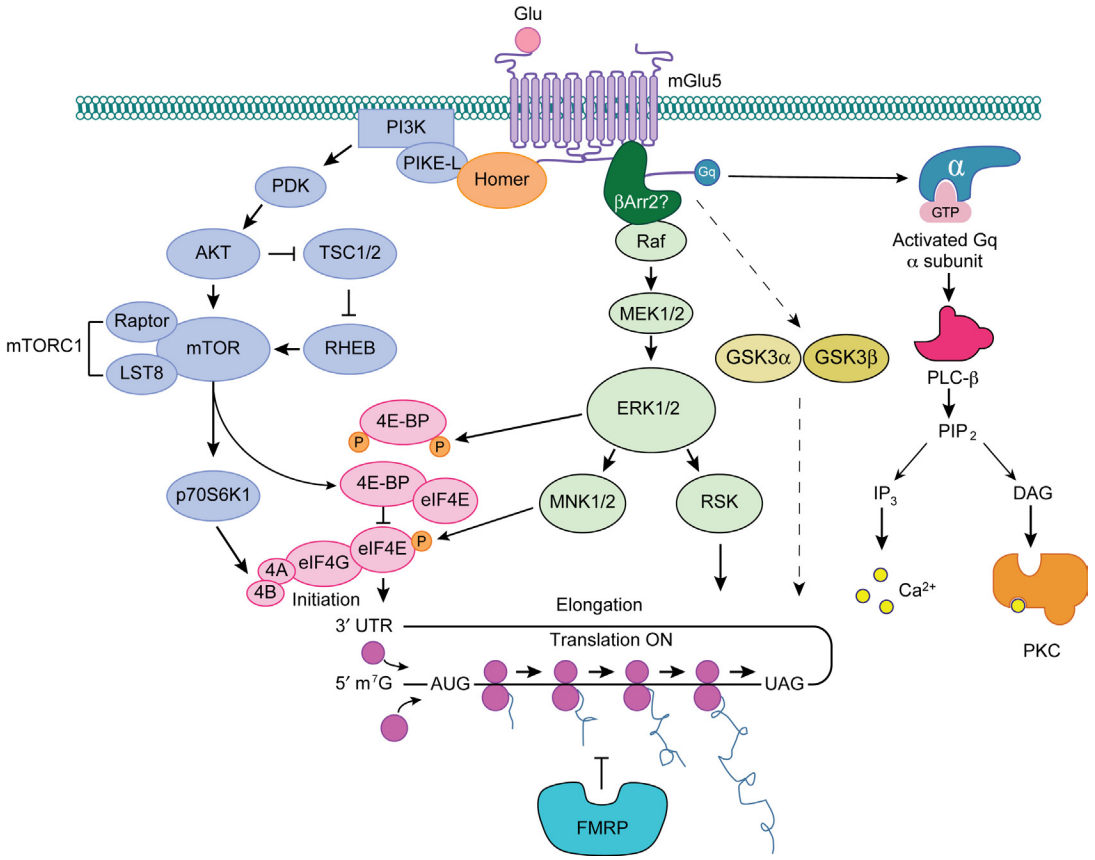

FIGURE 9.2 Signaling pathways mediate synaptic translation upon $mGlu_5$ activation. Glutamate binding to $mGlu_5$ activates three main pathways that couple the receptors to translational regulation: (1) the PLC/PKC pathway, (2) the mTOR pathway *(blue ovals)*, and (3) the ERK pathway *(green ovals)*. Key regulatory components of translation initiation are shown in *light pink*. *Arrows* indicate a positive consequence on downstream components; *perpendicular lines* indicate an inhibitory consequence. Abbreviations: Ca^{2+}, calcium release from intracellular stores; eIF4, eukaryotic initiation factor 4; ERK, extracellular signal–regulated kinase; FMRP, fragile X mental retardation protein; Gαq, heterotrimeric G proteins; IP_3, inositol-1,4,5-triphosphate; $mGlu_5$, metabotropic glutamate receptor 5; mTOR, mammalian target of rapamycin; PLC, phospholipase C; Raptor, regulatory-associated protein of mTOR; TSC, tuberous sclerosis complex.

Like other G-protein coupled receptors (GPCRs), $mGlu_5$ activates second messenger signaling cascades through association with a small G protein, in this case G_q. Stimulation of $mGlu_5$ leads to activation of G_q which activates phospholipase Cβ (PLCβ) to cleave PIP_2 into the intracellular signaling molecules IP_3 and DAG (Fig. 9.2). Subsequently, IP_3 activates receptors on the endoplasmic reticulum to raise intracellular Ca^{2+} while DAG activates protein kinase C (PKC) (for review see Luscher & Slesinger, 2010). Interestingly, early experiments revealed that the induction of mGluR-LTD is not blocked by PKC or PLCβ inhibitors, nor by depletion of intracellular Ca^{2+} stores. These experiments showed that "canonical" G_q signaling is not required for mGluR-LTD, and suggested that other signaling mechanisms were responsible

for inducing this protein synthesis-dependent form of plasticity (Fitzjohn et al., 2001; Mockett et al., 2011; Rush, Wu, Rowan, & Anwyl, 2002; Schnabel, Kilpatrick, & Collingridge, 1999).

In further studies, it was revealed that activation of $mGlu_{1/5}$ in hippocampal slices recruits two intracellular signaling pathways linked to cap-dependent translation: the Ras-extracellular signal regulated kinase 1/2 (ERK) pathway and the phosphoinositide 3 kinase (PI3K)-Akt-mammalian target of rapamycin (mTOR) pathway (Fig. 9.2) (Gallagher, Daly, Bear, & Huber, 2004; Hou & Klann, 2004). The activation of these pathways is critical for the initiation step of protein synthesis. Specifically, both ERK and mTOR signal to regulatory components of the initiation complex, primarily eukaryotic translation initiation factor 4E (eIF4E) and its inhibitor 4E binding protein (4E-BP) (for detailed review see Gingras et al., 2001; Waskiewicz et al., 1999). For translation to be initiated, multiple proteins must localize to the 5′ untranslated region (UTR) of mRNAs, including eIF4E and eIF4G, which together with eIF4A form the eIF4F complex. The eIF4F complex is essential for association of the mRNA with the small ribosomal subunit. Both ERK and mTOR facilitate eIF4F formation by phosphorylation of eIF4E and its inhibitor 4EBP, respectively (Fig. 9.2) (Proud, 2015). In addition, ERK and mTOR also stimulate protein synthesis by activating the ribosomal S6 kinase (S6K) pathway (Buffington, Huang, & Costa-Mattioli, 2014; Proud, 2015). Although it is clear that $mGlu_{1/5}$ can recruit both ERK and mTOR, the way in which these pathways regulate protein synthesis at FXS synapses remains an area of active investigation.

Targeting ERK

Application of the $mGlu_{1/5}$ agonist DHPG leads to a robust activation of the ERK pathway in several different preparations, including hippocampal slices (Berkeley & Levey, 2003; Ferraguti, Baldani-Guerra, Corsi, Nakanishi, & Corti, 1999; Garcia, Lopez, & Lopez-Colome, 2008). The ERK inhibitor U0126 disrupts the protein synthesis dependent portion of LTD induced by DHPG, suggesting that ERK is specifically required for the coupling of $mGlu_5$ to protein synthesis (Banko, Hou, Poulin, Sonenberg, & Klann, 2006; Gallagher et al., 2004). Supporting this idea, ERK inhibitors also block the prolonged bursting activity in hippocampal CA3 that is dependent on new protein synthesis induced by $mGlu_{1/5}$ activation (Merlin, Bergold, & Wong, 1998; W. Zhao, Bianchi, Wang, & Wong, 2004).

Although many groups have probed the involvement of signaling pathways in the pathophysiology of FXS, there have been conflicting observations in the literature. With respect to ERK activation, some have reported increased basal levels in the *Fmr1* KO mouse (Hou et al., 2006; Michalon et al., 2012; Price et al., 2007), while others have seen no difference between wild type (WT) and *Fmr1* KO mice (Gross et al., 2010; Hu et al., 2008; Z. H. Liu, Huang, & Smith, 2012; Osterweil et al., 2010). Still another group observed that $mGlu_{1/5}$ stimulation induced an aberrant dephosphorylation of ERK in *Fmr1* KO cortical synaptoneurosomes (Kim, Markham, Weiler, & Greenough, 2008). These differences likely arise from the different preparations used, the means and duration of stimulation, and the brain regions assayed. In any case, experiments using pathway specific inhibitors have consistently shown that reduction of ERK activity normalizes excessive protein synthesis in the FXS mouse hippocampus regardless of the basal activation state (Osterweil et al., 2013; Osterweil et al., 2010; Ronesi et al., 2012). It has been suggested that the excessive protein synthesis is not due to increased activation of $mGlu_5$-ERK, but rather an amplified response to normal levels of $mGlu_5$-ERK signaling (Osterweil et al., 2010).

Consistent with the correction of protein synthesis, several studies have established that ERK inhibitors ameliorate FXS pathology. In vitro studies of hippocampal slices show that application of ERK inhibitors eliminates epileptiform activity in the *Fmr1* KO mouse (Chuang et al., 2005). Additionally, injection of the brain penetrant ERK inhibitor SL327 greatly diminishes or completely eliminates the AGS phenotype in the *Fmr1* KO mouse (Osterweil et al., 2010; Wang et al., 2012). Several pathological phenotypes are also ameliorated in the *Fmr1* KO mouse by genetic reduction of ERK pathway signaling molecules. One study showed that disrupting ERK activation of eIF4E through genetic mutation of a key phosphorylation site was able to correct several phenotypes in the *Fmr1* KO mouse, including exaggerated protein synthesis, abnormal dendritic spine morphology, exaggerated mGluR-LTD, and deficiencies in social learning (Gkogkas et al., 2014). Genetic reduction of the ERK substrate Mnk kinase also corrects a number of electrophysiological and behavioral phenotypes in *Fmr1* KO mice, as does the eIF4E inhibitor cercosporamide (Bhattacharya et al., 2012; Gkogkas et al., 2014). Together, these studies point to the ERK pathway as a valid target for therapeutic intervention in FXS.

The fundamental involvement of the ERK pathway in diverse cellular functions makes it a difficult target for FXS-specific pharmacological manipulation. One approach has been to modify the upstream G protein Ras, which is a major regulator of ERK. Interestingly, classic studies in yeast revealed that the statin drug lovastatin could inhibit ERK signaling by reducing the farnesylation and membrane association of Ras (Schafer et al., 1989). This mechanism was tested in a clinical context by examining the ability of lovastatin to resolve phenotypes in the mouse model of Neurofibromatosis Type 1 (NF1), a disorder of hyperactive Ras (McKinney, Grossman, Elisseou, & Greenough, 2005). Recently, lovastatin was also proposed as a therapeutic strategy for downregulating ERK in FXS (Osterweil et al., 2013). Experiments using the *Fmr1* KO mouse showed that acute application of lovastatin corrects excessive hippocampal protein synthesis and mGluR-LTD, eliminates epileptiform activity in hippocampal CA3, and corrects hyperexcitability in visual cortical slices (Table 9.2). In addition, in vivo administration of lovastatin was seen to significantly reduce the incidence and severity of AGS, and correct deficits in visuospatial learning in the *Fmr1* KO mouse (Osterweil et al., 2013; Sidorov et al., 2014).

Targeting mTOR

The PI3K-mTOR signaling pathway has also been explored as a potential therapeutic option for FXS. This pathway is linked to $mGlu_{1/5}$ through association with the PI3K enhancing protein (PIKE) and the scaffolding protein Homer (Hou & Klann, 2004; Ronesi et al., 2012; Ronesi & Huber, 2008). Studies of *Fmr1* KO mice have revealed an alteration of PI3K-mTOR signaling due to destabilization of the $mGlu_5$-Homer complex at the postsynaptic density (Giuffrida et al., 2005; Ronesi & Huber, 2008). This disruption is thought to contribute to synaptic dysfunction in the *Fmr1* KO mouse, as genetically reducing the expression of the dominant-negative Homer1a isoform rescues several pathological phenotypes (Ronesi et al., 2012).

Interestingly, both PIKE and the p110β subunit of PI3K are mRNA targets of FMRP, and both proteins are reported to be overtranslated in the *Fmr1* KO brain (Darnell et al., 2011; Gross et al., 2015a; Gross et al., 2010). An upregulation of the PI3K-mTOR pathway has

TABLE 9.2 FX Phenotypes Corrected by Translation Control Pathway Manipulation

Fragile X phenotype (vs. WT)	ERK pathway manipulation	References
Exaggerated mGluR-LTD	Lovastatin (Ras farnesylation inhibitor)	Osterweil et al. (2013)
	eIF4E mutant (ERK substrate)	Gkogkas et al. (2014)
	Mnk mutant (ERK substrate)	Gkogkas et al. (2014)
	Cercosporamide (eIF4E inhibitor)	Gkogkas et al. (2014)
Increased protein synthesis	U0126 (ERK inhibitor)	Osterweil et al. (2010)
	U0126 (ERK inhibitor)	Ronesi et al. (2012)
	Lovastatin (Ras farnesylation inhibitor)	Osterweil et al. (2013)
	eIF4E mutant (ERK substrate)	Gkogkas et al. (2014)
	Mnk mutant (ERK substrate)	Gkogkas et al. (2014)
Altered visual cortical excitability	Lovastatin (Ras farnesylation inhibitor)	Osterweil et al. (2013)
Prolonged epileptiform discharges in hippocampus	PD98059 (ERK inhibitor)	Chuang et al. (2005)
	Lovastatin (Ras farnesylation inhibitor)	Osterweil et al. (2013)
AGSs	SL 327 (ERK inhibitor)	Osterweil et al. (2010)
	SL 327 (ERK inhibitor)	Wang et al. (2012)
	Lovastatin (Ras farnesylation inhibitor)	Osterweil et al. (2013)
	eIF4E mutant (ERK substrate)	Gkogkas et al. (2014)
	Mnk mutant (ERK substrate)	Gkogkas et al. (2014)
	Cercosporamide (eIF4E inhibitor)	Gkogkas et al. (2014)
Dendritic spine abnormalities	eIF4E mutant (ERK substrate)	Gkogkas et al. (2014)
	Mnk mutant (ERK substrate)	Gkogkas et al. (2014)
Impaired social interaction	eIF4E mutant (ERK substrate)	Gkogkas et al. (2014)
	Mnk mutant (ERK substrate)	Gkogkas et al. (2014)
	Cercosporamide (eIF4E inhibitor)	Gkogkas et al. (2014)
Impaired anxiety-like behaviors (open field activity)	eIF4E mutant (ERK substrate)	Gkogkas et al. (2014)
	Mnk mutant (ERK substrate)	Gkogkas et al. (2014)
	Cercosporamide (eIF4E inhibitor)	Gkogkas et al. (2014)
Reduced preference for social novelty	eIF4E mutant (ERK substrate)	Gkogkas et al. (2014)
	Mnk mutant (ERK substrate)	Gkogkas et al., 2014
	Cercosporamide (eIF4E inhibitor)	Gkogkas et al., 2014
Impaired visuospatial learning	Lovastatin (Ras farnesylation inhibitor)	Osterweil et al. (2013)
		Gkogkas et al. (2014)
		Gkogkas et al. (2014)
		Gkogkas et al. (2014)

Fragile X phenotype (vs. WT)	PI3K pathway manipulation	References
Exaggerated mGluR-LTD	$Tsc2^{+/-}$ cross (increased mTOR)	Auerbach et al. (2011)
Increased protein synthesis hippocampal slice	$Tsc2^{+/-}$ cross (increased mTOR)	Auerbach et al. (2011)
Increased protein synthesis synaptoneurosomes	Wortmannin/LY294002 (PI3K inhibitors)	Gross et al. (2010)
	P110b reduction (decreased PI3K)	Gross et al. (2015b)
Impaired anxiety-like behaviors (elevated plus maze, open field activity)	Temsirolimis (mTOR inhibitor)	Busquets-Garcia et al. (2013)

(*Continued*)

TABLE 9.2 FX Phenotypes Corrected by Translation Control Pathway Manipulation (*cont.*)

Fragile X phenotype (vs. WT)	PI3K pathway manipulation	References
Impaired hippocampal learning (novel object recognition)	Temsirolimis (mTOR inhibitor)	Busquets-Garcia et al. (2013)
Impaired hippocampal learning (contextual fear conditioning)	*Tsc2*$^{+/-}$ cross (increased mTOR)	Auerbach et al. (2011)
Dendritic spine abnormalities	PIKE reduction (decreased PI3K) P110b reduction (decreased PI3K) PTEN inhibitor (increased PI3K)	Gross et al. (2015a) Gross et al. (2015b) Boda et al. (2014)
Deficits in marble burying	PIKE reduction (decreased PI3K) P110b reduction (decreased PI3K)	Gross et al. (2015a) Gross et al. (2015b)
Impaired prefrontal-related cognition	P110b reduction (decreased PI3K)	Gross et al. (2015b)
AGSs	Temsirolimis (mTOR inhibitor) PIKE reduction (decreased PI3K) P110b reduction (decreased PI3K)	Busquets-Garcia et al. (2013) Gross et al. (2015a) Gross et al. (2015b)

Fragile X phenotype (vs. WT)	S6 kinase pathway manipulation	References
Exaggerated mGluR-LTD	S6K1 KO cross (decreased p70 S61 Kinase)	Bhattacharya et al. (2012)
Increased protein synthesis	PF-4708671 and FS-115 (p70 S61 Kinase inhibitors)	Bhattacharya et al. (2016)
Impaired novel object recognition	S6K1 KO cross (decreased p70 S61 Kinase)	Bhattacharya et al. (2012)
Increased dendritic spine/filopodia density	S6K1 KO cross (decreased p70 S61 Kinase) PF-4708671 and FS-115 (p70 S61 Kinase inhibitors)	Bhattacharya et al. (2012) Bhattacharya et al. (2016)
Decreased initial performance on rotorod	S6K1 KO cross (decreased p70 S61 Kinase)	Bhattacharya et al. (2012)
Deficits in the Y maze	S6K1 KO cross (decreased p70 S61 Kinase) PF-4708671 and FS-115 (p70 S61 Kinase inhibitors)	Bhattacharya et al. (2012) Bhattacharya et al. (2016)
Pubertal increase in body weight	S6K1 KO cross (decreased p70 S61 Kinase) FS-115 (p70 S61 Kinase inhibitor)	Bhattacharya et al. (2012) Bhattacharya et al. (2016)
Macroorchidism	S6K1 KO cross (decreased p70 S61 Kinase) PF-4708671 and FS-115 (p70 S61 Kinase inhibitors)	Bhattacharya et al. (2012) Bhattacharya et al. (2016)
Deficits in marble burying	FS-115 (p70 S61 Kinase inhibitor)	Bhattacharya et al. (2016)
Impaired social interaction	PF-4708671 and FS-115 (p70 S61 Kinase inhibitors)	Bhattacharya et al. (2016)

also been observed in the *Fmr1* KO hippocampus and cortex (Gross et al., 2010; Sharma et al., 2010). One group found that genetic reduction of PIKE or p110β restores normal levels of PI3K-mTOR signaling and corrects several phenotypes in the *Fmr1* KO, including abnormal dendritic spine morphology, mGluR-LTD and deficient cognition (Gross et al., 2015a,b). These results suggest that downregulation of the PI3K-mTOR pathway would be beneficial in FXS. However, other studies in *Fmr1* KO hippocampal slices show that neither application of the PI3K inhibitor wortmannin nor the mTOR inhibitor rapamycin is effective in normalizing exaggerated protein synthesis (Osterweil et al., 2010; Ronesi et al., 2012). The efficacy of mTOR inhibitors for ameliorating the AGS phenotype in the *Fmr1* KO mouse is also unclear,

with one group showing an amelioration and another group showing no significant effect (Busquets-Garcia et al., 2013; Osterweil et al., 2010). These seemingly contradictory findings may be due to experimental differences in preparation, mouse background strain, or acute versus chronic downregulation of PI3K-mTOR. However, considered together, the data suggest that alterations in mTOR signaling are a secondary consequence rather than a proximal cause of altered protein synthesis in FXS.

In apparent contradiction to studies of PIKE downregulation, studies using mouse models of tuberous sclerosis complex (TSC), a genetic disorder caused by the loss of the mTOR suppressor complex TSC1/2, suggest that increased mTOR activation could in fact be beneficial for FXS. Experiments performed in the hippocampus of the *Tsc2*$^{+/-}$ mouse model and in other mouse models of TSC reveal that both LTD and protein synthesis downstream of $mGlu_{1/5}$ are deficient (Auerbach, Osterweil, & Bear, 2011; Bateup, Takasaki, Saulnier, Denefrio, & Sabatini, 2011; Potter et al., 2013). Application of either the mTOR inhibitor rapamycin or the $mGlu_5$ positive allosteric modulator CDPPB will correct deficiencies in LTD and protein synthesis in the *Tsc2*$^{+/-}$ hippocampus (Auerbach et al., 2011). Moreover, a genetic cross of the *Tsc2*$^{+/-}$ mouse to the *Fmr1* KO mouse corrects deficits in hippocampal LTD and in behavioral measures of learning in both mutants (Auerbach et al., 2011). The suggestion from these studies is that pathological changes in the *Fmr1* KO brain might be corrected by *increasing* mTOR activity. Consistent with this idea, another group showed that application of an inhibitor to PTEN, a negative regulator of PI3K-mTOR signaling, corrected deficits in dendritic spine stability, LTP and learning in the *Fmr1* KO mouse (Boda, Mendez, Boury-Jamot, Magara, & Muller, 2014). Further studies are needed to fully understand how both the reduction and the increase in PI3K-mTOR can lead to similar corrections in *Fmr1* KO mouse phenotypes.

Targeting p70 S6K

Downstream of the ERK and mTOR signaling pathways, the ribosomal S6 kinases (S6Ks) facilitate protein synthesis through the activation of several translation factors. Activation of the 70 KDa S6K isoform (p70 S6K) by mTOR complex 1 (mTORC1) has been shown to engage both the initiation factor eIF4B and elongation factor eEF2 kinase (Proud, 2007). Recently, one group explored the consequences of genetically reducing p70 S6K in the *Fmr1* KO mouse (Bhattacharya et al., 2012). They found that protein synthesis was normalized, and deficits in dendritic spine morphology and behavior were rescued. In more recent work, this group showed that pharmacological inhibitors of p70 S6K are also effective in correcting pathological changes in the *Fmr1* KO mouse (see Table 9.2 for a full list) (Bhattacharya et al., 2016). These findings show that translational control is an effective target for treating FXS. In keeping with this idea, another study revealed that *Fmr1* KO phenotypes are ameliorated by genetic reduction of the translation regulator cytoplasmic polyadenylation element-binding protein (CPEB) (Udagawa et al., 2013). Thus, treatments that correct the aberrant elevation in protein synthesis have also consistently improved core FXS phenotypes.

Targeting GSK3α/β

Lithium, a mood stabilizer that had been traditionally used to treat bipolar disorder, has shown promise as a therapeutic intervention for patients with FXS in an open-label clinical

trial (Berry-Kravis et al., 2008). At the time, mechanistic benefit was misattributed to the effects of lithium as an inhibitor of inositol signaling downstream of $mGlu_5$ activation. It has since been revealed that the disease-relevant target of lithium is likely to be glycogen synthase kinase-3 (GSK3), a serine/threonine kinase initially identified as an enzyme important for glycogen synthesis (Embi, Rylatt, & Cohen, 1980; Chapter 13). It is now well established that GSK3 is involved in a number of important fundamental processes including gene transcription, apoptosis and microtubule dynamics, and altered GSK3 signaling has been implicated in the pathogenesis of a number of neuropsychiatric conditions including mood disorders, schizophrenia, and Alzheimer's disease (Grimes & Jope, 2001). GSK3 has two paralogs, GSK3α and GSK3β, which are negatively regulated by phosphorylation (GSK3α at Ser21, GSK3β at Ser9) (Woodgett, 1990).

Accumulating evidence suggests that GSK3 paralogs play a role in the pathogenesis of FXS phenotypes in the *Fmr1* KO mouse. GSK3β is a target of FMRP (Darnell et al., 2011), and the inhibitory serines of both GSK3α and GSK3β are hypophosphorylated in the *Fmr1* KO mouse, implying overactive GSK3α/β may be pathogenic in FXS (Guo et al., 2012; Liu et al., 2011; Min et al., 2009; Yuskaitis et al., 2010). Indeed, inhibition of GSK3α/β with either lithium or nonspecific GSK3 inhibitors corrects a multitude of synaptic and cognitive deficits (Table 9.3) in the *Fmr1* KO mouse, including excessive protein synthesis, indicating that GSK3α/β also participates in mRNA translational regulation (T. Chen et al., 2014b; X. Chen et al., 2013; Choi et al., 2011; Franklin et al., 2014; Guo et al., 2012; King & Jope, 2013; Z. H. Liu et al., 2011; Liu et al., 2012; Min et al., 2009; Mines, Yuskaitis, King, Beurel, & Jope, 2010; Yuskaitis et al., 2010). Interestingly, acute MPEP treatment reduces aberrant GSK3 signaling in *Fmr1* KO mice and dual administration of MPEP with lithium did not show additive benefit implying that MPEP and GSK3 may be acting within the same signaling pathway (Yuskaitis et al., 2010).

CORRECTING FXS: OTHER TARGETS

Other rescue strategies have targeted proteins regulating $mGlu_5$ (Homer1a) or GPCRs more generally (RGS4) (Guo et al., 2016; Pacey, Heximer, & Hampson, 2009; Ronesi et al., 2012) or proteins that are excessively translated, including APP and Matrix Metalloproteinase 9 (MMP9) (Chapter 15; Gkogkas et al., 2014; Sidhu, Dansie, Hickmott, Ethell, & Ethell, 2014; Westmark et al., 2011). Thus far, only MMP9 has been pursued as a candidate for pharmacological testing, using the drug minocycline as a potential treatment strategy for FXS (Paribello et al., 2010).

Inhibiting $mGlu_5$ has proven to be more efficacious than inhibiting other Gq-coupled receptors known to be dysregulated in FXS (Volk, Pfeiffer, Gibson, & Huber, 2007), including $mGlu_1$, or muscarinic receptors (Thomas et al., 2011; Thomas et al., 2012; Veeraragavan et al., 2011a; Veeraragavan, Bui, Perkins, Yuva-Paylor, & Paylor, 2011; Veeraragavan et al., 2012). However, another strategy that has seen success in the Fmr1 KO mouse targets gamma-aminobutyric acid (GABA) signaling (Chapter 10). Impaired $GABA_A$ expression and GABAergic signaling has been implicated in the pathophysiology of FXS (Adusei, Pacey, Chen, & Hampson, 2010; Braat et al., 2015; Centonze et al., 2008; Curia, Papouin, Seguela, & Avoli, 2009; D'Hulst et al., 2006; D'Hulst et al., 2009; El Idrissi et al., 2005; Q. He, Nomura, Xu, & Contractor, 2014; Heulens, D'Hulst, Van Dam, De Deyn, & Kooy, 2012; Martin, Corbin, & Huntsman, 2014;

TABLE 9.3 FX Phenotypes Corrected by GSK3α/β Manipulation

Fragile X phenotype (vs. WT)	GSK3 manipulation	References
Exaggerated mGluR-LTD	Lithium	Choi et al. (2011)
Impaired signaling	Lithium	Liu et al. (2012)
LTP, reduced stead-state depolarization in the dentate gyrus	Lithium, CT99021	Franklin et al. (2014)
Increased protein synthesis	Lithium	Liu et al. (2012)
Impaired L-LTP in the anterior cingulate cortex	CT99021, SB415286	Chen et al. (2014b)
Increased glycogen synthase kinase-3 activity	Lithium SB415286	Min et al. (2009), Yuskaitis et al. (2010), Liu et al. (2011) Guo et al. (2012)
Adult hippocampal neurogenesis and maturation deficits	SB415286	Guo et al. (2012)
Increased dendritic spine/filopodia density	Lithium	Liu et al. (2011)
Exaggerated inhibitory avoidance extinction	Lithium	Liu et al. (2011), Yuskaitis et al. (2010)
Impaired trace fear memory and/or AMPAR GluA1 upregulation	CT99021 SB415286	Chen et al. (2014b) Guo et al. (2012)
Increased AGS	Lithium	Yuskaitis et al. (2010)
Deficits in categorical and/or spatial processing tasks	TDZD-8, VP0.7 Lithium	Franklin et al. (2014) King and Jope (2013)
Deficits in the elevated plus maze	Lithium	Yuskaitis et al. (2010), Liu et al. (2011), Chen et al. (2013)
Impaired novel object recognition	TDZD-8, VP0.7	Franklin et al. (2014), King and Jope (2013)
Increased open-field activity	Lithium	Min et al. (2009), Yuskaitis et al. (2010), Liu et al. (2011), Chen et al. (2013)
Abnormal social interaction with unfamiliar mouse	Lithium	Liu et al. (2011), Mines et al. (2010)
Macroorchidism	Lithium (partial rescue)	Liu et al. (2011)

Abbreviations: LTP, long term potentiation; L-LTP, late-long term potentiation; GluA1, glutamate ionotropic receptor AMPA type subunit 1.
Lithium = direct and indirect inhibitory action at GSK3; CT99021, SB415286 = ATP-competitive GSK3 inhibitors; TDZD-8, VP0.7 = noncompetitive ATP binding site inhibitors.

Selby, Zhang, & Sun, 2007; Wahlstrom-Helgren & Klyachko, 2015; W. Zhao, Wang, Song, Li, & Yuan, 2015). Reduced GABAergic inhibition increases network excitability and, consequently, the release of glutamate at excitatory synapses. Thus, there is a logic consistent with the assumptions of the mGluR theory for strategies to augment inhibition and decrease excitability. Indeed, it has been shown that the $GABA_B$ agonist R-baclofen will also correct the excessive basal protein synthesis in hippocampal slices from *Fmr1* KO mice and improve many phenotypic deficits in the *Fmr1* KO mouse (Table 9.4) (Henderson et al., 2012; Qin et al., 2015).

TABLE 9.4 FX Phenotypes Corrected by Treatment With the $GABA_B$ Agonist R-Baclofen

Fragile X phenotype (vs. WT) corrected by R-baclofen	References
Impaired signaling	Qin et al. (2015)
Elevated AMPAR internalization	Henderson et al. (2012)
Increased protein synthesis	Henderson et al. (2012), Qin et al. (2015)
Increased dendritic spine/filopodia density	Henderson et al. (2012)
Increased AGS	Henderson et al. (2012)
Abnormal social interaction with unfamiliar mouse	Qin et al. (2015)

In a parallel line of research, unrelated to the mGluR theory, it was discovered that large-conductance calcium-activated K^+ (BK) channel open probability is reduced by loss of FMRP, leading to prolongation of presynaptic action potentials, increased hippocampal network excitability, and excessive glutamate release. Genetic and pharmacological upregulation of BK activity in the *Fmr1* KO mouse can ameliorate some phenotypes that overlap with those corrected by targeting $mGlu_5$ and downstream signaling (Deng & Klyachko, 2016; Hebert et al., 2014). Thus, a BK channel opener also has promise for correcting FXS phenotypes caused by excessive protein synthesis in addition to those related to action potential width.

FROM MICE TO MEN: CLINICAL TRIALS FOR FXS

The overwhelming success in correcting a wide range of phenotypic deficits in the *Fmr1* KO mouse in preclinical studies has motivated a number of early proof-of-concept clinical trials and subsequent larger, later phase trials investigating similar targeted interventions in patients with FXS. Two open-label studies, one investigating the safety and efficacy of lithium, the other lovastatin in FXS, were inspired by studies in *Fmr1* KO animal models (E. Berry-Kravis et al., 2008; Caku, Pellerin, Bouvier, Riou, & Corbin, 2014; McBride et al., 2005; Osterweil et al., 2013; Pellerin et al., 2016). Chronic lithium treatment resulted in significant improvement in behavioral scales, verbal memory, and abnormal ERK activation rates, all secondary outcome measures, but failed to show more than mild improvement on the ABC-C Irritability Subscale, the study's primary endpoint (Berry-Kravis et al., 2008). Chronic administration of lovastatin has shown more promising outcomes. Significant improvement was demonstrated on predefined behavioral scales, including a recently developed behavioral scale tailored to patients with FXS, with the study meeting both primary and secondary endpoints (Caku et al., 2014). Additionally, elevated phosphorylated ERK1/2 levels in FXS platelets were normalized upon treatment with lovastatin (Pellerin et al., 2016). Despite promising preliminary outcomes and observations that the drug was well tolerated with minimal side effects, others have cautioned the use of this targeted treatment. Lovastatin is an FDA-approved statin, originally intended to manage high cholesterol. Lowering cholesterol in males with FXS, a group observed to have pathologically low baseline cholesterol levels, could lead to unintended negative consequences (Berry-Kravis et al., 2015). Further, it is important to emphasize that the lithium and lovastatin trials were open label, without placebo controls.

It is now well understood that there is a large placebo effect using the endpoints employed in these studies. At best, the results to date suggest that placebo-controlled studies are worth pursuing.

A pilot randomized, double blind, placebo-controlled crossover trial using minocycline, an antibiotic that inhibits overexpressed synaptic MMP9, a known target of FMRP, has also shown promise in the clinic. Chronic minocycline treatment demonstrated mild global clinical improvement, as well as a significant reduction in MMP levels in the blood of responders (Dziembowska et al., 2013; Leigh et al., 2013). Treatment with the $GABA_B$ agonist arbaclofen (STX209) in a phase II double-blind placebo-controlled crossover trial showed improvement over placebo in measures of social withdrawal and parent-identified "problem behaviors," prompting a larger phase III placebo-controlled trial in adults and adolescents with FXS (Berry-Kravis et al., 2012). Despite showing significant improvements on various behavioral scales and meeting secondary endpoints, the study fell short of meeting its primary outcome measure of social avoidance. Perhaps most disappointing, based on the optimism generated by the multitude of studies providing preclinical validation in the *Fmr1* KO mouse model, has been the failure of $mGlu_5$ inhibitors in clinical trials. Treatment with mavoglurant (AFQ056; Novartis), or basimglurant (RO4917523; Roche), both negative allosteric modulators of $mGlu_5$, have demonstrated no therapeutic benefit in patients with FXS (Scharf et al., 2015).

FAILURE IN THE CLINIC AND WHAT WE CAN LEARN

Thus far, identification of preclinical targets by investigation of animal models of FXS has not been successfully translated to therapeutically beneficial treatments for behavioral and cognitive impairments in patients with FXS. This raises a number of questions that are important to evaluate prior to the development and evaluation of additional drugs for FXS. The history of drug development for complex idiopathic neuropsychiatric disorders based on animal testing is replete with failures (Hyman, 2012), and it is tempting to conclude that the latest fragile X experience is just one more reason to discard animal models. We strenuously disagree with this point of view. Unlike previous attempts to model psychiatric disease in rodents based on phenocopy (e.g., learned helplessness in a forced swim test as a model for depression), fragile X experiments have been conducted on model organisms that have been engineered to reproduce the same genetic etiology as the human disease (a particular advantage of a monogenic disorder). Moreover, genetically validated models for fragile X include not only mice, but also *Drosophila*, zebrafish, and most recently rats, separated by tens of millions of years of evolution. It seems reasonable to conclude that if all these divergent species demonstrate the same core pathophysiology (e.g., excessive protein synthesis) that responds to the same treatment strategies, then it is very likely that this is also a feature of the human disease. Of course, how this pathophysiology is expressed structurally and behaviorally will vary depending on the species and the niche it occupies, and this is where translating insight from one species to another is particularly fraught. In the design of human clinical trials, best guesses must be made on drug dosing, treatment durations, patient selection (ages, symptom severity) and endpoints for the evaluation of efficacy. These choices are constrained by the potential for drug toxicity and side effects, the substantial costs of conducting well-powered and long duration trials, and precedents for regulatory approval.

The recent clinical study of mavoglurant clearly illustrates these challenges (Berry-Kravis et al., 2016). The youngest subjects were 12 years old, the treatment duration was 3 months, and the primary endpoint was improvement on a parent-rated aberrant behavior scale, now understood to be strongly responsive to placebo. These choices were all reasonable considering the various constraints, but none were clearly guided by the animal work except for the choice of target ($mGlu_5$). 30+ days of chronic treatment in mice, which successfully reversed many fragile X phenotypes, corresponds to years in a human. Moreover, the most striking and consistent behavioral improvements in the animal models, reduced seizure incidence and improved cognition, were not explicitly tested in the human study. It is also important to recognize that treatment based on the mGluR theory and normalization of aberrant protein synthesis is meant to be disease-modifying, not palliative. Improvement in most symptoms would be expected to take time, and be aided by concurrent cognitive-behavioral therapy. The clinical investigators are well aware of these limitations, and perhaps the most encouraging outcome of this first large human study is the commitment of these clinicians to repeat the trial with a much longer (20 month) treatment period and quantitative, cognitive endpoints (Berry-Kravis & Hagerman, 2016). At this time, the jury is still out on the applicability of the mGluR theory to humans.

The success of the theory in animals has come from the fact that much of the aberrant protein synthesis apparently is downstream of a single receptor, $mGlu_5$. The human forebrain expresses $mGlu_5$ at a very high level (Brown et al., 2008), but it is entirely possible that there is functional redundancy in the neurotransmitter systems linked to FMRP-regulated protein synthesis. In this context, it is interesting to note that although many fragile X phenotypes in mice are corrected by chronic inhibition of $mGlu_5$, some (e.g., AGS) show evidence of rapid drug tolerance (tachyphylaxis) (Yan et al., 2005). The possibility of redundancy or compensation could be addressed with combination therapies targeting more than one receptor (e.g., $mGlu_1$ and $mGlu_5$), or therapies that target points of signaling convergence (e.g., Ras-ERK or GSK3). Moreover, as discussed previously, activation of $mGlu_5$ engages multiple signaling pathways, of which only a subset are relevant to the pathophysiology of FXS. Global inhibition of $mGlu_5$ signaling will interfere with physiological processes unrelated to synaptic mRNA translation, and this might offset behavioral improvements in fragile X or give rise to side effects that constrain drug dosages. For instance, $mGlu_5$ is known to modulate NMDA receptors, which are vital for synaptic function and plasticity (H. H. Chen, Liao, & Chan, 2011; Collett & Collingridge, 2004; Okubo, Kakizawa, Hirose, & Iino, 2004). Ideally, one would like to target exclusively the pathway that physically couples glutamatergic activation of $mGlu_5$ to the translational machinery, bypassing effects on unrelated "canonical" signaling pathways.

NEW DIRECTIONS

$mGlu_5$ and the downstream signaling pathways that couple the receptor to FMRP-regulated protein synthesis are extraordinarily well-validated targets in the animal models of fragile X (see Bhakar et al., 2012 and Tables 9.1 and 9.2). As mentioned, there are a number of issues that need to be solved empirically before we know the extent to which these insights apply to human fragile X. In the meantime, however, work continues in the animal models to

identify new targets in anticipation of the possibility that the obstacles encountered in human trials with existing validated approaches cannot be overcome. Here we discuss some of the ongoing efforts.

Beta Arrestins: a Scaffold for Ras-ERK and Modulator of Signaling

Honing in on the disease-relevant targets in the pathology of FXS could lead to better targeted-drug development and greater success in restoring social, behavioral and cognitive impairments in clinical trials. Indirect inhibition of the ERK signaling pathway has yielded promising results in preclinical and clinical studies (Caku et al., 2014; Osterweil et al., 2013; Pellerin et al., 2016). However, there are significant concerns regarding global inhibition of Ras farnesylation in patients with FXS (Berry-Kravis et al., 2015). The ideal target would be one that couples $mGlu_5$ activation to synaptic translation but leaves G-protein-dependent signaling unaltered. β-arrestins are adaptor proteins that are important for the regulation of GPCRs, and have been shown to be directly involved in G-protein-independent signaling pathways. Recently, many studies have shown a functional divergence of β-arrestin isoforms in GPCR function (Srivastava, Gupta, Gupta, & Shukla, 2015). Specifically, at the angiotensin II receptor, β-arrestin2 has been shown to recruit the ERK pathway in a manner that is both temporally and spatially distinct from G-protein dependent ERK activation (Ahn, Kim, Hara, Ren, & Lefkowitz, 2009; DeWire et al., 2008), and to stimulate protein synthesis (DeWire et al., 2008). It is now understood that β-arrestin- and G protein-dependent cellular signaling are pharmacologically separable, and β-arrestin-biased allosteric modulators of mGluRs are feasible (Hathaway et al., 2015; Iacovelli, Felicioni, Nistico, Nicoletti, & De Blasi, 2014; Sheffler, Gregory, Rook, & Conn, 2011). The next generation of $mGlu_5$ inhibitors for fragile X might be those that specifically modulate β-arrestin-dependent signaling, and leave G_q signaling intact.

GSK3α and GSKβ

Inhibition of GSK3α/β with either lithium or nonspecific GSK3 inhibitors has been used to treat fragile X phenotypes in the mouse model (see Table 9.3) in experiments that were motivated by early findings in *Drosophila* (McBride et al., 2005). Although GSK3α and GSK3β are commonly referred to as isoforms, they are actually paralogs, derived from different genes. GSK3α and GSK3β share 85% overall sequence homology and 98% amino acid sequence identity within their kinase domains; however, GSK3α has an extended N-terminal region (Dajani et al., 2001; Woodgett, 1990). Both GSK3α and GSK3β are highly expressed in the mouse hippocampus postnatally, in both neurons and glia (Ferrer, Barrachina, & Puig, 2002; Perez-Costas, Gandy, Melendez-Ferro, Roberts, & Bijur, 2010; Woodgett, 1990; Yao, Shaw, Wong, & Wan, 2002). Paralog-specific GSK3 KO animals have been generated. GSK3β KO mice die late in fetal development, however GSK3β heterozygous mice are viable and have been extensively characterized (Beaulieu et al., 2004; Hoeflich et al., 2000; O'Brien et al., 2004). GSK3α KO mice are viable and exhibit similar phenotypes as GSK3β heterozygous mice that include memory impairments (Beaulieu et al., 2004; Bersudsky et al., 2008; Kaidanovich-Beilin et al., 2009; Kimura et al., 2008; MacAulay et al., 2007; O'Brien et al., 2004). Importantly, there are notable differences in the contribution of GSK3α

and GSK3β, especially in the involvement of neuropsychiatric disease (Kaidanovich-Beilin et al., 2009). For example, genetic reduction of GSK3α specifically reverses abnormalities in prepulse inhibition (Cooper et al., 2011), hyperactivity, and dendritic spine development in the Disc1-L100P mouse model of schizophrenia (Lee, Kaidanovich-Beilin, Roder, Woodgett, & Wong, 2011; Lipina et al., 2011).

Intriguingly, the *Fmr1* KO mouse shares similar deficits in PPI, hyperactivity and spine abnormalities, suggesting that paralog-specific inhibition of GSK3 could offer enhanced therapeutic benefit by specifically targeting the enzyme which is relevant to the pathophysiology of FXS. It was once hypothesized that development of paralog-specific small molecule inhibitors would be a near impossibility due to the high sequence identity of the GSK3α and GSK3β kinase domains (Kaidanovich-Beilin & Woodgett, 2011). In fact, most currently available GSK3 inhibitors lack true selectivity, most often nonspecifically acting on cyclin-dependent kinases as well (O'Leary & Nolan, 2015). However, highly selective inhibitors of both GSK3α and GSK3β have recently been developed, and it will be interesting to test whether these compounds can correct alterations in the *Fmr1* KO mouse model. The appeal of GSK3 as a target is that it provides another way to correct excessive protein synthesis in fragile X, but may do so via a mechanism that is distinct from that recruited by $mGlu_5$.

Novel Targets From the FXS Translatome

The identification of the mRNA targets of FMRP has been the focus of several important studies (Brown et al., 2001; Darnell et al., 2011). The conclusion from this work is that there are hundreds of mRNAs regulated by FMRP, many of which are important for synaptic function. However, it is not clear that these are the same mRNAs altered in FXS. Indeed, as the developed FXS brain is the result of FMRP loss that occurred much earlier in development, it would not be unexpected if the resulting changes in the translatome represent a number of compensatory changes that allow for some functional stability in the absence of FMRP. The current challenge is to understand the functional ramifications of such a substantial loss of translation regulation so that new targets for treatment can be identified.

The complete list of mRNAs that are aberrantly translated in fragile X is not known. This information is critical for understanding how altered translation leads to pathology in FXS. Previous work using proteomic approaches in *Fmr1*$^{-/y}$ cultured neurons, whole brain, and isolated synaptic (synaptoneurosome) fractions have found subtle changes in the expression of some proteins (Klemmer et al., 2011; Liao, Park, Xu, Vanderklish, & Yates, 2008; Tang et al., 2015; Vanderklish & Edelman, 2005). While these studies have been informative about the cellular disturbances in FXS, it is not clear how many of the observed changes are the result of altered translation per se. It is also not clear that the proteins identified are responsible for functional changes in the FXS brain. Given the abundance of mRNA targets of FMRP, and the variety of neuronal circuits that are abnormal in the FXS brain, it is highly likely that the group of aberrantly translated proteins contributing to pathological changes in FXS will vary between different neuronal populations. Therefore, new strategies to identify cell type specific changes in translation could be particularly useful for understanding how changes in proteins synthesis lead to disrupted function in different areas of the FXS brain. There is little doubt that the next few years will reveal a substantial amount of new information about the nature of translational dysfunction in FXS.

CONCLUDING REMARKS

The mGluR theory has had a major impact on the FXS field. What was once viewed as an intractable developmental disorder, is now approached as a disease that can be substantially corrected with targeted therapeutic intervention. Testing the theory in animals has provided a wealth of information on the synaptic control of neuronal protein synthesis and the pathophysiology of FXS. Human studies inspired by the theory have provided many glimmers of hope that insights from animal models can indeed be translated into new therapies for genetically defined diseases, and have informed the design of new and improved clinical trials. There is good reason to remain optimistic that disease-modifying treatments for FXS are within reach.

What began as an "mGluR theory" has been broadened in the past decade to include the signaling pathways that control aberrant protein synthesis and its consequences. Further research is required to establish the mechanisms that link neuronal activity and mRNA translation, as well as to understand the contribution of dysregulated mRNA translation to the pathology of FXS.

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CHAPTER

10

The GABAergic System Contributions to the Fragile X Syndrome Phenotype

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INTRODUCTION

FXS is caused by the expansion of an unstable CGG tri-nucleotide repeat in the regulatory (5'UTR) region of the *Fmr1* gene located on the X-chromosome. When the expansion reaches greater than 200 CGG repeats a silencing of the *Fmr1* gene occurs as a result of hypermethylation (Hagerman et al., 2009; Penagarikano, Mulle, & Warren, 2007). This restriction leads to extreme reduction or elimination of the protein product fragile X mental retardation protein (FMRP). Both the severity and physical manifestation of behavioral symptoms is linked to the amount of FMRP reduction (Loesch, Huggins, & Hagerman, 2004). *Fmr1* knock out (KO) mice have a deletion of the *Fmr1* gene, which results in a total lack of FMRP protein in the animal (Chapter 7). FMRP is widely expressed throughout the brain, in both glia and neurons—including inhibitory interneurons (Olmos-Serrano et al., 2010; Wang et al., 2004; Devys, Lutz, Rouyer, Bellocq, & Mandel, 1993; Feng et al., 1997). The *Fmr1* KO mice display many of behavioral alterations and neuroanatomical deficiencies compatible with the human condition making them an ideal model to study cellular and synaptic abnormalities (Bakker & Oostra, 2003; Kooy, 2003). In *Fmr1* KO mice, a complete loss of this broad-spectrum protein indicates a disruption in normal maturation and function in both cells and synapses.

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For example, FMRP likely regulates the expression of a number of synaptic proteins including: cation channels, adhesion molecules, neurotransmitter receptors, and components of vesicle transport and vesicle release machinery (Liao, Park, Xu, Vanderklish, & Yates, 2008; Brown et al., 2001; Schütt, Falley, Richter, Kreienkamp, & Kindler, 2009). Therefore, these studies suggest broad cellular and synaptic alterations in the FXS brain. This chapter will explore how the lack of FMRP affects the expression and function of key components of the GABAergic system from interneurons to inhibitory synapses and finally to GABA receptors. The identification of the different sources of dysfunction may provide potential additional avenues of therapeutic rescue.

INHIBITORY INTERNEURON DYSFUNCTION IN FXS

FMRP expresses in both excitatory and inhibitory interneurons. Therefore, the absence of FMRP is expected to affect both excitatory and inhibitory cellular function. While outnumbered by excitatory neurons, local circuit inhibitory interneurons exert a wide reaching control over large populations of excitatory networks. Interneurons control the excitability, temporal integration, and the rhythmic output of both excitatory principal cells, as well as other populations of inhibitory interneurons (Cobb, Buhl, Halasy, Paulsen, & Somogyi, 1995; Klausberger & Somogyi, 2008; Freund & Katona, 2007). Electrical and chemical synapses provide for the neuronal responses that are time-locked in the same temporal window of their preferential oscillatory frequency and thus show direct involvement in the synchronization and control of excitatory networks (Klausberger & Somogyi, 2008). Thus it is likely that a causal relationship exists between interneuron dysfunction and disorders with altered synchronization, such as epilepsy, FXS and schizophrenia (Lewis, Hashimoto, & Volk, 2005; Huntsman, Porcello, Homanics, Delorey, & Huguenard, 1999; Sohal, Keist, Rudolph, & Huguenard, 2003; Paluszkiewicz, Olmos-Serrano, Corbin, & Huntsman, 2011; Goncalves, Anstey, Golshani, & Portera-Cailliau, 2013; Braat & Kooy, 2015a; Contractor, Klyachko, & Portera-Cailliau, 2015).

In the Fragile X brain, multiple studies reveal that neuronal activity is both hypersynchronous and hyperexcitable (Goncalves et al., 2013; Rotschafer & Razak, 2013; Paluszkiewicz et al., 2011; Gibson, Bartley, Hays, & Huber, 2008). This could be causal to the increased incidence of seizures and sensory integration dysfunctions associated with FXS (reviewed in Hagerman & Stafstrom, 2009; Hagerman et al., 2009; Musumeci et al., 1999). Based on the ability to synchronize the activity of large neuronal networks it is likely that inhibitory interneurons play important roles in this phenotype. For example, inhibitory interneurons can target either the soma or dendrites of the postsynaptic cell. Soma targeting interneurons have a role in the synchronization of network circuits by imposing a rhythm while dendritic-targeting cells participate in the propagation of synchronized activity waves throughout the network. For example, interneurons that exclusively target the soma, such as the parvalbumin positive basket cell mainly controls pyramidal cell excitability by regulating Na^+-dependent action potential initiation (Freund & Katona, 2007). In contrast, the dendritic targeting somatostatin positive cells strongly affect local integration by regulating dendritic Ca^{2+}-dependent spike initiation and propagation (Miles, Tóth, Gulyás, Hájos, & Freund, 1996).

SYNAPTIC COMPONENTS AT GABAergic SYNAPSES ARE DYSREGULATED IN FXS

As FMRP is widely expressed throughout the brain (Olmos-Serrano et al., 2010; Wang et al., 2004; Devys et al., 1993; Feng et al., 1997) the deletion of this broad spectrum protein results in widespread alterations in the expression of mRNA and proteins in both interneurons and glia (Fig. 10.1, Table 10.1). These proteins range from a number of functional categories, including: cation channels, adhesion molecules, neurotransmitter receptors, and components of the vesicular transport and release machinery (Liao et al., 2008; Brown et al., 2001; Schütt et al., 2009). Therefore, FMRP is a broad range functional protein with the capability of imposing widespread effects on cellular and synaptic alterations of both presynaptic and postsynaptic components in the FXS brain (Christie, Akins, Schwob, & Fallon, 2009; Gantois et al., 2006). Alterations of glutamic acid decarboxylase (GAD) have been reported in *Fmr1* KOs (D'Hulst, Atack, & Kooy, 2009; Adusei, Pacey, Chen, & Hampson, 2010; Olmos-Serrano et al., 2010; Braat et al., 2015) along with decreased presynaptic terminals indicating lower vesicular GABA levels and quantal content (Vislay et al., 2013). In addition, proteins required for GABA transport (GAT) and catabolism (GABA-T, SSADH) also exhibit decreased expression in a number of brain regions (D'Hulst et al., 2009; Adusei et al., 2010; Liao et al., 2008). Importantly, FMRP is broadly expressed in multiple subtypes of GABAergic interneurons (Olmos-Serrano et al., 2010; Feng et al., 1997), indicating that it is involved in normal interneuron maturation and function.

A heterogeneous population of interneuronal subtypes synthesizes and releases GABA, which exerts its effects through three distinct forms of GABAergic neurotransmission. The first two forms are mediated by ionotropic $GABA_A$ receptors via synaptically located receptors that mediate "phasic" inhibition and extrasynaptically located receptors that mediate "tonic" inhibition. The third form of inhibition is mediated through metabotropic G-protein-coupled $GABA_B$ receptors. The components of the GABAergic system were first implicated in the pathogenesis of FXS based on the observation of reduced $GABA_A$ receptor expression in *Fmr1* KO mice (Gantois et al., 2006; D'Hulst et al., 2006; El Idrissi et al., 2005). Functional $GABA_A$ receptors are hetero-pentameric structures nonrandomly composed of 19 subunits, including $\alpha_{1–6}$, $\beta_{1–3}$, $\gamma_{1–3}$, δ, ε, θ, π, $\rho_{1–3}$ (Farrant & Nusser, 2005). This results in a wide variety of subtypes where subunit composition determines subcellular localization, response kinetics, and sensitivity to a number of clinically relevant compounds (Rudolph & Möhler, 2006; Hevers & Lüddens, 1998; Sieghart & Sperk, 2002; D'Hulst et al., 2009). For instance, the receptor subtypes receptors that contain an $\alpha_{1–3}$, $\beta_{2/3}$, and a γ_2 subunit are mainly synaptic, whereas $\alpha_{4–6}$- and δ-containing receptors are mainly perisynaptically or extrasynaptically located.

In mature neurons, $GABA_A$ receptors permit the influx of chloride ions in the presence of GABA resulting in hyperpolarization of the postsynaptic membrane. Phasic inhibition is mediated in response to relatively high concentrations (mM) of synaptically-released neurotransmitter. Extrasynaptic $GABA_A$ receptors produce a slow, persistent tonic conductance in response to very low neurotransmitter concentrations (nM to low μM) in the extrasynaptic space (for review see Farrant & Nusser, 2005). Conversely, postsynaptic $GABA_B$ receptor activation hyperpolarizes the postsynaptic membrane by activating G-protein inwardly rectifying K^+ channels (GIRKs) to produce slow inhibitory currents (Padgett & Slesinger, 2010; Newberry & Nicoll, 1984). Presynaptic $GABA_B$ receptors control neurotransmitter release via

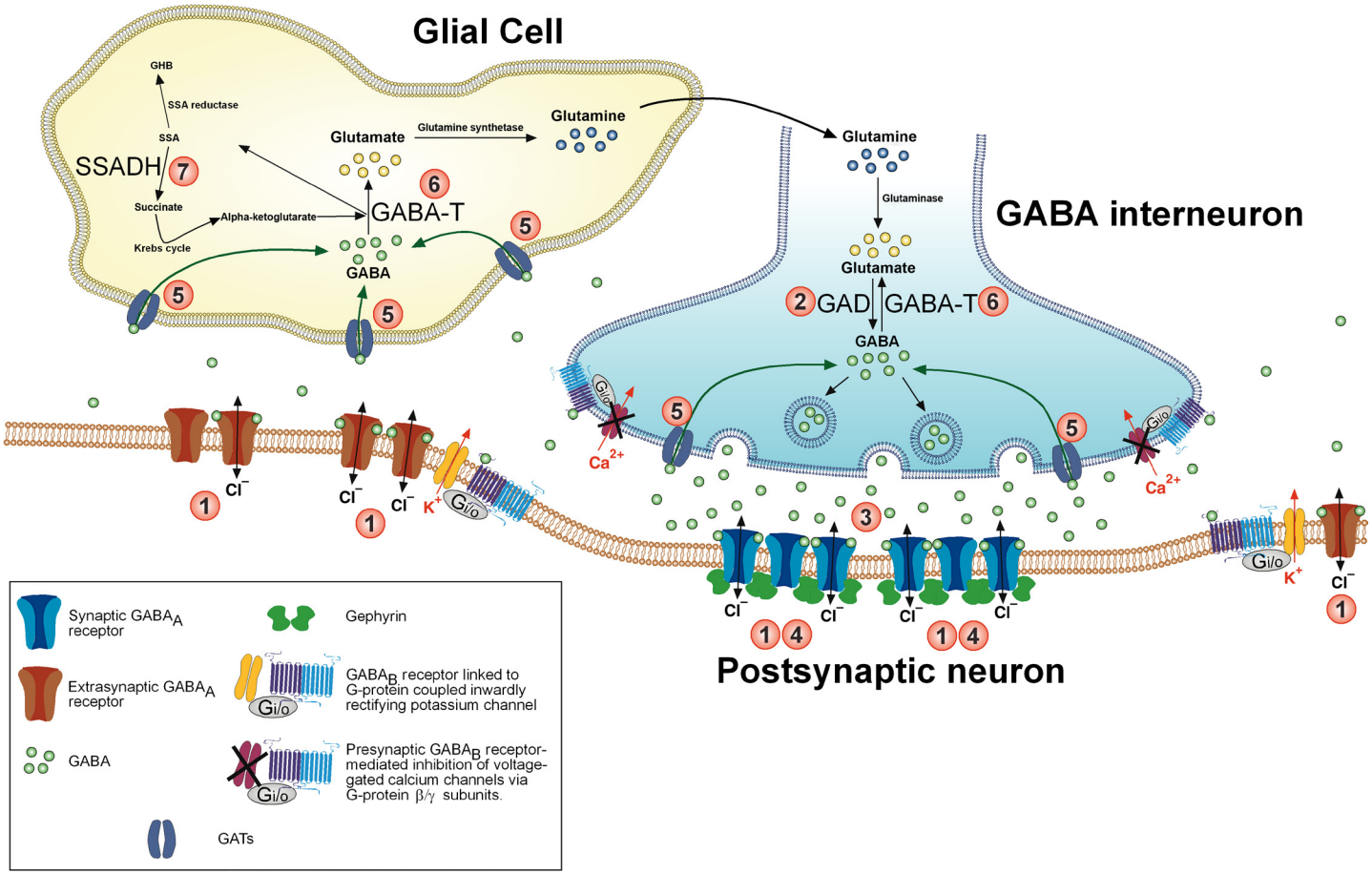

FIGURE 10.1 **Several GABAergic synapse components exhibit altered expression in the *Fmr1* KO mouse model of FXS.** Numbers identify key synaptic proteins disrupted in *Fmr1* KOs, including $GABA_A$ receptors, enzymes involved in GABA production and catabolism (see inset legend and Table 10.1). *GAD*, Glutamic acid decarboxylase; *GAT*, GABA transporters; *GHB*, gamma hydroxy butyrate; *GABA-T*, GABA transaminase; *SSA*, succinyl semialdehyde; *SSADH*, succinyl semialdehyde dehydrogenase. *Source: Modified from Paluszkiewicz, S., Martin, B., & Huntsman, M. (2011). Fragile X Syndrome: the GABAergic system and circuit dysfunction.* Developmental Neuroscience, *33, 349–364.*

TABLE 10.1 GABAergic Synapse Components With Altered Expression in *Fmr1* KO Mice

Serial number	Molecule	Function	Brain region	Expression	References
1	$GABA_A$ receptor subunits (α, β, γ, δ)	Ionotropic GABA receptor function, localization	Cortex, subiculum, hippocampus	↓ mRNA, protein	El Idrissi et al. (2005); D'Hulst et al. (2006); Gantois et al. (2006); Curia et al. (2009); Adusei et al. (2010)
2	GAD	GABA synthesis	Cortex, cerebellum	↓ mRNA	D'Hulst et al. (2009);
			Amygdala	↓ protein	Olmos-Serrano et al. (2010);
			Whole forebrain, cortex	↑ protein	El Idrissi et al. (2005); Adusei et al. (2010)
3	GABA	Ligand	Amygdala	↓ release	Olmos-Serrano et al. (2010)
4	Gephyrin	GABA receptor clustering	Cortex	↓ mRNA	D'Hulst et al. (2009)
5	GAT1,4	GABA reuptake	Whole forebrain, cortex, cerebellum	↓ mRNA, protein	Liao et al. (2008); D'Hulst et al. (2009); Adusei et al. (2010)
6	GABA-T	GABA catabolism	Cortex	↓ mRNA, protein	D'Hulst et al. (2009); Adusei et al. (2010)
7	SSADH	GABA catabolism	Cortex, cerebellum	↓ mRNA, protein	D'Hulst et al. (2009); Adusei et al. (2010)

voltage dependent calcium channels (Chen & van den Pol, 1998). In addition to its role as a postsynaptic inhibitory neurotransmitter, GABA can also modulate neurotransmitter release in an autocrine or paracrine fashion, via distinct mechanisms at presynaptic $GABA_A$ and $GABA_B$ receptors (Bettler, Kaupmann, Mosbacher, & Gassmann, 2004; Trigo, Marty, & Stell, 2008). Presynaptic $GABA_B$ receptors are expressed on GABAergic and glutamatergic terminals to inhibit neurotransmitter release and as such have the ability to alter inhibitory connections and excitatory synapses (Chen & van den Pol, 1998; Isaacson & Hille, 1997).

The lack of FMRP affects the expression and function of key components of GABAergic neurons, synapses, and GABA receptors. Broad reductions of GABA synthesis and release affect the strength of synaptic inhibitory transmission that may contribute to hyperexcitability in brain areas relevant to the behavioral phenotype of FXS (Vislay et al., 2013). Reduced GABA availability reduces GABA concentration in the synaptic cleft (Olmos-Serrano et al., 2010; Braat et al., 2015; Davidovic et al., 2011). Additionally, reduction of GABA in the extrasynaptic space regulates a powerful GABAergic tonic conductance that can significantly affect excitability and excitatory/inhibitory balance of a neuronal network (Martin, Corbin, & Huntsman, 2014). GABAergic tonic inhibition powerfully controls cellular excitability (Brickley, Cull-Candy, & Farrant, 1996; Hamann, Rossi, & Attwell, 2002; Mitchell & Silver, 2003; Semyanov, Walker, & Kullmann, 2003; Krook-Magnuson & Huntsman, 2005; Bright, Aller, & Brickley, 2007) and may therefore affect thresholds for excitatory neuronal activity in multiple

brain regions. FMRP has been demonstrated to bind directly to several subunits of the $GABA_A$ receptor, including the δ-subunit, a major extrasynaptic subunit in brain regions associated with behavioral symptoms observed in FXS (Dictenberg, Swanger, Antar, Singer, & Bassell, 2008; Braat et al., 2015). In these regions, δ subunit expression (mRNA, protein, or both) is reduced (D'Hulst et al., 2006; Curia, Papouin, Seguela, & Avoli, 2009) in the *Fmr1* mouse model of FXS. Future electrophysiological studies are needed to examine the state of excitatory/inhibitory balance. The involvement of the GABAergic system in maintaining that balance may provide potential avenues of therapeutic rescue.

TARGETING DEFICIENCIES OF THE GABAergic SYSTEM IN FXS AS VIABLE TREATMENT OPTIONS

Complementary deficits in excitatory and inhibitory function support a prevalent causal theory for many of the symptoms of FXS. Several marked symptoms of FXS indicate decreased inhibitory function of the GABAergic system – most notably anxiety, autistic behaviors and epilepsy (Berry-Kravis et al., 2010; McNaughton et al., 2008; Cordeiro, Ballinger, Hagerman, & Hessl, 2011; Grigsby et al., 2007; D'Hulst & Kooy, 2007). Therefore, the GABAergic system remains a potentially viable, underexplored complement to FXS treatments currently mostly aimed at dampening excessive mGluR signaling (Braat & Kooy, 2014, 2015b). Rescuing inhibition via tonically active $GABA_A$ receptors may provide a better option that lacks the sedative side effects associated with especially the α_1 subunit of the phasic $GABA_A$ receptors located in synapses. Tonic conductance is stronger than phasic inhibitory conductance in magnitude and therefore can more effectively and dynamically control neuronal excitability (Semyanov et al., 2003; Mitchell & Silver, 2003). Benzodiazepine sensitive $GABA_A$ receptor subtypes mediate different aspects of receptor that may result in unwanted side effects, such as sedative and addictive actions, which are largely based on subunit composition and regional expression patterns (reviewed in Mohler, Fritschy, Crestani, Hensch, & Rudolph, 2004). However, most of these side effects are a consequence of the direct binding with the α_1 subunit. Over the last years, drugs have been developed that specifically bind to various other subunits and that are devoid of the sedative and addictive actions, but that maintain the anxiolytic properties (reviewed in D'Hulst et al., 2009). Nevertheless, targeting extrasynaptic receptors, such as δ subunit-containing receptors may provide a more isolated pharmacological target. For example, neuroactive steroids often have a much greater specificity for extrasynaptic receptors than they have for synaptically located phasic receptors (Reddy, 2010). Most of these compounds are effective at reducing hyperexcitability in FXS as demonstrated by the reduction of audiogenic seizures and dose-dependent correction of marble burying behavior in mice dosed with the neuroactive steroid ganaxolone (Heulens, D'Hulst, Van Dam, De Deyn, & Kooy, 2012; Braat et al., 2015). In addition as described earlier, the δ-subunit preferring agonist THIP can rescue cellular and behavioral phenotypes in the *Fmr1* KO mouse (Olmos-Serrano, Corbin, & Burns, 2011; Olmos-Serrano et al., 2010). The pharmacological targeting of extrasynaptic receptors may carry reduced potential for unwanted side effects by enabling targeting of a more specific $GABA_A$ receptor pool. Targeting extrasynaptic receptors to modulate neuronal networks thus is a promising strategy for therapeutic intervention for a myriad of FXS symptoms (Lozano, Hare, & Hagerman, 2014; Braat & Kooy, 2015b).

Direct support for the potential of GABAergic treatment in FXS theory comes from studies in *Drosophila* deficient in the fragile X proteins (Chapter 7). In an elegant study, flies were reared on food containing a significant amount of glutamate, as a result of which the flies died before full development (Chang et al., 2008). The flies were screened for survival after adding a collection of, at that time, 2000 different FDA-approved drugs in combination with natural products. After thorough validation studies, nine compounds appeared capable of rescuing fly lethality. Three of those are directly linked to the GABAergic system, including GABA itself, nipecotic acid (a GABA reuptake inhibitor), and creatinine (a potential $GABA_A$ receptor activator). These compounds appeared able to correct specific phenotypes of *dfmr1* deficient *Drosophila*. All three compounds reduced overexpression of *Futsch*, a known Fmrp mRNA target and *Drosophila* MAP1B orthologue. Moreover, GABA improved structural defects in the mushroom body, axon-like fiber structures that terminate at the midline in control flies. However, in *dfmr1* mutants these cross through the midline. GABA, nipecotic acid, and creatinine were also able to improve additional mushroom body deficits. The compromised courtship behavior of *dfmr1* deficient males could also be rescued by the addition of GABA to the food. However, GABA or nipecotic acid food supplementation was not able to restore mushroom body-dependent olfactory learning defects in *dfmr1* mutant flies (Gatto et al., 2014).

PREVENTING DEPOLARIZING GABAergic POTENTIALS IN DEVELOPING CIRCUITS

While $GABA_A$ receptors are inhibitory neurotransmitter receptors during adulthood, there is overwhelming evidence that the same receptors are excitatory in early life (reviewed by Ben-Ari et al., 2012). Depolarizing of GABA acting on $GABA_A$ receptors has been known to occur in developing and early postnatal circuits for quite some time (Ben-Ari, Cherubini, Corradetti, & Gaiarsa, 1989). The switch from excitatory to inhibitory occurs following changes in intracellular chloride concentration, mediated by changes in expression of the two ion transporters Na^+–K^+–$2Cl^-$ cotransporter 1 (NKCC1) and K^+–Cl^- cotransporter 2 (KCC2). The intracellular chloride concentration is the result of the balance between the activity and amount of the importer NKCC1 and the exporter KCC2. Early in development, the NKCC1 cotransporter activity results in maintainance of a high intracellular chloride concentration and consequently to GABA-mediated depolarization. During maturation, the expression of the cotransporter NKCC1 is reduced, while the expression of the cotransporter KCC2 is increased, resulting in decreased intracellular chloride concentrations and hyperpolarizing GABA signaling. This phenomenon is required for proper circuit development (Cherubini & Ben-Ari, 2011) and has been implicated in the autistic behavioral phenotype (Cherubini, Griguoli, Safiulina, & Lagostena, 2011). A recent study illustrates that the developmental switch from depolarizing to hyperpolarizing GABA signaling is delayed in the Fragile X brain (He, Nomura, Xu, & Contractor, 2014). In cortical neurons, hyperpolarizing and thus inhibitory GABAergic signaling occurs within the first week (P8). However in the *Fmr1* KO mouse model of FXS, this switch does not occur until P13–14. In cortical circuits this coincides with the critical period of plasticity. Any disruption during this period could result in altered sensory responsiveness, cognitive impairments, and cortically originating epilepsies in FXS patients (Hagerman & Stafstrom, 2009). In the hippocampus, the driving force for depolarizing GABA

is elevated intracellular chloride levels (Tyzio et al., 2014). In this study, prenatal treatment with bumetanide, an inhibitor of NKCC1 activity that works primarily on extrasynaptic $GABA_A$ receptors rescues hyperexcitability and autistic behaviors. These recent studies provide a platform to look more deeply into the control of chloride homeostasis and thus depolarizing GABAergic signaling in developing circuits. Prevention and correction of improper inhibitory circuit formation is likely to be more effective than to correct circuits in the fully mature brain. Identification of key developmental time windows may prove to be effective in treating FXS and other neurodevelopmental disorders (Meredith, Dawitz, & Kramvis, 2012; Contractor et al., 2015).

CONCLUSIONS

Defective GABAergic system components in adult and developing brain circuits appear to be an emerging theme in neurodevelopmental disorders and clinical trials with the neurosteroid drug ganaxolone are in progress (Chapter 19). *Fmr1* KO mice have proven to be an effective model to reveal these deficiencies as they reveal disturbances of the GABAergic system and functional inhibitory neurotransmission in a number of brain regions that are highly relevant to the FXS phenotype. Thus, the GABAergic system presents an important pharmacological target for the treatment of a number of neurological manifestations of FXS. The biophysical aspects of synaptic and extrasynaptic localization of tonically active $GABA_A$ receptors makes these particular GABAergic components intriguing candidates to improve function of abnormally developed networks by regulating circuit excitability and output at key time points in development.

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CHAPTER

11

Intracellular Signaling Networks in Fragile X Syndrome: Approaches to Drug Discovery and Therapeutics

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INTRODUCTION

Signal transduction has been the focus of extensive research in virtually all areas of biomedical science. Pharmacological interventions targeting diverse signaling pathways have been developed and used in many basic, preclinical, and clinical studies. Dysregulated membrane receptor–mediated signaling, one of the major characteristics of fragile X syndrome (FXS), is therefore an area of particular interest for developing therapeutic strategies for this disease.

One of the first molecular signaling defects associated with loss of fragile X mental retardation protein (FMRP) was exaggerated signaling through group 1 metabotropic glutamate receptors (mGlu1/5) (Huber, Gallagher, Warren, & Bear, 2002), a class of Gq-coupled seven-transmembrane receptors that are important for activity-dependent protein synthesis and long-term synaptic plasticity. This finding by Huber et al. (2002) initiated the formulation of the "mGluR theory of FXS," which suggested that altered signaling through mGlu1/5 causes impaired neuronal function, and therefore is a promising therapeutic target in FXS (Bear, Huber, & Warren, 2004). The mGluR theory has been supported by many studies in animal models, (Dolen et al., 2007; Levenga et al., 2011; McBride et al., 2005; Michalon et al., 2012; Yan, Rammal, Tranfaglia, & Bauchwitz, 2005). However, clinical trials in patients with FXS

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based on this work have not been successful so far. Details about mGluR signaling in FXS, possible reasons for the difficulties encountered with recent clinical trials using mGluR5 inhibitors, and potential solutions to improve the outcome of future trials are further discussed in Chapters 9, 20, and 21 of this book.

Today, more than a decade after the mGluR theory of FXS was first published, it is becoming more and more evident that signaling through mGlu1/5 receptors is by far not the only membrane receptor–dependent signaling pathway that is dysregulated in FXS. Soon after the seminal finding of exaggerated mGlu1/5-signaling in *Fmr1* knockout (KO) mice, Volk, Pfeiffer, Gibson, and Huber (2007) reported that signaling through other Gq-coupled receptors is similarly upregulated in the absence of FMRP (also confirmed by others, e.g., Veeraragavan, Bui, Perkins, Yuva-Paylor, & Paylor, 2011). Later, other classes of seven-transmembrane receptors, such as endocannabinoid and dopamine receptors were shown to be altered in FXS mouse models (Busquets-Garcia et al., 2013; Wang, Kim, & Zhuo, 2010; Wang et al., 2008; Zhang & Alger, 2010). Moreover, signaling through entirely different types of membrane receptors, for instance the receptor tyrosine kinase TrkB and its ligand BDNF (Lauterborn et al., 2007; Osterweil, Krueger, Reinhold, & Bear, 2010; Uutela et al., 2014) or the interleukin-2 receptor (Gross & Bassell, 2012) are stimulus-insensitive or dysregulated in FXS mouse models and cells from individuals with FXS. Notably, altered intracellular signaling was also observed in FMRP-deficient nonneuronal cells (Gross and Bassell, 2012; Gross et al., 2010; Hoeffer et al., 2012; Jeon et al., 2011; Kumari et al., 2014). These recent discoveries suggest that impaired neuronal function in FXS is not merely caused by deficiencies in the receptors itself, but in fact is mediated by defects in the intracellular signaling pathways the membrane receptors converge on. Dysregulated intracellular signaling also provides a direct link to altered protein synthesis in the absence of FMRP, which is a central characteristic of FXS.

This chapter will summarize published evidence for defects in intracellular signaling pathways mediated by phosphoinositide 3-kinase (PI3K), extracellular regulated kinase 1/2 (ERK1/2), the small GTPase Ras, tuberous sclerosis complex 1–2 (TSC1–2), mammalian target of rapamycin (mTOR), p70 ribosomal S6 kinase 1 (S6K1), and eukaryotic initiation factor 4E (eIF4E) in the absence of FMRP, will review potential underlying molecular causes and discuss their use as therapeutic targets and biomarkers in FXS.

DYSREGULATED PI3K SIGNALING IN FXS

Aberrant activity and downstream signaling of PI3K have been frequently observed in fragile X animal models and in peripheral cells from patients with FXS. PI3K is a phospholipid kinase that catalyzes the phosphorylation of phosphoinositides at the third hydroxyl group of the inositol ring (Hawkins, Anderson, Davidson, & Stephens, 2006). The products of PI3K activity, mainly phosphoinositide-(3,4,5)-trisphosphates (PIP3), have multiple functions in the cell. They not only activate downstream signaling pathways important for protein synthesis, cell proliferation, and apoptosis (Vanhaesebroeck, Guillermet-Guibert, Graupera, & Bilanges, 2010), but also directly affect synaptic function by regulating AMPA receptor trafficking (Arendt et al., 2010). Here, we summarize findings of dysregulated PI3K signaling in FXS, describe the potential underlying causes, and discuss its contributions to the FXS phenotype and possible use as a therapeutic target.

PI3K Downstream Signaling is Defective in FXS Mouse Models

Activation of the PI3K pathway is often assessed by phosphorylation of the PI3K downstream target, Akt. Akt phosphorylation is induced by increased PIP3 levels at the membrane leading to recruitment of Akt and its upstream activator phosphoinositide-dependent kinase 1 (PDK1) to the membrane via their PIP3-responsive Pleckstrin homology (PH) domains (Downward, 1998). The first studies of altered Akt phosphorylation in *Fmr1* KO mice reported lack of histamine- or mGlu1/5-induced activation in hippocampal slices from *Fmr1* KO mice (Hu et al., 2008; Ronesi & Huber, 2008). A similar pattern of absent mGlu1/5-induced PI3K activation in *Fmr1* KO mice was observed in at least two other studies (Gross et al., 2010; Sharma et al., 2010). Another group found that shRNA-mediated reduction of *FMR1* in HeLa cells blunted the cellular stress-induced upregulation of Akt phosphorylation (Jeon et al., 2011). Together, these studies build strong support for a defect in stimulus-induced PI3K signaling in FXS.

At first view, studies quantifying basal levels of PI3K signaling in *Fmr1* KO mice have been less consistent, reporting unchanged (Osterweil et al., 2010; Ronesi & Huber, 2008), reduced (Hu et al., 2008; Jeon et al., 2011), and increased (Busquets-Garcia et al., 2013; Gross et al., 2010; Sharma et al., 2010; Sidhu, Dansie, Hickmott, Ethell, & Ethell, 2014) phosphorylation levels of the downstream target Akt. However, a closer analysis of this literature reveals that studies using freshly dissected hippocampal or cortical tissue consistently detected increased basal levels of Akt phosphorylation (Busquets-Garcia et al., 2013; Gross et al., 2010; Osterweil et al., 2010; Sharma et al., 2010; Sidhu et al., 2014), whereas analyses performed on hippocampal slices, which had been equilibrated in artificial cerebrospinal fluid for several hours prior to lysis, showed no changes or slight reductions of phosphorylated Akt (Hu et al., 2008; Osterweil et al., 2010; Ronesi & Huber, 2008). Observations of increased basal PI3K signaling in FXS may thus be sensitive to tissue preparation or divergent proteomic homeostasis in slices.

In summary, this supports a model in which elevated basal activity of the PI3K/Akt pathway leads to aberrant stimulus-induced signaling through PI3K/Akt. This notion is supported by the role FMRP plays in regulating the expression of components of the PI3K complex, and more recent observations of increased PI3K activity and signaling in cell lines, peripheral blood cells, and postmortem brain tissue from individuals with FXS, which will be discussed further in this chapter.

FMRP Regulates PI3K Activity by Controlling mRNA Translation and Protein Expression of PI3K Catalytic and Regulatory Subunits

Several screens identified components of the PI3K catalytic complex as potential mRNA targets of FMRP, pointing toward a direct role of FMRP in regulating PI3K expression and activity (Ascano et al., 2012; Brown et al., 2001; Darnell et al., 2011; Miyashiro et al., 2003). Candidate targets include mRNAs coding for PI3K catalytic subunits (p110α and p110β) and PI3K regulatory subunits [p85 and PI3K enhancer (PIKE)]. Of those, p110β and PIKE were confirmed as mRNA-binding partners of FMRP in an independent study (Gross et al., 2010). Loss of the translational suppressor FMRP in *Fmr1* KO mice leads to elevated mRNA translation of p110β, increased p110β and PIKE protein expression, and enhanced and stimulus-insensitive p110β-associated PI3K enzymatic activity (Gross et al., 2010; Sharma et al., 2010). In line with

an important role for increased PI3K/Akt signaling in FXS, broad spectrum PI3K inhibitors correct several molecular and cellular defects in vitro in cortical synaptic fractions or cultured hippocampal neurons from *Fmr1* KO mice (Gross et al., 2010; Swanger, Yao, Gross, & Bassell, 2011). More recently, genetic studies supported the importance of enhanced expression of the PI3K subunits p110β and PIKE for FXS-associated molecular, cellular, behavioral, and cognitive defects in vivo in mouse models (Gross et al., 2015a,b). Reduction of p110β or PIKE protein levels in *Fmr1* KO mice that were heterozygous for *Pik3cb* (p110β) or *Cntg1* (PIKE) decreased protein synthesis rates and dendritic spine density, improved behavior, and lowered susceptibility to audiogenic seizures. Interestingly, two phenotypes, stimulus insensitivity of protein synthesis and prolonged UP states, a form of spontaneous neocortical activity (Hays, Huber, & Gibson, 2011), were only rescued by reduction of PIKE, but not p110β. PIKE couples PI3K catalytic subunits to mGluRs (Rong et al., 2003), suggesting that PIKE's effect on other PI3K catalytic subunits, such as p110α, a reported target of FMRP (Ascano et al., 2012), might explain the more comprehensive rescue of phenotypes by genetic reduction of PIKE compared to p110β. Both strategies improved cognitive function in FXS animal models: heterozygous reduction of *CENG1A*, the *Drosophila* homolog of *Centg1*, restored courtship memory in a fly model of FXS (Gross et al., 2015a), and adult-onset knockdown of p110β in the prefrontal cortex of two different FXS mouse models rescued impaired goal-directed behavior and decision making, which are considered higher-order cognitive tasks (Gross et al., 2015b). Impaired higher-order cognition is a pivotal problem reducing quality of life in individuals with FXS and their caretakers. Improvement of cognition by targeting p110β in the adult animal therefore corroborates the PI3K complex as a promising therapeutic target in FXS.

When considering components of the PI3K enzymatic complex as therapeutic targets in FXS, it is noteworthy that isoform-selective inhibitors to PI3K catalytic subunits have already been developed and are currently in use in clinical trials with cancer patients (Cui, Cai, & Zhou, 2014). These isoform-specific inhibitors have the advantage of leaving other parts of the PI3K signaling complex intact, which reduces the probability of unwanted side effects. A p110β-selective inhibitor rescues increased protein synthesis in cortical synaptic fractions from *Fmr1* KO mice and in patient cells (Gross & Bassell, 2012; Kumari et al., 2014), supporting the potential efficacy of PI3K catalytic subunit-specific inhibition to rescue FXS-associated phenotypes. Further preclinical studies in animal models using pharmacological approaches are needed to evaluate the potential of PI3K catalytic subunit-specific inhibitors to treat FXS.

Enhanced PI3K Activity in Peripheral Blood Cells and Tissue From Individuals with FXS

As alluded to in the previous paragraph, increased PI3K enzymatic activity and downstream signaling were also observed in peripheral patient cells. In lymphoblastoid cell lines from individuals with FXS, p110β expression and activity, and Akt phosphorylation are increased (Gross & Bassell, 2012). In contrast, Akt phosphorylation in fibroblasts from individuals with FXS was not elevated (Kumari et al., 2014). Notably, a p110β-selective inhibitor reduces elevated protein synthesis rates in lymphoblastoid cell lines and fibroblasts (Gross & Bassell, 2012; Kumari et al., 2014). Whereas the p110β-selective inhibitor reduced protein synthesis rates in both wildtype (WT) and FXS fibroblasts, there was no effect on WT in lymphoblastoid cell lines. It is therefore unclear if increased protein synthesis rates in fibroblasts from

patients with FXS are due to increased p110β activity, as it seems to be the case in lymphoblastoid cells. Elevated Akt phosphorylation compared to healthy controls was also detected in a small study using blood lymphocytes isolated from individuals with FXS detected (Hoeffer et al., 2012). In addition, Hoeffer et al. (2012) showed increased Akt phosphorylation in frontal lobe homogenates of postmortem tissue from patients with FXS. These studies represent a valuable confirmation of the observations made in the FXS mouse model, and support an important role of increased PI3K signaling in the disease etiology of FXS.

DYSREGULATED ERK1/2 SIGNALING IN FXS

Similarly as PI3K, ERK1/2 [a.k.a. mitogen-activated protein kinase (MAPK)] is a central signal transductor that integrates information from many neurotransmitter membrane receptors, including tyrosine receptor kinases such as growth factor receptors, cytokine receptors, and G protein–coupled receptors. While there is so far no confirmed evidence for a direct role of FMRP in regulating ERK1/2, several studies have shown that absence of FMRP in FXS affects ERK1/2 signaling. Perhaps the most exciting and clinically relevant part of these findings is their implications for ERK1/2 as a blood biomarker in FXS. The following paragraph summarizes findings of aberrant ERK1/2 signaling in FXS and their potential roles in FXS-associated phenotypes, and briefly discusses the recent use of altered ERK1/2 signaling as a molecular outcome measure in clinical trials.

Impaired Stimulus-Induced ERK1/2 Activation: A First Potential Biomarker in FXS

One of the initial experimental evidence for a defect in intracellular signaling in FXS was provided by Weiler et al. (2004). They reported that in cortical synaptic fractions from *Fmr1* KO mice neither stimulation of the membrane receptor mGlu1/5, nor activation of the intracellular signal transducer protein kinase C (PKC) resulted in a shift of mRNAs into polysomal fractions, an indicator of increased mRNA translation, as observed in WT (Weiler et al., 2004). The same group later showed that mGlu1/5-mediated activation of the PKC downstream signal transducer ERK1/2 was absent in cortical synaptic fractions from *Fmr1* KO mice (Kim, Markham, Weiler, & Greenough, 2008). Instead, mGlu1/5 activation led to a significant decrease in ERK phosphorylation in the absence of FMRP. This defect was not compensated using phorbol myristate acetate (PMA), a direct activator of the mGlu1/5 downstream signal transducer PKC (Castagna et al., 1982). Notably, a similar phenotype of altered stimulus-induced ERK1/2 signaling was also observed in peripheral blood cells from FXS mice and from individuals with FXS, the first evidence for a potential molecular biomarker in FXS (Weng, Weiler, Sumis, Berry-Kravis, & Greenough, 2008).

Does Defective ERK1/2 Signaling Contribute to the FXS Phenotype?

Despite these promising early results, the role of altered basal or neuronal activity–induced ERK1/2 activity in the FXS phenotype is unclear, and potential molecular mechanisms leading to aberrant ERK1/2 activation are not yet fully understood. Analyses of basal ERK1/2

phosphorylation in the FXS mouse model and in patient cells have yielded variable results, suggesting that ERK1/2 activity is more indirectly affected by loss of FMRP and depends on neuronal activity, which is influenced by brain region, age, and tissue type and preparation. In fact, there are reports of unchanged, increased, and decreased ERK1/2 phosphorylation in the absence of FMRP. The majority of studies did not detect differences in basal levels of ERK1/2 phosphorylation in cortical synaptic preparations or hippocampal slices from *Fmr1* KO mice (Gross et al., 2010; Osterweil et al., 2010; Ronesi et al., 2012; Ronesi & Huber, 2008), or in lymphoblastoid cell lines from patients with FXS (Kovács, Bánsági, Kelemen, & Kéri, 2014). Others reported a decrease of ERK1/2 activity in fibroblasts (Matic, Eninger, Bardoni, Davidovic, & Macek, 2014) or in the CA1 region of *Fmr1* KO hippocampal slices (Osterweil et al., 2010). Lastly, a few studies found increased basal ERK1/2 phosphorylation levels in cortical brain tissue form patients with FXS and/or from *Fmr1* KO mice (Michalon et al., 2012; Sawicka et al., 2016; Wang et al., 2012).

Despite these varying results regarding the basal activity of ERK1/2 in the absence of FMRP, several pharmacological studies support a role of ERK1/2 dysregulation in FXS phenotypes. A MEK inhibitor rescued elevated protein synthesis rates in hippocampal slices from *Fmr1* KO mice without affecting WT, suggesting that increased sensitivity of mRNA translation to mGlu1/5–ERK1/2 activation underlies excessive protein synthesis and neuronal dysfunction in FXS (Osterweil et al., 2010). Notably, another study showed that the same MEK inhibitor used at a higher concentration reduced protein synthesis rates in both WT and *Fmr1* KO cortical synaptic fractions, supporting a role of ERK1/2 in protein synthesis regardless of absence or presence of FMRP (Gross et al., 2010). However, a brain-permeable MEK inhibitor significantly reduced susceptibility to audiogenic seizures in *Fmr1* KO mice in two independent studies (Osterweil et al., 2010; Wang et al., 2012), which suggests a role of aberrant ERK1/2 signaling in certain aspects of the FXS phenotype. This hypothesis was further supported by a very recent study showing that pharmacological reduction of increased activity of p90-ribosomal S6 kinase (RSK), which is downstream of ERK1/2, reduced susceptibility to audiogenic seizures in FXS mice (Sawicka et al., 2016).

There are at least two mechanisms that could contribute to defective ERK1/2 activity. First, FMRP may directly regulate the protein expression of ERK1/2 or its upstream regulators, such as MAPK/ERK kinase (MEKs) or MEK kinases (MEKKs), which could lead to increased expression and activity of these enzymes in the absence of FMRP. So far, there is little experimental evidence that FMRP directly regulates ERK1/2 or MEK expression. A few mRNAs coding for upstream regulators of ERK1/2 were found to be associated with FMRP in screens (Ascano et al., 2012; Darnell et al., 2011), but no published study showed that the expression of ERK1/2 or of one of the MEKs or MEKKs is directly regulated by FMRP. Second, the observed defects in ERK1/2 signaling might be an effect of dysregulated receptor–mediated upstream signaling or other signaling pathways, such as PI3K/mTOR, which cross-react with ERK1/2 signaling. This second hypothesis is supported by the variable results that were reported with respect to receptor-mediated activation of ERK1/2. A few studies showed that mGlu1/5 stimulation increased ERK1/2 phosphorylation in WT and *Fmr1* KO cortical synaptic fractions and hippocampal slices (Osterweil et al., 2010; Ronesi et al., 2012; Ronesi & Huber, 2008), suggesting that mGlu1/5-mediated activation of ERK1/2 is functional in the absence of FMRP. This is in contrast to the results by Greenough and coworkers discussed earlier, and may be explained by different brain regions, tissue preparations, and timing. Interestingly, while

neuregulin 1–induced ERK1/2 stimulation seems to be normal in FXS (Kovács et al., 2014), stimulation with glycine decreased ERK1/2 phosphorylation in *Fmr1* KO neurons, but no effect was observed in WT (Shang et al., 2009). In summary, these studies corroborate that certain characteristics of ERK1/2 signaling are affected by loss of FMRP. To support the idea of a central role for the ERK1/2 pathway in the FXS phenotype and to further develop ERK1/2 signaling as potential therapeutic target in FXS, it will be important to fully understand how FMRP regulates basal and receptor-mediated ERK1/2 enzymatic activity.

Aberrant ERK1/2 Activation as Potential Biomarker in Clinical Trials

Regardless of the limited understanding of the underlying mechanisms leading to defective ERK1/2 signaling in FXS, there are promising results suggesting that ERK1/2 signaling could be used as biomarker in peripheral blood from individuals with FXS to monitor molecular effects of therapeutic treatments and interventions in FXS. As mentioned earlier, membrane receptor–activated ERK1/2 phosphorylation was delayed in nonneuronal cells from *Fmr1* KO mice and individuals with FXS compared to healthy controls (Weng et al., 2008). This was one of the first indications of a potential molecular biomarker in peripheral blood cells, which could be used to monitor the efficiency of therapeutic treatments. Several clinical studies have since then used aberrant ERK1/2 phosphorylation in blood as one of many outcome measures to test drugs for the treatment of FXS. In particular, open-label treatments with lithium (Berry-Kravis et al., 2008), riluzole (Erickson et al., 2011), and lovastatin (Pellerin et al., 2016) were promising, suggesting that improvements in behavioral outcomes in response to drug treatment are accompanied by correction of the molecular defect in ERK1/2 activation in blood cells. Several ongoing clinical trials in FXS use quantification of ERK1/2 activation in peripheral blood as one of their outcome measures (www.clinicaltrial.gov), and it will be interesting to see if the promising results of the open-label studies can be recapitulated in these large, blinded, and placebo-controlled studies.

TARGETING THE SIGNALING HUB RAS TO CORRECT ALTERED SIGNALING IN FXS

In view of the observed defects in two major intracellular signaling pathways and the altered signaling downstream of diverse membrane receptors, signaling molecules that act as integrators for all of these pathways are attractive targets to correct aberrant signal transduction in FXS. One of these intracellular signaling hubs is the small GTPase Ras, which can activate both PI3K and ERK1/2 signaling (Castellano & Downward, 2011; also see Fig. 11.1). The highly conserved family of Ras GTPases comprises H-Ras, K-Ras, and N-Ras, which are crucial regulators of cell proliferation, differentiation, and apoptosis, and are often mutated in cancer (Downward, 2003). Germline mutations in the RAS signaling pathway have been implicated in many diseases, coining the term "RASopathies" (Rauen, 2013). These disorders, which include neurofibromatosis 1, Costello syndrome, and Noonan syndrome, have craniofacial, cardiac and muscular defects, and increased risk for cancer, but are also associated with cognitive defects and autistic-like traits (Adviento et al., 2014), which parallels the FXS phenotype. Interestingly, one study has reported defects in Ras signaling in *Fmr1* KO mice, which led to increased basal activity of Ras,

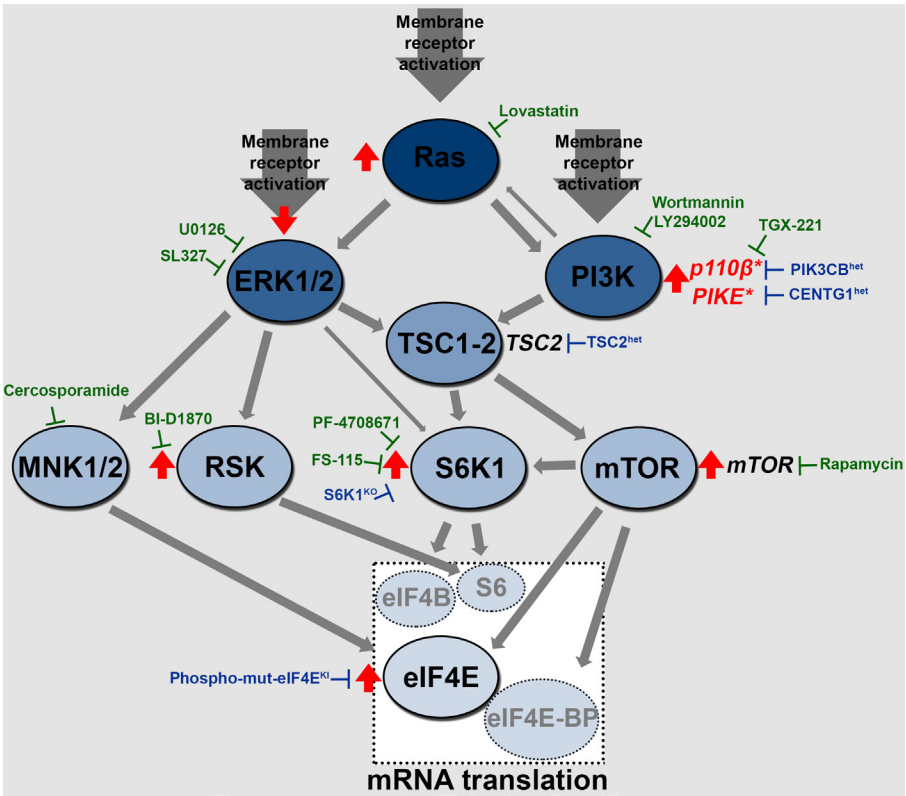

FIGURE 11.1 Overview of dysregulated intracellular signaling pathways in fragile X syndrome (FXS) and genetic or pharmacological rescue strategies to correct these deficits. Ras, extracellular regulated kinase 1/2 (ERK1/2), and phosphoinositide-3 kinase (PI3K) receive direct input from membrane receptors. Ras functions as signaling hub that relays signals to both ERK1/2 and PI3K. ERK1/2 and PI3K activate overlapping, but not identical sets of downstream effectors, including tuberous sclerosis complex 1–2 (TSC1–2), MAP kinase interacting protein kinase (MNK1/2), p70 ribosomal S6 kinase 1 (S6K1), p90 ribosomal S6 kinase (RSK), and mammalian target of rapamycin (mTOR). Ras and PI3K have been shown to be overactive, whereas only receptor-mediated activation of ERK1/2, but not basal activity, seems to be altered in the absence of fragile X mental retardation protein (FMRP) (indicated by *red arrows* next to or above the signaling molecules, respectively). TSC1-2 functions as a central signaling node that integrates inputs from both ERK1/2 and PI3K. All of these pathways control mRNA translation *(dashed white box)*. Translation initiation factors or ribosomal proteins that are regulated by the signaling pathways affected in FXS, but are not altered in FXS animal models or human cells, are *shaded*. TSC2, mTOR, and the PI3K subunits, PI3K enhancer (PIKE) and p110β, are mRNA targets of FMRP (indicated in *italics* next to the respective signaling molecules); p110β and PIKE have been confirmed as mRNA-binding partners of FMRP in an independent study (indicated by *asterisks*); PIKE and p110β were shown to be upregulated in FXS mouse models and human cells or tissue (indicated in *red*). *Gray arrows* indicate regulation/activation; *thin gray* arrows (from ERK1/2 to S6K1 and PI3K to Ras) show less prevalent regulation pathways. Genetic rescue strategies are shown in *blue*, pharmacological rescue strategies in *green*. Refer to the text for additional information. *het*, Heterozygous; *ki*, knockin; *ko*, knockout.

but impaired stimulus-induced activation of Ras and the PI3K/Akt pathway (Hu et al., 2008). This study also provided electrophysiological evidence that deficiencies in Ras-mediated PI3K activation may underlie impaired synaptic delivery of AMPA receptor subunits and reduced LTP in hippocampal CA1 neurons of *Fmr1* KO mice. In contrast to the studies described earlier, in which PI3K signaling was *reduced* in FXS mice to rescue phenotypes (Gross et al., 2015a,b), Hu et al. (2008), *enhanced* the Ras–PI3K–Akt pathway by either overexpressing active Ras or a dominant-negative mutant of PTEN, which in its active form dephosphorylates PIP3 and thus reduces PI3K downstream signaling. Overexpressing active Ras or the dominant-negative PTEN mutant in cultured hippocampal neurons rescued impaired synaptic delivery of AMPA receptors. Moreover, viral expression of the active Ras in vivo in the CA1 region restored synaptic delivery of AMPA receptors and LTP in hippocampal slices of *Fmr1* KO mice to WT levels. It is noteworthy that in both approaches taken by Hu et al. (2008), upregulation of the PI3K pathway is only one of the expected consequences. Overactive Ras will induce ERK1/2 and PI3K downstream signaling and may change the cellular GTP/GDP ratio, which can induce a plethora of secondary effects. A dominant-negative PTEN still retains its protein phosphatase activity (Myers et al., 1997) and enzymatic activity–independent functions that are mediated by protein–protein interactions (Tang & Eng, 2006). Similarly as with overactive Ras, it is difficult to predict what the impact of dominant-negative PTEN on cellular function is. Hu et al. (2008) did not analyze the effects of Ras and PTEN manipulations on Akt phosphorylation, and the underlying mechanisms of the observed rescue are therefore largely unclear.

It is also unknown if increasing Ras activity improves behavior, cognition, or neuronal hyperexcitability in FXS. The latter would be particularly interesting in view of results from a recent study by Osterweil et al. (2013) who took the opposite approach to test the signaling hub Ras as a therapeutic target to correct phenotypes in FXS. Interestingly, both, Hu et al. (2008) and Osterweil et al. (2013), observed increased Ras activity; yet, the interpretation and the rescue strategy were different. Whereas Hu et al. (2008) argue that the relay of Ras activity to downstream signaling pathways is deficient, justifying their approach to increase PI3K signaling, Osterweil et al. (2013) reduced Ras GTPase activity by treating *Fmr1* KO mice with lovastatin, an inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, which blocks the rate-limiting step of the biosynthesis of cholesterol and isoprenoid. Lower levels of isoprenoid reduce the farnesylation of Ras, which is essential for membrane localization and subsequent activation of Ras (Ahearn, Haigis, Bar-Sagi, & Philips, 2011). As expected, lovastatin decreased Ras activity and ERK phosphorylation in both *Fmr1* KO and WT hippocampal slices (Osterweil et al., 2013). Lovastatin also specifically reduced elevated protein synthesis rates and exaggerated mGluR-LTD in *Fmr1* KO slices with no effect on WT. Moreover, it reduced neuronal hyperexcitability in hippocampal slices and the visual cortex of *Fmr1* KO mice, and reduced susceptibility to audiogenic seizures. In contrast, lovastatin did not rescue impaired visuospatial discrimination and extinction in *Fmr1* KO mice (Sidorov et al., 2014), suggesting that these defects in higher cognition depend on other molecular mechanisms, such as exaggerated PI3K signaling (Gross et al., 2015b). One caveat for the interpretation of these results is that, as an inhibitor of cholesterol and isoprenoid synthesis, lovastatin most likely affects many other cellular mechanisms apart from ERK1/2 and Ras signaling, similarly as overactive Ras or dominant-negative PTEN. Nevertheless, lovastatin is a particularly attractive drug to treat FXS because it is already FDA approved and regularly prescribed for adults and children to lower cholesterol levels (Krukemyer &

Talbert, 1987; Vuorio et al., 2014). The efficacy of lovastatin to improve behavior in patients with FXS was confirmed in an open-label trial in 15 individuals (Çaku, Pellerin, Bouvier, Riou, & Corbin, 2014). These results are encouraging; however, larger studies are needed to corroborate these initial findings. Lovastatin's function as an HMG-CoA reductase inhibitor is not specific for Ras, but has pleiotropic, disease-mitigating effects on, for example, cardiovascular, pulmonary, and rheumatologic disorders, possibly by regulating an array of cellular mechanisms including vasoconstriction, platelet aggregation, cytokine production, and autophagy (Mihos & Santana, 2011). Therefore, to fully understand the benefits and potential disadvantages of this drug for the treatment of FXS, it will be necessary to identify the exact cellular mechanism(s) that lovastatin is targeting to mediate its beneficial effects in FXS.

TSC–mTORC1–S6K1–4EBP NEXUS: A MAJOR mRNA TRANSLATION CONTROL NODE IN FXS

Given the key role of translation in FXS, there was a major emphasis on understanding the activity status of a major regulator of this process, the mechanistic target of rapamycin complex 1 (mTORC1). Upstream of mTORC1: TSC1–2, and downstream of mTORC1: S6K1, and eIF4E-binding protein 1 or 2 (4E-BP 1 or 2) form another nexus of signaling that is critical to protein synthesis across different organisms and genera. mTORC1 can also be thought to be downstream of PI3K and interacts with ERK1/2, thereby forming a link from cell surface receptors to actual translation machinery (Fig. 11.1). In the past 5 years, a phenomenal amount of evidence linking TSC1-2, mTORC1, S6K1, and eIF4E/4E-BP to pathology of autism and other neurodevelopmental disorders has accumulated (Betancur, 2011; Richter, Bassell, & Klann, 2015; Sahin & Sur, 2015; Santini & Klann, 2014; Tang et al., 2014). The central role of this nexus in integrating signals to translation control has been deeply studied in FXS, with strong genetic proof of principle studies and some pharmacological intervention. The use of these as biomarkers has also been explored. Further, we describe the current knowledge and emerging issues in TSC–mTOR–S6K1–4E signaling in FXS and their potential as treatment targets.

TSC 1–2 COMPLEX IS A VITAL, BUT UNDERSTUDIED SIGNALING NODE FOR FXS

TSC has actually been the focus of medical research for over 100 years and is one of the other well-studied syndromic forms of autism spectrum disorders (ASD) (for a comprehensive review, see Jülich & Sahin, 2014). The molecular complex of TSC1 and TSC2 together function as dimer called the TSC1–2 complex. TSC1–2 complex through its GTPase-activating protein (GAP) activity toward the small G-protein Ras homologue enriched in brain (Rheb), is a key negative regulator of the major signaling scaffold/hub called the mTORC1. TSC1–2 also activates the Rictor-associated mTORC2, independently of Rheb via currently unresolved mechanisms, and is indirectly responsible for phosphorylation of Akt via this signaling arm (Han & Sahin, 2011). Hence in terms of signaling TSC1/2 forms a nodal regulation point downstream of Akt, GSK-3, ERK-RSK1, AMPK, and inhibitory κB kinase β (IKK3 β); funneling inputs from these points into either suppressing mTORC1 or activating mTORC2.

At a biochemical level, TSC1–2 complex in FXS has not been clearly shown to be misregulated. In the HITS-CLIP screen of Darnell et al. (2011), TSC2 and TSC domain family 2 (TSDD2) mRNA are listed to specifically associate with FMRP–ribosome complex. As FMRP negatively regulates the expression of most of its target mRNAs, the expectation was that TSC2 levels would be upregulated in FXS (Hoeffer & Klann, 2010). However, the report that FXS causes increased phosphorylation and activity of mTORC1 contradicts this (Sharma et al., 2010). The PARS-CLIP study by Ascano et al. (2012), reported that TSC2 levels were largely lowered when FMRP was overexpressed in HEK cells, but are variable in different areas of the brain in human patients. The implication is that translational control of TSC2 by FMRP enhances its synthesis rather than depressing it. A key study by Auerbach, Osterweil, and Bear (2011) sheds light on the electrophysiology and fear memory mechanisms between TSC2 and FXS. First, they showed that TSC2 heterozygous (het) mice have impaired DHPG- and PP-LFS–mediated LTD likely due to deficient postsynaptic properties. Further they showed a small, but significant decrease in global rates of basal translation in the hippocampus and impaired stimulus-driven translation of Arc mRNA. Surprisingly these deficits in TSC2 het could be overcome by either rapamycin treatment or stimulation of mGluR signaling using a positive allosteric modulator, CDPPB, alluding to the fact that flux through TSC1–2 complex is indeed impaired. The surprising finding was that crossing *TSC2* het with *Fmr1* KO mice normalized hippocampal LTD, translation, and fear memory in the resultant double transgenic. This was perhaps the first report demonstrating that two syndromic deletions can cancel each other out and needs to be verified in humans with FXS and TSC together, the coincident occurrence of which is ultrarare.

Direct pharmacological targeting of the TSC1–2 complex in FXS has not been attempted, likely because it is difficult to target specifically those phosphosites that would affect downstream mTORC1 signaling alone. To date no study has investigated the relative levels of different phospho-sites of TSC1 or 2 in various brain areas of FXS animal models.

mTOR IS A WELL-STUDIED CANDIDATE IN FXS, BUT MAY NOT BE SUITED FOR DIRECT THERAPEUTIC INTERVENTION

The kinase mTOR is an AGC family kinase (like PI3K, Akt, and S6K1) and forms the core of two multiprotein complexes, mTORC1 and 2. mTORC1 comprises of Raptor, Pras40, and mLST8 and is a critical hub kinase complex regulating protein anabolism, amino acid sensing, autophagy, etc. (Costa-Mattioli, Sossin, Klann, & Sonenberg, 2009; Hoeffer & Klann, 2010; Lipton & Sahin, 2014; Tan & Miyamoto, 2016). With respect to translation, mTORC1 activates S6K1 and represses 4E-BP, both of which are discussed further below. mTORC2 is made up of Rictor, mLST8, mSIN1, as well as Deptor, TEL2, TTI1, and Protor1/2, is rapamycin-insensitive in shorter timescales (but sensitive following prolonged or chronic exposure), and has been shown to be important for actin remodeling, gene transcription, etc. (Cybulski & Hall, 2009; Huang & Fingar, 2014). Both pharmacologically using rapamycin, and with genetic studies in behavior and synaptic plasticity paradigms, such as LTP and LTD, mTORC1 has been established to be critical for multiple different forms of memory, including acquisition, retrieval, consolidation, and extinction (Hoeffer & Klann, 2010; Lipton & Sahin, 2014). In contrast, it is only relatively recently that mTORC2

via its regulation of actin dynamics has been demonstrated to be important for memory formation (Huang et al., 2013).

One of the most seminal papers in the FXS field, Qin, Kang, Burlin, Jiang, and Smith (2005), measured regional rates of protein synthesis in WT and *Fmr1* KO mice and incontrovertibly proved that loss of FMRP leads to elevated rates of protein synthesis. By this time, impaired mGluR-dependent LTD and behavioral abnormalities had been reported in FXS model mice (D'Hooge et al., 1997; Huber et al., 2002; Musumeci et al., 2000; Paradee et al., 1999; Yan, Asafo-Adjei, Arnold, Brown, & Bauchwitz, 2004; The Dutch-Belgian Fragile X Consortium, 1994). This, coupled to the fact that rapamycin inhibits hippocampal mGluR-LTD in WT mice (Hou & Klann, 2004), made a strong case for disturbances in mTORC1 signaling in FXS. However, it was only in 2010, that Sharma et al. (2010) showed that in FXS hippocampus, not only is mTORC1 activity higher, but it also causes elevation of activity of the downstream S6K1 and increases formation of eIF4E–eIF4G, all of which in concert increases protein synthesis. In 2010, two additional papers were published showing disturbances in ERK1/2 and PI3K signaling as well (mentioned earlier in the text), which along with the studies of Sharma et al. (2010), firmly established the biochemical basis of the signaling deficits in FXS (Gross et al., 2010; Osterweil et al., 2010). These two studies found opposing results regarding the effect of rapamycin treatment on protein synthesis rates in different brain regions of *Fmr1* KO mice, showing no effect in either WT or *Fmr1* KO hippocampal slices (Osterweil et al., 2010), but a decrease to WT levels in cortical synaptoneurosomes of *Fmr1* KO mice (Gross et al., 2010). Biochemical analyses apart, Sharma et al. (2010) also showed that the mGluR-LTD in FXS was protein synthesis-independent and rapamycin-insensitive. Further in 2012 and 2014, two additional papers published using human postmortem tissues and patient-derived fibroblasts, showed that mTOR phosphorylation at serine 2448 (believed to be coincident to association of Raptor with mTOR) was higher in FXS versus controls (Hoeffer et al., 2012; Kumari et al., 2014). Kumari et al. (2014) showed a negative correlation of phospho-mTOR levels with age in FXS patients alone, which may allude to slowdown of the pathway dynamics in older FXS individuals. This has important implications in selecting subjects for clinical trials. While activity levels of mTORC1 are changed in FXS, the actual levels of mTOR protein are not found to be any different in the FXS mouse model (*unpublished observations*), even though mTOR is detected as an FMRP target by the PAR-CLIP study (Ascano et al., 2012) and was reported to be variable in expression levels in the postmortem tissue used in that study. This contrasts with the finding of mTOR being a low-probability FMRP target in a HITS-CLIP screen (Darnell et al., 2011).

Given how firmly the phenotype of dysregulated mTORC1 signaling is established in FXS, the reader may find it curious that there were no clinical trials for the use of rapamycin or associated rapalogs in FXS. This stems from the fact that any therapeutic strategy would involve prolonged treatment using rapalogs spanning over several days, which would likely start affecting the nutrient sensing, transcription, autophagy, and mRNA-degradation functions of mTORC1 (Diaz-Troya, Perez-Perez, Florencio, & Crespo, 2008; Proud, 2009; Rosenbluth & Pietenpol, 2009) and other anabolic arms of mTOR signaling. In addition, long-term use of rapalogs has been shown to inhibit mTORC2 as well (Sarbassov et al., 2006), which considerably complicates matters. This scenario is akin to the lack of trials using ERK1/2 inhibitors directly to manage FXS in humans. Acute rapamycin exposure neither normalized excessive protein synthesis seen in FXS hippocampal slices nor the audiogenic seizures and mGluR-LTD (Osterweil et al., 2010; Sharma et al., 2010). However, rapamycin did rescue wayward

protein synthesis in cortical synaptic preparations (Gross et al., 2010), and a different mTOR inhibitor, temsirolimus, reduced audiogenic seizures (Busquets-Garcia et al., 2013). Hence, the answer lay in targeting downstream signaling molecules from mTORC1, which is discussed in the following sections. An interesting fact to be considered is that rapamycin treatment is beneficial for TSC1–2 and PTEN mutant mice and in humans (Jülich and Sahin, 2014, www.clinicaltrials.gov). This implies that in TSC1–2 and PTEN conditions, the signaling flux to translation is heavily weighted/dependent on mTORC1, whereas in FXS, as there are disturbances in ERK1/2 and GSK-3β signaling, the translation control may be shared among these major hub kinases. An indirect support of this fact is provided by a recent article, in which Kong et al. (2014) looked at genomewide gene expression in the cerebellum and blood of *Fmr1* KO and TSC-2 het mice, and found that different gene clusters were enriched for FXS versus TSC-2. In the FXS cerebellum, clusters of genes associated with synaptic plasticity were the most significantly perturbed, in the TSC2 condition gene sets for immune system–related pathways, ribosomal subunits, and glycolipid metabolism were the most changed.

S6K1: A SIGNAL INTEGRATOR AND TRANSLATIONAL REGULATOR WITH THERAPEUTIC POTENTIAL IN FXS

It is widely accepted that FMRP loss, causing FXS, wreaks havoc mainly at the level of translation, signaling to translation, and activity of large-conductance membrane channels. The first two conditions usually cause FXS to be categorized as a "translationopathy." While it would appear that for such a condition the best approach would be to either directly manipulate *Fmr1* expression by gene editing (discussed in Chapter 17) or intervene at the level of translation initiation and elongation, it is important to remember that translation is vital to an organism's survival, and there is immense pressure to not alter the stoichiometry of these critical components. For instance, KOs of components of cap-binding complex, elongation, and ribosome release factors are usually lethal (Gandin et al., 2008) and overexpression has ceiling effects (Ruggero et al., 2004). The matter is complicated by the paucity of small molecule agents that disrupt translation factors directly. This makes a strong case for targeting immediate downstream targets of mTORC1 as promising avenues of investigation. These are S6K1 and 4E-BP 1/2. Each has been targeted in FXS and ASD and lead to some conserved effects and differ in some aspects that are discussed here.

S6K1 Shows Subtly Different Effects in Genetic Deletion and Pharmacological Inhibition Studies

S6K1 is an enduring target of mTORC1 and one most commonly used as a readout of its activity. Interestingly, the kinase was discovered independently and was named after its downstream effector, ribosomal protein S6. Activation of S6K1 is a multistep process that requires phosphorylation at many sites; in cell culture–based experiments phosphorylation at Thr389 by mTORC1 and Thr229 by PDK1 is deemed critical for kinase activity (Magnuson, Ekim, & Fingar, 2012). Recently however, it was shown that in neural systems, Ser421/422 phosphorylation downstream of ERK1/2 activation seems to mediate fear memory and extinction (Huynh, Santini, & Klann, 2014). Therefore, it is likely that S6K1 activity is downstream of

ERK 1/2, Akt1, and mTORC1 activation, funneling signaling to translation control. S6K1 influences translation by promoting initiation and elongation. By phosphorylating and activating eIF4B, it enhances the helicase activity of eIF4A, which is crucial for the cap-binding initiation complex to jump-start translation (Özeş, Feoktistova, Avanzino, & Fraser, 2011). By activating ribosomal protein S6 on the small ribosomal subunit, S6K1 is thought to promote ribosomal processivity, but this notion is controversial (Magnuson et al., 2012). Most directly by negatively affecting the kinase activity of eEF2 kinase, S6K1 allows for elongation factor 2 (eEF2) to associate with the ribosome and perform its function on translocating the newly forming peptide chain from A to P site (Browne & Proud, 2002). Additionally Narayanan et al. (2008) had reported that S6K1 is "the only known FMRP kinase," and by phosphorylating it could decrease the binding and hence translation repression wielded by FMRP. This has been contradicted by a more thorough work by Bartley, O'Keefe, & Bordey (2014) where in spite of in vitro kinase phosphorylation, the in vivo effect is missing. However in an unrelated paper, Spencer, Mulholand, and Chandler (2016) show that FMRP is phosphorylated in an S6K1-sensitive manner in alchohol vapor-stimulated mouse brains and hence the effect may be case-specific. Casein kinase II has been shown to phosphorylate dFMRP at S406 (Siomi, Higashijima, Ishizuka, & Siomi, 2002). Very recently, Bartley et al. (2016) reconfirmed Casein Kinase as phosphorylating FMRP at Ser 499, which then promotes its secondary phosphorylation.

In two separate papers (Bhattacharya et al., 2012, 2016) the notion of targeting S6K1 to ameliorate phenotypes of FXS was evaluated in the mouse model. In the 2012 study, a global S6K1 KO was crossed with FXS model mice to yield a double KO that showed decreased activation of S6, eIF4B, and decreased basal rates of hippocampal protein synthesis. In addition, ablation of S6K1 also rescued the hippocampal LTD phenotype, as well as aberrant dendritic spine density and spine morphology of FXS mice. Most importantly, in a diverse behavioral test battery, genetic deletion of S6K1 normalized inappropriate social preference, object discrimination, and behavioral inflexibility of FXS model mice. There were some improvements reported from rota rod memory and hyperactivity in the open field; however, stereotypic behaviors measured by marble burying remained unaffected. This is interesting because genetic reduction of either p110β or PIKE rescued enhanced marble burying, suggesting that this behavior is regulated by S6K1-independent signaling pathways (Gross et al., 2015a,b). As the knockdowns of both genes were global, and S6K1 KO mice are known to have a smaller mouse phenotype owing to smaller cell size (Shima et al., 1998), it was not surprising to see an effect on increased weight gain and macroorchidism in the double KO. Finally, upon testing for normalization of a host of candidates from the Darnell et al. (2011) HITS-CLIP list, the authors found normalization in levels of Shank3, eEF2, CamkIIα, and eIF4G levels with no change in PSD-95 and Arc (both previously shown to be under translation regulation by FMRP) (Muddashetty, Kelic, Gross, Xu, & Bassell, 2007; Park et al., 2008).

The notion of dysregulated S6K1 was shown earlier in Sharma et al. (2010), but was also supported by subsequent reports by Hoeffer et al. (2012) and Kumari et al. (2014) in patient postmortem tissue and patient-derived primary fibroblasts, respectively. Importantly, acute treatment of fibroblasts with an S6K1 specific blocker, PF-4708671, decreased rates of protein synthesis in the same fibroblasts. Hence altered activity of S6K1 is a significant phenotype in humans with FXS, and has the potential to serve as a biomarker.

The search for small molecule inhibitors to S6K1 have lagged behind in the past likely because S6K1 was considered more as a readout to mTORC1, and because of the lack of any specific disease with a pure S6K1 basis. This is changing with a clear implication of S6K1 in

pancreatic, colon, and rectal cancers and in neurological conditions, such as Alzheimer's disease and depression (Alliouachene et al., 2008; Caccamo et al., 2015; Dwyer, Maldonado-Aviles, Lepack, DiLeone, & Duman, 2015; Slattery, Lundgreen, Herrick, & Wolff, 2011). In the pharmacological targeting study in FXS (Bhattacharya et al., 2016), two experimental drugs PF-4708671 and FS-115, were compared head-to-head to evaluate how subchronic inhibition of S6K1 after the manifestation of the FXS (in adult male mice) could be corrected to some degree. This was important to note, as genetic KO studies are at best a proof of concept and do not have relevance to human clinical trials, where most of the recruited individuals with FXS have been adults or teenagers. As S6K1 blockade effects were not known in the CNS, the study was the first preclinical characterization of the acute and subchronic application in vivo and aimed at identifying a conserved set of behaviors or biochemical profiles that S6K1 inhibition would address. PF-4708671 and FS-115 are structurally dissimilar, yet acute application in WT and FXS hippocampal and cortical tissue decreased basal translation and S6 phosphorylation. The two drugs also differed in the routes of delivery and accumulation in the brain, which determined different subchronic dosing periods. Regardless, eIF4B phosphorylation remained unaffected and elevated in FXS samples, contrasting with the genetic rescue data and alluding to more developmentally persistent regulation of this translation factor. Chronic treatment impacted eEF2 levels most profoundly, implying that the resetting of translation homeostasis was likely more heavily weighted at the elongation phase. Despite different dosing times and amounts, both inhibitors were able to rescue aberrant social interaction and behavioral inflexibility in the Y-maze. However, effects on hyperactivity were muddled by an across groups effect of hypoactivity, which is well documented with S6K1 KO (Antion et al., 2008). An interesting and perhaps off-target effect seen with FS-115 was the rescue of marble burying phenotype that even the genetic reduction of S6K1 was not able to normalize in FXS model mice. FS-115 does have cross-reactivity with other AGC family kinases, and it is possible that continued brain exposure would have inhibited those as well. Peripheral effects of the two drugs also provided novel insights into S6K1 signaling in FXS and its control on body weight and macroorchidism. The more peripherally localized PF-4708671 was able to correct macroorchidism, but did not affect weight gain in FXS, which FS-115 did. This was surprising, as PF-4708671 was able to rescue increased filopodial spines in the hippocampus. In summary the conserved set of phenotypes rescued by both inhibitors will likely inform future drug development efforts and future clinical trial design for S6K1 blockers in FXS and other related disorders.

MODULATION eIF4E VIA Mnk1 OFFERS AN ALTERNATIVE TO MANAGING FXS PHENOTYPES

Two independent studies (Gkogkas et al., 2013; Santini et al., 2013) showed that either genetically ablating 4E-BP or increasing net levels of eIF4E cause more translation and manifestation of a large number of ASD-like behaviors in mice. Additionally, Aguilar-Valles et al. (2017) show that group I metabotropic receptor antagonism can rescue many phenotypes of the 4E-BP2 mice. This sets a clear precedent for targeting this complex formation or activity of eIF4E as a therapeutic strategy for intervention in FXS, which is characterized by aberrant protein synthesis and elevated mGluR signaling. In the absence of a phosphomimetic for 4E-BP and a way to sequester away eIF4E reliably, an indirect strategy to reset translation homeostasis in FXS

was to interfere with eIF4E phosphorylation (p-eIF4E).The Ras/ERK1/2 pathway mentioned earlier activates MAP kinase interacting protein kinase (Mnk1 and Mnk2). Mnk 1 and 2 in turn stimulate translation via phosphorylation of eIF4E on Ser209 (Waskiewicz, Flynn, Proud, & Cooper, 1997). This phosphorylation on eIF4E correlates with increased rates of cap-dependent translation initiation and has been implicated in learning and memory (Kelleher, Govindarajan, Jung, Kang, & Tonegawa, 2004). ERK inhibition blocks neuronal activity–induced translation, as well as phosphorylation of eIF4E (Banko, Hou, & Klann, 2004; Kelleher et al., 2004).

Given that ERK1/2 signaling is perturbed in FXS (Osterweil et al., 2010), Gkogkas et al. (2014) explored the status of eIF4E activation and how impacting this node could rescue deficits seen in FXS model mice. The study found that not only were p-eIF4E levels elevated in frontal cortex and hippocampus in postmortem patient samples, but also the same held true in multiple brain areas in the FXS mouse. In an earlier study of genomewide translation profiling in a specially designed mouse having a Ser209Ala knockin in eIF4E ($eIF4E^{ki}$), a major candidate with elevated translation was matrix metalloprotease 9 (MMP-9, Furic et al., 2010). MMP-9 misexpression has been well established to underlie many phenotypes in FXS (discussed in Chapter 15) and Gkogkas et al. (2014) also found elevated levels of MMP-9 in FXS human and mouse samples. Upon crossing the $eIF4E^{ki}$ with FXS mice, the double mutant showed decreased MMP-9 levels, dendritic spine density, and hippocampal LTD. The double KO also showed improved survival to audiogenic seizures, social interaction preference, discrimination in the light-dark box, and improved hyperactivity measured in open field. Interestingly, the results were entirely recapitulated by decreasing Mnk1 levels in the FXS model mice, underscoring the mechanism. Finally using cercosporamide, a broad-spectrum antifungal that also affects eIF4E phosphorylation by inhibiting Mnk1 (Konicek et al., 2011), the authors showed rescue in MMP-9 translation and social behavior and LTD. Finally the authors stress that elevated translation of MMP-9 mRNA downstream of Mnk/eIF4E is sufficient for inappropriate social behaviors. This claim is bolstered by the result that using cercosporamide in *Mmp9* KO rescues inappropriate social behaviors. Cercosporamide was in preclinical trials for lung and colon carcinomas; however, to be adapted to FXS, authors recommend a revised dosage in keeping with the updated pharmacokinetic and pharmacodynamics data.

CHALLENGES AND FUTURE OUTLOOK

Aberrant translation is one of the core molecular causes of FXS and this arises due to a coincidence of loss of FMRP, a critical translation regulator, and insult caused to the protranslation signaling downstream of a wide range of cell surface signal transducers (Fig. 11.1). It is noteworthy that several of the intracellular signaling nodes that are dysregulated in FXS (and have been covered here) have also been implicated in other ASD, for example, tuberous sclerosis, autism associated with certain copy number variations, and several "RASopathies," including neurofibromatosis 1 (Cusco et al., 2009; Lipton & Sahin, 2014; Plasschaert et al., 2015). What is more pertinent is that the pathways affected are critical in regulating many other functions outside the central nervous system. This is a bane and a boon at the same time. The advantage lies in the fact that these molecules are implicated in the pathology of other human diseases and in most cases, proven therapeutics agents are available or are being designed aggressively. Hence small molecules that perturb or target the system are available for preclinical testing.

The downside is that any pharmacological intervention will likely have off-target effects and general body side effects that need to be monitored closely in a clinical trial setting.

An important area of exploration is their use as biomarker for patient stratification and treatment outcome measures. For instance, PI3K/mTOR signaling in lymphoblastoid cell lines and fibroblasts from patients with FXS was increased compared to healthy controls (Gross & Bassell, 2012; Kumari et al., 2014). While these studies are encouraging, for this to be used as a quantitative blood biomarker, further work is required to address variability of PI3K and mTOR–S6K1 kinase activity in blood, which is susceptible to external factors, such as nutrition status, history of infections, and method of drawing/storing the blood sample. Nonetheless, a pilot study using lymphocytes isolated from whole blood from nine patients with FXS detected increased Akt phosphorylation and downstream signaling compared to seven healthy controls (Hoeffer et al., 2012). It is hoped that larger-scaled studies will clearly evaluate the usability of the activation levels of these signaling molecules as biomarkers in blood lymphocytes from individuals with FXS. As discussed above, work to evaluate dysregulated ERK1/2 activation as blood biomarker in FXS is already more advanced, yielding promising results.

A third area that merits further investigation is the developmental trajectory of these signaling pathways. In almost all studies discussed earlier, the investigators have used one specific age to do most of their experiments, and rescue experiments have either been in global KOs or at ages that do not entirely overlap between all studies. This poses problems in scaling for human studies because FXS clinical trials are not likely to be approved for young children. Autism and FXS likely occur due to problems in critical periods of plasticity and no mouse study has tested drug administration in a specific development window, with effects being evaluated several weeks later in a drug-free condition. This is again critical because agents, such as lovastatin and S6K1 inhibitors, are known to have effects on metabolism, and can potential impact general development in children.

To summarize, the wealth of work establishing that several aspects of FXS are mediated by wayward PI3K/ERK/mTORC1 signaling, provides a solid platform to address the future challenge of providing tangible leads for biomarker development and tractable therapeutic approaches to yield the maximal results in humans.

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CHAPTER

12

The Endocannabinoid System in Fragile X Syndrome

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INTRODUCTION

The Endocannabinoid System

The endocannabinoid (eCB) system, named after that notorious group of compounds, is a neuromodulatory hub. Among modulatory molecules, the eCB system does not rely on afferent innervation to generate signaling, but instead is a locally driven signaling module present at the majority of both inhibitory and excitatory central synapses. eCB neuromodulation functions through the local synthesis of bioactive lipid derivatives in or near the synapse and the activation of neighbouring eCB receptors. Stimulation of eCB synthesis is an active process, dependent on neuronal state and synaptic input. In the most common scenario, eCB molecules function as retrograde messengers; synthesized in the postsynapse and acting as ligands to receptors localized in the presynaptic bouton, where invariably their function is to inhibit neurotransmitter release. This allows the eCB system to act as both a homeostatic and phasic modulator of neurotransmission. The dynamic nature of the eCB system makes it a powerful mechanism in the integration of neuronal inputs and network modulation. Unsurprisingly therefore the eCB system is thought to have an important role in cognition, as well as learning and memory. Dysfunction of the eCB system profoundly affects neuronal function and is associated with many neuropsychiatric disorders notably anxiety and depression (Mechoulam & Parker, 2013), but also addiction and genetic disorders (Chakrabarti, Persico, Battista, & Maccarrone, 2015). This list has recently expanded to include fragile X syndrome (FXS), where changes in the eCB system are being actively explored in the *Fmr1* KO mouse model. What is becoming clear is that the eCB system is profoundly affected in FXS. However

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due to the modular nature of the eCB system, this also opens opportunities for pharmacological treatments (Ligresti, Petrosino, & Di Marzo, 2009).

In the CNS two principal eCBs are thought to be responsible for most neuromodulatory function: 2-arachildonoylglycerol (2-AG) and anandamide (István Katona & Freund, 2012). Both molecules are synthesized on-demand and can signal to a variety of receptors. Most prominent in neuronal function is the CB_1 receptor, found concentrated at many presynaptic specializations. Upon ligand binding, CB_1 receptors couple to $G_{i/0}$ linked G-protein complexes and act to inhibit neurotransmitter release (Fig. 12.1). The strength and duration of inhibition depends on the expression and coupling of the CB_1 receptor, but also critically on the local concentration of eCBs. Since eCBs rely on passive diffusion the local concentration reflects the distance from the source, however both 2-AG and anandamide are party to rapid degradation by local selective lipases; notably monoacylglycerol Lipase (MAGL) and ABHD6 for 2-AG and fatty acid amide hydrolase (FAAH) for anandamide. Thus the activation of CB_1 receptors is tightly controlled by the turnover of eCB ligand. Other eCB receptors are present in the CNS, but their localization and importance are debated. CB_2 receptors are principally associated with immune system cells, but are also reported in the CNS although their function and

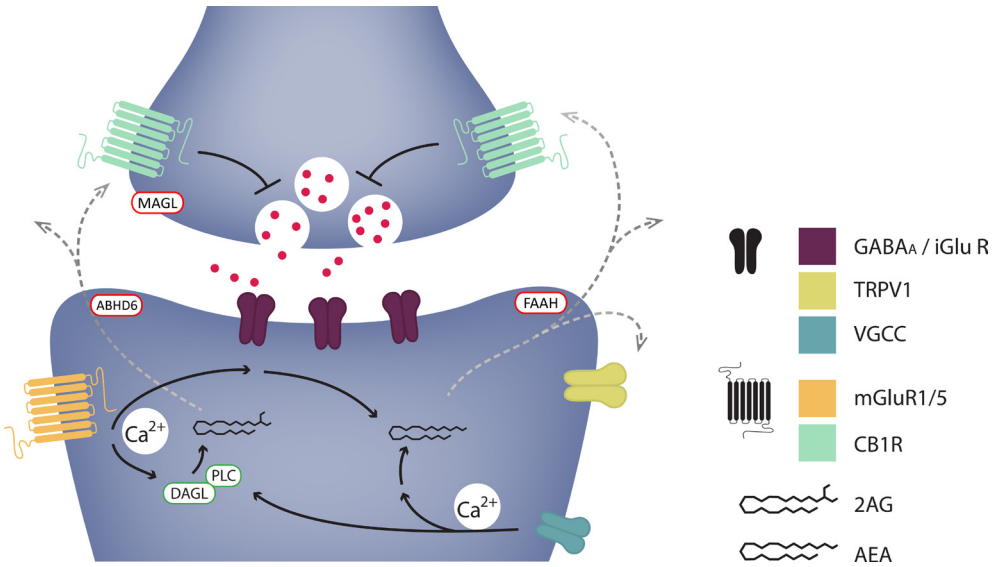

FIGURE 12.1 Principal endocannabinoid system components at fast central synapses. Endocannabinoids (eCB) primarily function as retrograde messengers in the CNS, mediating the local depression of both inhibitory and excitatory synaptic neurotransmission. eCB synthesis is initiated in the postsynaptic compartment via activation of either G_q coupled GPCRs *(mGluR1/5)* and/or voltage-gated calcium channels *(VGCC)*, both leading to an increase in postsynaptic calcium concentrations. The steps leading to 2-AG synthesis culminate in the hydrolysis of the lipid precursor diacylglycerol (DAG) by DAG lipase *(DAGL)* releasing 2-AG. In contrast multiple mechanisms may potentially lead to anandamide synthesis, whose regulation is poorly understood. Both eCBs locally diffuse *(dashed grey arrows)*, resulting in presynaptic activation of CB_1 receptors. The activated CB_1 receptor reduces neurotransmitter release, thus inhibiting the synapse. Furthermore, anandamide may also significantly activate TRPV1 receptors. Signaling is terminated by metabolism of the eCBs by specific lipases; 2-AG by monoacylglycerol lipase *(MAGL)* in the presynaptic compartment and ABHD6 in the postsynapse, and anandamide by fatty acid amide hydrolase *(FAAH)*.

coupling are unclear. Orphan G-protein coupled receptors (GPCRs) notably GPR55 may too be of importance. Finally it also appears that eCBs may directly couple to ion channels, particularly the TRPV1 receptor of the vanilloid family (Castillo, Younts, Chávez, & Hashimotodani, 2012). In this mode, a nonretrograde mechanism is proposed wherein anandamide production leads to postsynaptic depolarization due to TRPV1 channel opening. Functionally, this too may lead to depression of synaptic transmission only via a postsynaptic mechanism.

The pathways leading to the biosynthesis of 2-AG and anandamide are complex and in the case of anandamide incompletely described (István Katona & Freund, 2012). Specific molecules and enzymes in the context of FXS are described later; otherwise the reader is directed to some excellent reviews (Kano, Ohno-Shosaku, Hashimotodani, Uchigashima, & Watanabe, 2009). However it is worth noting that two distinct forms of 2-AG synthesis exist (Ohno-Shosaku & Kano, 2014). The first, in response to increased neuronal depolarization/firing, leads to a calcium dependent activation of phospholipase C β (PLCβ) and the release of the 2-AG from its precursor diacylglycerol (DAG) by DAG lipase. Generally this leads to a transitory global decrease in synaptic activity. The second mechanism depends on the activation of G_q coupled G-proteins, most prominently through Group I mGlu GPCRs in the postsynapse, again coupling to PLCβ and DAG lipase. Synaptic activation of group I mGlu receptors leads to a synaptic specific depression of neurotransmission that either alone or coupled with postsynaptic depolarization may lead to prolonged depression of synaptic activity (Robbe, Kopf, Remaury, Bockaert, & Manzoni, 2002). This long-term depression (LTD) is widely expressed in the CNS and is important in learning and memory functions and ultimately behavior.

MOLECULAR ALTERATIONS IN FXS

Fragile X Mental Retardation Protein (FMRP) is thought to modulate the translation of over 6000 mRNAs including a third of the postsynaptic proteome and a quarter of the presynaptic proteome (Ascano et al., 2012; Darnell et al., 2011). Of the eCB signaling complex prominently the principal 2-AG synthesizing protein DGLα is included on the list of FMRP targeted mRNAs (Darnell et al., 2011). In most neurons DGLα is found in the postsynaptic region and dendrites where it catalyzes the conversion of DAG to 2-AG (Tanimura et al., 2010). FMRP directly binds *DGLα* mRNA and leads to its enrichment in the postsynapse (Jung et al., 2012). In the *Fmr1* KO mouse this association is lost and leads to abnormalities in DGLα function (Straiker, Min, & Mackie, 2013). Notwithstanding the highly adaptive nature of the eCB system (Martin, Sim-Selley, & Selley, 2004), other components of the eCB system are remarkably stable in the *Fmr1* KO. Notably CB_1 receptor expression is unaffected (Zhang & Alger, 2010) and coupling to downstream mechanisms leading to inhibition of release are equivalent to wild-type mice at both inhibitory and excitatory forebrain synapses (Jung et al., 2012; Maccarrone et al., 2010; Zhang & Alger, 2010), although in vitro neuronal cultures do show an age-linked desensitization of CB_1 receptors (Straiker et al., 2013).

In contrast to 2-AG, the enzymatic pathways responsible for the production and degradation of anandamide appear normal in the *Fmr1* KO (Busquets-Garcia et al., 2013). However, there is potential for alternative parallel synthetic pathways and a complete audit of anandamide synthesis is awaiting (István Katona & Freund, 2012). This too applies to the

secondary eCB receptors where FXS related data is lacking. Notably TRPV1 and CB_2 receptors which have been recently identified as potential therapeutic targets (Busquets-Garcia et al., 2013).

Research has expanded the role of FMRP in the presynapse beyond its translation regulatory function and suggests FMRP has an important role in modulating presynaptic ion channel function and neurotransmitter release (Ferron, Nieto-Rostro, Cassidy, & Dolphin, 2014). Though there does not appear to be an overlap in CB_1 receptor mechanisms and FMRP regulation. However both systems are incompletely described particularly regarding presynaptic CB_1 receptor long-term plasticity changes (Castillo et al., 2012), so a presynaptic interaction should not be excluded.

INHIBITORY NEUROTRANSMISSION

Hippocampal DSI

In response to short postsynaptic depolarizing steps, GABAergic synaptic transmission onto CA1 pyramidal neurons in the hippocampus is rapidly inhibited (Pitler & Alger, 1992). The process, coined as depolarization induced suppression of inhibition (DSI), has subsequently been observed at many central synapses and demonstrated in response to natural neuronal activity (Kano et al., 2009). Critically, the phenomenon in the hippocampus and other brain regions is eCB mediated, requiring retrograde signaling of an eCB messenger to presynaptic CB_1 receptors (Kreitzer & Regehr, 2001; Ohno-Shosaku, Maejima, & Kano, 2001; Wilson & Nicoll, 2001). DSI depends on the postsynaptic synthesis of the eCB 2-AG in a calcium-dependent process, however under subthreshold conditions 2-AG synthesis and DSI may be achieved with coactivation of G_q linked GPCRs (Kim, Isokawa, Ledent, & Alger, 2002; Ohno-Shosaku et al., 2003; Varma, Carlson, Ledent, & Alger, 2001). Notably in the hippocampus, activating G_q coupled M_1 mACh receptors or $mGlu_5$ receptors (mGluR5) with coincident repeated DSI protocols leads to an extended depression of inhibition (iLTD), suggestive of a dose dependent effect of 2-AG signaling to CB_1 receptors (Younts, Chevaleyre, & Castillo, 2013). This form of inhibitory long-term depression is physiologically relevant and has an important role in gating CA1 long-term potentiation (Thomazeau, Bosch-Bouju, Manzoni, & Layé, 2016; Younts & Castillo, 2014).

Early after development of the *Fmr1* KO mouse it was clear that deficits in hippocampal function were prominent (The Dutch-Belgian Fragile X Consortium, 1994; Chapter 7). This led to the subsequent seminal discovery that CA1 pyramidal neurons from *Fmr1* KO mice have a lower threshold to glutamatergic mGluR-LTD induction due to an enhancement of mGluR5 function (Huber, Gallagher, Warren, & Bear, 2002). The history and implications of this finding are covered elsewhere, however it is important to emphasize that this mGluR-LTD as described in the hippocampus is mechanistically distinct. Induction of mGluR-LTD at CA1 glutamatergic synapses requires local protein translation and involves the postsynaptic internalization of glutamate receptors (Huber, Kayser, & Bear, 2000; Snyder et al., 2001). It has been proposed that in FXS it is the loss of regulated translation of mGluR5 activity targeted mRNAs by FMRP that leads to the enhanced coupling to mGluR5 mediated LTD (Waung & Huber, 2009). In contrast, both short-term and long-term depression of synaptic transmission

by endocannabinoids is independent of protein translation in both wild type and *Fmr1* KO mice (Yin, Davis, Ronesi, & Lovinger, 2006). Thus it was initially unclear how the loss of regulated translation by FMRP in FXS would impact endocannabinoid signaling in the hippocampus.

In the *Fmr1* KO mouse hippocampus there is evidence that CA1 inhibitory neurotransmission is altered, both in the number of inhibitory synapses and GABA receptor subunit composition (Paluszkiewicz, Martin, & Huntsman, 2011). However the core eCB signaling machinery at these synapses does appear to be maintained (Jung et al., 2012; Zhang & Alger, 2010). Short depolarizing steps in CA1 pyramidal neurons induces a like-for-like short term depression of evoked inhibitory postsynaptic currents (IPSCs) in *Fmr1* KO mice and wild-type littermates (Zhang & Alger, 2010). As such the induction of DSI appears unaffected in FXS. Direct CB_1 receptor activation produces a similar depression of IPSCs at these synapses and global expression levels of CB_1 receptor are unaffected (Busquets-Garcia et al., 2013; Zhang & Alger, 2010). Nevertheless coupling between mGluR5 and eCB signaling does appear changed. Measure of mGluR5 agonist depression of IPSCs suggests a dose-response shift in the depression of inhibitory synaptic signaling. Using the archetypal mGluR5 agonist DHPG, Zhang and Alger (2010) report an increased sensitivity to DHPG induced depression of IPSCs (Fig. 12.2), but a similar maximal inhibition, is also reported (Busquets-Garcia et al., 2013). At threshold concentrations of DHPG, *Fmr1* KO mice but not wild-type littermates express iLTD and disinhibition of LTP at CA1 synapses, suggesting a lower threshold to synaptic plasticity (Zhang & Alger, 2010). However, heterosynaptic iLTD protocols do not appear to show the same sensitivity and result in a similar depression of GABAergic transmission in wild type and *Fmr1* KO animals (Busquets-Garcia et al., 2013). Likely differences in synaptic (i.e., localized) versus broad agonist stimulation of mGluR5 receptors are responsible for these inconsistencies. Induction of iLTD is dependent on the local concentration of 2-AG, which due to its rapid degradation by MAG lipases is limited to a small volume estimated around 10 μm radius (Chevaleyre & Castillo, 2004; Younts et al., 2013). DHPG stimulated 2-AG production couples mGluR5 and mGluR1 activation to DAGα throughout the dendritic tree. In contrast, synaptically evoked 2-AG is limited to mGluR5-DGLα coupling in the perisynaptic region of activated glutamatergic synapses. Thus, the findings indicate a selective enhancement of extrasynaptic group I mGluR coupling eCB mediated depression of inhibitory synaptic transmission in the *Fmr1* KO.

Modulation of mGluR5-Coupled Function by Homer

Further interpretation of these findings is complicated by the complex hippocampal endophenotype of FXS. Prominently hippocampal synapses and spines are changed in the *Fmr1* KO mouse, notably a reduction in the head size and length of CA1 pyramidal neuron spines and possibly number (Lauterborn, Jafari, Babayan, & Gall, 2013; Sidhu, Dansie, Hickmott, Ethell, & Ethell, 2014). In the wild-type hippocampus, mGluR5 is principally concentrated in the perisynaptic annulus surrounding the active zone at CA1 glutamatergic synapses, although this is not uniformly the case and significant extrasynaptic populations do exist (Lujan, Nusser, Roberts, Shigemoto, & Somogyi, 1996; Luján, Roberts, Shigemoto, Ohishi, & Somogyi, 1997). In FXS there is no gross change in mGluR5 expression in the hippocampus (Dölen et al., 2007; Giuffrida et al., 2005). Furthermore, ultrastructural studies in adult *Fmr1*

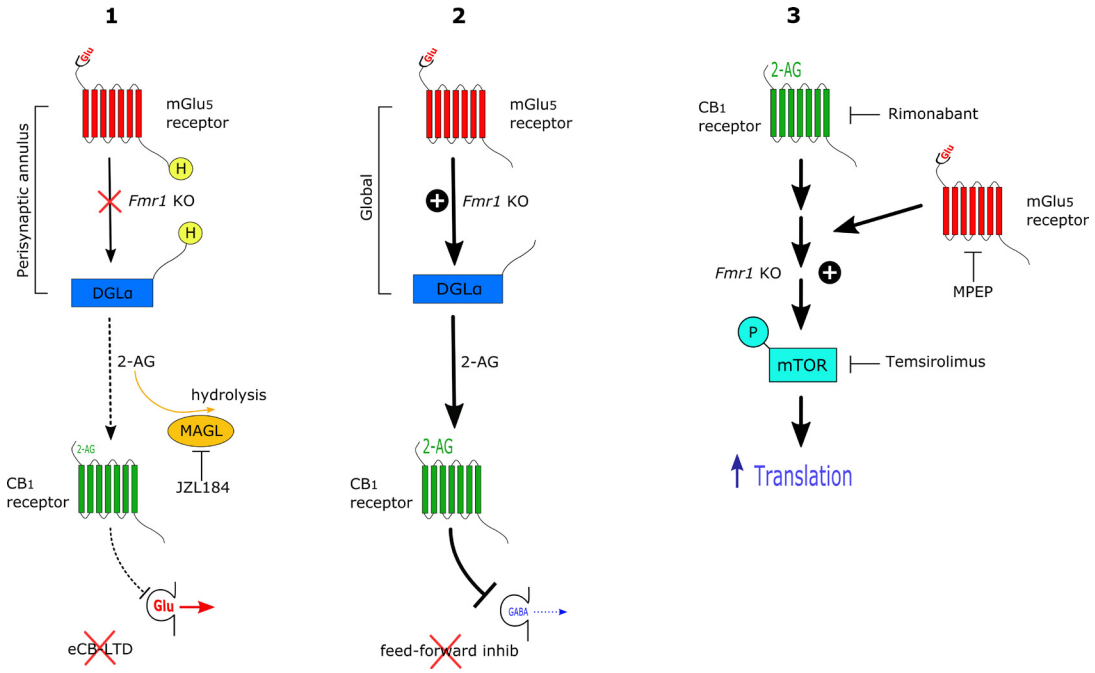

FIGURE 12.2 **Multiple deficits in endocannabinoid mediated signaling in the *Fmr1* KO mouse.** Schematic of the three principal mechanisms in which endocannabinoid (eCB) signaling is affected in the *Fmr1* KO mouse (**1**) At excitatory synapses *Fmr1* KO leads to a loss of the tight coupling between mGluR5 and DGLα in the perisynaptic region necessary for the on demand synthesis of the eCB 2-AG (Jung et al., 2012), possibly due to a loss in long form homer (H) interactions. Consequently activation of mGluR5 results in reduced eCB production and diminished activation of presynaptic CB_1 receptors. This loss of CB_1 receptor activation is manifested as an absence of eCB mediated long-term depression *(eCB-LTD)* and consequently a principal mechanism of regulating the strength of synaptic connections. At these synapses induction of eCB-LTD is lost. Function may however be restored via inhibition of 2-AG hydrolysis by MAGL with the selective antagonist JZL184. (**2**) Global activation of mGluR5 and coupling to inhibitory synapse CB_1 receptors may in contrast be enhanced in *Fmr1* KO mice. Treatment with mGluR5 agonist (DHPG) leads to increased inhibition of GABA release at a subset of inhibitory synapses (Maccarrone et al., 2010; Zhang & Alger, 2010). At CA1 pyramidal neurons this may contribute to decreased feed-forward inhibition. (**3**) In the hippocampus CB_1 receptor activation may contribute to the exaggerated translation endophenotype found in the *Fmr1* KO. Antagonists of both CB_1 receptors and mGluR5 reduce the *Fmr1* KO dependent increase in basal mTor activity and improve performance in a hippocampus related task (Busquets-Garcia et al., 2013).

KO CA1 indicate the receptor distribution is unchanged (Jung et al., 2012). Notwithstanding the unchanged mGluR5 distribution, clearly the coupling of mGluR5 to its effector signaling pathways is profoundly affected in *Fmr1* KO mice.

A clue comes from reported changes in the postsynaptic protein homer. Homer proteins are a group of principally postsynaptic scaffold proteins that exist in two important splice variants; a long constitutively expressed form and a short inducible form (Shiraishi-Yamaguchi & Furuichi, 2007). All homer isoforms contain an N-terminal EVH1 domain which binds the intracellular tail of mGluR5 and mGluR1a. All long homer forms also contain a C-terminal coiled-coil domain that allows multimerization of homer long forms. This is important since

the EVH1 domain also binds to other postsynaptic signaling molecules and scaffold proteins, notably Shank, IP_3 receptor, PI3 Kinase enhancer and DGLα (Sala, Roussignol, Meldolesi, & Fagni, 2005). Thus long homer isoforms both correctly localize group I mGluRs in the synapse, but also couple it to effector mechanisms. In contrast short homer isoforms, prominently Homer1a, lack a coiled-coil domain and effectively dissociate mGluR5 from other homer associated signaling molecules. This has the effect of uncoupling mGluR5 from downstream pathways (Kammermeier & Worley, 2007; Ronesi & Huber, 2008). However, Homer1a binding to group I mGluRs also results in the constitutive activation of the receptor and leads to changes in activity in coupled ion channels (Ango et al., 2001; Hu et al., 2010). Notably, Homer1a is an immediate early gene and its expression is rapidly modulated in response to changes in neuronal activity and as such offers a mechanism of homeostatic scaling of neuronal inputs (Guo, Ceolin, Collins, Perroy, & Huber, 2015; Hu et al., 2010). However, changes in homer expression are also found in many neuropathological states, including addiction and ASD (Szumlinski, Kalivas, & Worley, 2006).

In FXS it appears that some of the changes in mGluR5 function may be attributed to homers. In the hippocampus the association of mGluR5 with Homer1a is increased in *Fmr1* KO mice, and the association with long form homer isoforms is correspondingly reduced (Giuffrida et al., 2005; Guo et al., 2015). The change in homer isoform binding in *Fmr1* KO is mediated by a hyperphosphorylation of long-form homer by CaMKIIα leading to a loss of mGluR5 binding (Guo et al., 2015). Correction of the increased Homer1a association in Homer1a knockout mice restores mGluR5 coupling to synaptic downstream signaling cascades, principally the PI3K-mTor pathway and regulated protein translation in the hippocampus (Ronesi et al., 2012). Furthermore, either Homer1a knockdown or correction of long-form homer hyperphosphorylation remedies a number of neurological and behavioral phenotypes in *Fmr1* KO mice, although notably not aberrant mGluR-LTD (Guo et al., 2015; Ronesi et al., 2012). Likewise disruption of mGluR5 binding to homers in wild-type mice has the effect of mimicking some of the synaptic deficits in *Fmr1* KO mice (Ronesi et al., 2012; Ronesi & Huber, 2008; Tang & Alger, 2015). Significantly the effect of peptide disruption of homer-group I mGluR interaction with a blocking peptide is to shift the DHPG-mediated depression of IPSCs dose response curve in a similar manner to the *Fmr1* KO (Tang & Alger, 2015). Furthermore, DHPG-induced eCB-mediated depression of IPSCs by the peptide is occluded in *Fmr1* KO mice, suggesting a shared mechanism. Similar to the *Fmr1* KO, the peptide has no effect on DSI in wild-type mice (Tang & Alger, 2015). The implication is that loss of mGluR5 association with long homer isoforms augments functional coupling between mGluR5 and eCB-mediated depression of CA1 IPSCs. Critically however, the effects of mGluR5-Homer1a association on eCB synaptic signaling are unknown.

Studies from the nucleus accumbens hint at a loss of mGluR5-DGLα coupling due to increased association with homer isoforms (Fourgeaud et al., 2004), and in vitro experiments measuring instead eCB mediated depression of excitatory neurotransmission (DSE) suggest that Homer1a expression reduces mGluR5 synaptic depression (Li, Krogh, & Thayer, 2012; Roloff, Anderson, Martemyanov, & Thayer, 2010). Thus the effect of mGluR5 association with Homer1a on eCB signaling may be detrimental to eCB signaling. However, interpretation is further complicated by the binding of DGLα to homers (Jung et al., 2007). In contrast to mGluR5, the ultrastructural distribution of DGLα suggests it is profoundly affected in the *Fmr1* KO hippocampus. In wild-type mice DGLα is found concentrated in the perisynaptic

annulus near mGluR5 (Jung et al., 2012; Yoshida et al., 2006), however in *Fmr1* KO neurons, DGLα concentration at the synapse is lost in both the hippocampus and nucleus accumbens (Jung et al., 2012). Group I mGluR and DGLα share the same homer binding motif, thus expression of Homer1a may have the effect of dissociating DGLα from the synaptic endocannabinoid signaling complex resulting in reduced synaptic 2-AG synthesis (Jung et al., 2012). How this relates to extrasynaptic mGluR1/5 coupling to DGLα is uncertain. It is worth mentioning that global expression levels of DGLα are increased in *Fmr1* KO mice and that *Dglα* mRNA is FMRP associated; hinting at perhaps enhanced extrasynaptic 2-AG synthesis (Darnell et al., 2011; Jung et al., 2012). However without direct measurement of eCB levels in the hippocampus or knowledge of the relative activity of other eCB machinery components in *Fmr1* KO at these synapses, this remains speculative.

Endocannabinoid Modulation of Striatal Neurotransmission

A notable neuroanatomical feature of FXS is an enlarged striatum (Eliez, Blasey, Freund, Hastie, & Reiss, 2001; Menon, Leroux, White, & Reiss, 2004). Significantly there is an overlap in striatal-associated behaviors and FXS clinical features, suggesting dysfunction in this brain structure. Endocannabinoids also prominently regulate short and long term plasticity in this brain region at both glutamatergic (Robbe et al., 2002) and GABAergic synapses (Calabresi, Picconi, Tozzi, Ghiglieri, & Di Filippo, 2014; Gerdeman, Ronesi, & Lovinger, 2002; Kano et al., 2009). Medium spiny neurons (MSN) make up the majority of neurons in the striatum and are also the principal projection neurons. In the *Fmr1* KO mouse the intrinsic properties of these neurons are unaltered (Centonze et al., 2008; Neuhofer et al., 2015). Nevertheless synaptic inhibitory inputs appear upregulated compared to wild-type mice, possibly reflecting an increased GABAergic innervation of medium spiny neurons (Centonze et al., 2008). Much like the CA1 pyramidal neurons, MSNs express a presynaptic CB_1 receptor mediated DSI in response to postsynaptic depolarization (Narushima, Uchigashima, Hashimoto, Watanabe, & Kano, 2006), which is enhanced with concomitant activation of mGluR5 or M_1 mAChR (Narushima et al., 2007; Uchigashima et al., 2007). Challenging MSNs with high doses of the mGluR5 agonist DHPG depresses spontaneous IPSC in the absence of postsynaptic depolarization, an effect that is enhanced in the *Fmr1* KO (Maccarrone et al., 2010). Suggesting at least in response to bulk activation of mGluR5 that coupling to eCB mediated synaptic depression of GABAergic signaling is enhanced in the striatum of *Fmr1* KO mice (Fig. 12.2), much like the hippocampus (Maccarrone et al., 2010; Zhang & Alger, 2010). However since basal inhibitory synaptic activity is higher in the *Fmr1* KO, the effect of DHPG is to normalize spontaneous activity compared to DHPG challenged wild-type mice. Consistent with *Dglα* mRNA regulation by Fmrp, basal DGLα activity is enhanced in the *Fmr1* KO striatum and forebrain (Jung et al., 2012; Maccarrone et al., 2010). However this is not reflected in an increase in 2-AG concentrations, nor in tonic effects on synaptic transmission (Jung et al., 2012; Maccarrone et al., 2010); possibly due to compensatory increased MAGL activity (Maccarrone et al., 2010). A further confounding factor may be an increase in mGluR5 expression in the *Fmr1* KO striatum (Maccarrone et al., 2010), however this has not been observed by other groups (Jung et al., 2012; Michalon et al., 2014).

A recent study of synaptic iLTD in the striatum may hint at an alternative interpretation of changes in eCB modulation of inhibitory transmission in FXS (Mathur, Tanahira, Tamamaki,

& Lovinger, 2013). An intriguing observation made by Maccarrone et al. (2010), is that only a proportion of cells respond to DHPG in *Fmr1* KO. Induction of iLTD in MSNs proceeds via two mutually exclusive mechanisms depending on the membrane potential, linked possibly to changes in "up" and "down" state in the intact striatum. In the "up" state eCB-iLTD engages DGLα and 2-AG mediated activation of CB_1 receptor, while in the "down" state eCB-iLTD instead engages anandamide synthesis again leading to CB_1 receptor activation and iLTD (Mathur et al., 2013). A similar segregation of eCB-LTD mechanisms is found in the extended amygdala (Puente et al., 2011). Both forms of striatal iLTD depend on mGluR activation, however in contrast to "up" state, "down" state iLTD is found only in one subpopulation of MSNs. Nominally the recordings of Maccarrone et al. (2010) of spontaneous IPSCs appear to be recorded in the "down" state and approximately half of recorded MSN do not respond to DHPG, suggesting that the DHPG effect on inhibitory transmission may be anandamide mediated. Such a mechanism would reconcile the confounding observation that DHPG mediated 2-AG synthesis is absent in *Fmr1* KO forebrain and striatum preparations (Jung et al., 2012; Maccarrone et al., 2010). Whether coupling to anandamide-mediated mechanisms is actually enhanced in the *Fmr1* KO is undetermined, however unpublished observations (Manzoni and Martin) in the prefrontal cortex suggest that in the absence of 2-AG mediated mechanisms that anandamide may have a compensatory role in the *Fmr1* KO mouse.

EXCITATORY NEUROTRANSMISSION

Endocannabinoid-Mediated Long-Term Depression (eCB-LTD)

In FXS studies of endocannabinoid regulation, inhibitory neurotransmission has predominated perhaps due to eCB-iLTD plasticity dominating in the hippocampus. However eCB regulation of excitatory neurotransmission is equally prominent in the CNS (Kano et al., 2009). Significantly monosynaptic eCB-LTD can readily be induced in many brain regions in response to synaptic stimulation (Heifets & Castillo, 2009). In the ventral striatum, notably the nucleus accumbens, eCB-LTD is expressed at glutamatergic afferents synapsing onto MSNs and requires mGluR5 activity (Robbe et al., 2002). This LTD is 2-AG mediated and results in a sustained depression of glutamate release. In *Fmr1* KO mice the LTD is absent (Jung et al., 2012). Unlike inhibitory neurotransmission, *Fmr1* KO MSNs show no changes in basal excitatory activity and tonic eCB signaling is unaffected (Jung et al., 2012; Neuhofer et al., 2015). Thus modulation of MSN inputs is lost in the *Fmr1* KO.

Serial electron microscopy has allowed reconstruction of the MSN glutamatergic synapses allowing detailed understanding of the ultrastructural changes occurring in *Fmr1* KO. However in common with other brain regions the structural phenotype is moderate in adult tissue; an increase in synapse density and lengthening of the spine neck (Neuhofer et al., 2015). It is only when the ultrastructural synaptic components are analyzed that a molecular deficit in the eCB system becomes apparent, notably a loss in DGLα concentration in the perisynaptic annulus region and an intracellular retention in the neck region (Jung et al., 2012). Similar to the hippocampus mGluR5 localization does not appear affected. Compared to inhibitory synapses, glutamatergic afferents onto striatal MSN contain fewer CB_1 receptors, which appears

to be related to a higher DSE induction threshold compared to DSI (Uchigashima et al., 2007). Thus, a loss in the precise coupling between mGluR5 and DGLα in the spine might be expected to dramatically affect local 2-AG production and thus, CB_1 receptor activation (Fig. 12.2). As such despite increased expression of DGLα in the *Fmr1* KO, mGluR5-mediated 2-AG production is impaired (Jung et al., 2012; Maccarrone et al., 2010).

If it is the failure to achieve threshold concentrations of 2-AG that impairs eCB-LTD, a reasonable prediction is that pharmacological treatments that increase 2-AG concentrations might restore eCB function in the *Fmr1* KO. Two pharmacological routes present themselves: enhancement of 2-AG synthesis and inhibition of 2-AG degradation/hydrolysis. The duration and strength of 2-AG signaling is particularly sensitive to hydrolysis by MAGL (Blankman, Simon, & Cravatt, 2007; Kano et al., 2009). Consequently we reported that blocking 2-AG degradation with the specific MAGL inhibitor JZL184 leads to a rapid recovery of eCB-LTD not only in the nucleus accumbens, but also prefrontal cortex of the *Fmr1* KO mouse (Jung et al., 2012). This finding not only confirms that it is a decoupling in mGluR5-mediated 2-AG synthesis which underlies deficits in excitatory eCB-LTD, but also suggests the eCB system may be a worthy clinical target in FXS (discussed later). An alternative, but paradoxical approach in light of the mGluR theory of FXS, would be to enhance mGluR5 function to restore DGLα coupling in the *Fmr1* KO. While such a treatment might be controversial, in other nonsyndromic models of autism and cognitive dysfunction enhancement of mGluR5 function with a positive allosteric modulator normalizes physiology and behavior (Won et al., 2012). Preliminary results have shown that treatment of prefrontal cortex *Fmr1* KO neurons with the mGluR5 positive allosteric modulator CDPPB restores eCB-LTD (Martin and Manzoni, unpublished). Thus in the context of the endocannabinoid system, treatments that inhibit mGluR5 function might be expected to aggravate deficits in 2-AG signaling.

Findings in Other Nonsyndromic Models

Changes in eCB function are linked to many of the cognitive and behavioral phenotypes of FXS including: intellectual disability (Lysenko et al., 2014; Thomazeau et al., 2014), anxiety (Larrieu, Madore, Joffre, & Layé, 2012), and autism (Kerr, Downey, Conboy, Finn, & Roche, 2013). Recent work studying the role of tonic eCB signaling in the ASD-linked neuroligin mutations in mice are particularly noteworthy, since the model shares deficits in mGluR-LTD (Baudouin et al., 2012).

Neuroligin 3

Neuroligins and their presynaptic partners, neurexins, have important roles in synaptic adhesion and the determination of inhibitory and excitatory synaptic properties (Bemben, Shipman, Nicoll, & Roche, 2015). Numerous mutations have been identified in neurexins and neuroligins that are associated with autistic features, which variably effect both inhibitory and excitatory neurotransmission (Bang & Owczarek, 2013; Bemben et al., 2015). In this context an accentuated feature is the transsynaptic signaling of neuroligins wherein mutations may affect both post and presynaptic function. Mutations affecting the expression or function of neuroligin 3 (NL3), have similar physiological and behavioral deficits to the *Fmr1* KO (Baudouin et al., 2012; Tabuchi et al., 2007).

One unusual feature of ASD linked *NL3* mutations is the synapse selective effect it has on basal GABAergic neurotransmission, leading to localized increases or decreases in inhibitory drive (Földy, Malenka, & Südhof, 2013; Speed, Masiulis, Gibson, & Powell, 2015; Tabuchi et al., 2007). An important determining factor in the direction of this change is the tonic eCB signal at the synapse. CA1 pyramidal neurons receive somatic inhibitory drive from parvalbumin (PV) and cholecystokinin (CCK) basket cells (Klausberger & Somogyi, 2008). In *NL3* knockout mice IPSCs are enhanced solely in connections arising from CCK positive interneurons and not from PV interneurons (Földy et al., 2013). PV basket cell interneurons lack CB_1 receptors and thus a tonic eCB signal, whereas near all CCK interneurons are CB_1 receptor positive (Katona et al., 1999). Unexpectedly in the NL3 knockout model tonic CB_1 receptor inhibition of IPSCs is lost at CA1 synapses, yet both DSI and iLTD are maintained indicating that the core eCB machinery is functional. How NL3 affects tonic eCB signaling is unclear and it should be emphasized that the loss of tonic eCB signaling does not account for enhanced IPSCs at all mutant synapses (Rothwell et al., 2014). However, tonic eCB signaling is thought to have an important homeostatic role in mediating hippocampal networks (Kim & Alger, 2010), thus deficits are likely to impact hippocampal excitability.

An initial test of changes in eCB tonic signaling in the *Fmr1* KO failed to detect any gross changes at CA1 inhibitory synapses (Busquets-Garcia et al., 2013). However it is possible any tonic effect is hidden in specific interneuron inputs. A related *NL3* gain of function mutation (R451C) which has autism related behaviors similar to the *NL3* knockout lacks a tonic eCB signaling at CCK interneuron-CA1 pyramidal neuron synapses, however in this case overall inhibitory drive is compensated by increased PV interneuron IPSCs (Földy et al., 2013). Finally it is noted that changes in tonic eCB signaling need not be limited to inhibitory synapses. Studying the *Neurexin β* knockout ASD model there are reported changes at excitatory CA1 afferents in the subiculum (Aoto, Földy, Ilcus, Tabuchi, & Südhof, 2015). Here a decrease in miniature EPSCs is connected to an upregulation of tonic eCB function possibly due to enhanced 2-AG production (Anderson et al., 2015). Thus in neuroligin models the principal synaptic endophenotype is linked to deficits in tonic rather than phasic endocannabinoid signaling.

ENDOCANNABINOID SYSTEM INTERVENTIONS

An uncoupling between glutamatergic signaling and DGLα mediated 2-AG synthesis is currently the strongest eCB related endophenotype in the *Fmr1* KO mouse model (Jung et al., 2012; Maccarrone et al., 2010). The subsequent loss of eCB-LTD at glutamatergic synapses is not only robust, but also found in multiple brain areas (Jung et al., 2012, Fig. 12.2). As discussed earlier, presynaptically localized MAGL has a critical role in determining the strength of 2-AG signaling to CB_1 receptors via the rapid hydrolysis of 2-AG. Importantly inhibition of MAGL by the highly selective drug JZL184 has the power to transform nominally subthreshold eCB signaling to supra-threshold CB_1 receptor mediated signaling (Martin et al., 2015). JZL184 has therapeutic potential, it is highly selective for MAGL and systemically tolerated (Long et al., 2009). Furthermore in mouse models of anxiety and depression JZL184 has anxiolytic effects and reduces depressive-like behavior (Busquets-Garcia et al., 2011; Zhong et al., 2014).

Focusing on the acute restoration of eCB function with JZL184 Jung et al. (2012) have assayed single intraperitoneal injections of JZL184 in *Fmr1* KO mice at concentrations that do not affect activity in wild-type littermates. Single JZL184 treatment results in a rapid increase in 2-AG concentrations, reaching a maximum after 8 h and remaining elevated over 24 h (Jung et al., 2012; Long et al., 2009). *Fmr1* KO mice become less hyperactive with JZL184 treatment, such that locomotion is normalized 8 h after injected (Jung et al., 2012). Furthermore aberrant behavior on the elevated plus maze is corrected with JZL184. *Fmr1* KO mice are less anxious than wild-type mice in this generalized test of anxiety, although there may be increased social anxiety (Liu & Smith, 2009; Yuskaitis et al., 2010). JZL184 corrects both the number of entries into open arms, but also the time spent in open arms in *Fmr1* KO mice (Jung et al., 2012). Thus enhancement of 2-AG signaling ameliorates some of the FXS linked phenotypes in the *Fmr1* KO mouse. Drawing a link to the disruption of mGluR5 postsynaptic signaling complexes in FXS, a similar correction of behavior has been reported in *Fmr1* KO mice lacking Homer 1a (Ronesi et al., 2012). Crossing *Fmr1* KO mice with a *Homer 1a* KO normalizes elevated plus maze behavior and reduces audiogenic seizures (Ronesi et al., 2012). Notwithstanding these promising results, an extended behavioral assay of JZL184 in the *Fmr1* KO is pending. Furthermore whether JZL184 might be tolerated in chronic protocols is uncertain in light of reports of functional antagonism of the eCB system in wild-type mice (Schlosburg et al., 2010). Thus exploration of alternative methods of 2-AG pharmacological modulation is also warranted (Jung et al., 2012; Marrs et al., 2010).

The core behavioral phenotypes of FXS, hyperactivity and anxiety, are consistent with hypoactivity of the eCB system, however in the *Fmr1* KO mouse there is evidence that inhibition of the eCB signaling may improve hippocampus-linked cognitive dysfunction (Busquets-Garcia et al., 2013). Increased basal protein synthesis is a hallmark of FXS (Qin, Kang, Burlin, Jiang, & Smith, 2005). Likely multiple endogenous signaling cascades contribute to the enhanced translation phenotype, however the mTor and ERK1/2 signaling cascades are thought to be particularly important (Bhakar, Dölen, & Bear, 2012). Through an indirect mechanism hippocampal CB_1 receptors localized on interneurons are able to modulate both mTor and ERK1/2 activity in principal cells (Derkinderen et al., 2003; Puighermanal et al., 2009). In this context activation of CB_1 receptors may contribute to the negative effects of cannabinoids on memory, as well as be mechanistically therapeutic (Puighermanal et al., 2009; Zhong et al., 2014). Busquets-Garcia et al. (2013) made the observation that if enhanced mTor activity is responsible for the hippocampal FXS endophenotype (Bhakar et al., 2012; Sharma et al., 2010) interventions that down regulate the mTor pathway may normalize hippocampal function (Fig. 12.2). Indeed, inhibitors of upstream and downstream molecules in the mTor pathway normalize hippocampal protein synthesis and correct behavior in the *Fmr1* KO mouse (Bhattacharya et al., 2012, 2015; Busquets-Garcia et al., 2013; Gross et al., 2010). As discussed earlier there may be enhanced activity-dependent eCB signaling at interneuron-CA1 pyramidal synapses in the *Fmr1* KO hippocampus (Zhang & Alger, 2010), thus CB_1 receptor antagonists could downregulate pathological mTor activity. Confirming this hypothesis, chronic treatment with the CB_1 receptor antagonist rimonabant reduces hyperactivity in the mTor signaling cascade (Busquets-Garcia et al., 2013). Correspondingly, rimonabant improves *Fmr1* KO mouse performance in a hippocampal-based object memory task, both after chronic and surprisingly acute treatment during the consolidation stage with a similar profile to direct mTor inhibition (Busquets-Garcia et al., 2013). Critically these effects were observed with doses of rimonabant

that are not anxiogenic in wild-type mice (Zhong et al., 2014), although given the confused *Fmr1* KO anxiety phenotype it is unclear if this also applies to FXS where there is already heightened anxiety. Whether CB_1 receptor inhibition is effective at ameliorating other *Fmr1* KO phenotypes especially in the ASD domain is largely undetermined. However in light of the JZL184 results of Jung et al. (2012) care should be taken in applying these findings to other brain structures.

Recent evidence investigating the effects of the anesthetic propofol suggest that anandamide, in contrast, may have a therapeutic role in improving behavior in the *Fmr1* KO. Propofol promotes $GABA_A$ receptor function, but also has FAAH inhibitory activity and thus is expected to augment anandamide concentrations (Patel et al., 2003). Testing the consolidation phase of a passive avoidance task in which *Fmr1* KO show significant deficits, treatment with either propofol or the established FAAH inhibitor URB597 improved performance in *Fmr1* KO mice (Qin et al., 2015). Importantly improvement in this hippocampus-based task did not rely on the GABAa receptor properties of propofol and instead was dependent on CB_1 receptor function. Interestingly the alternative augmenting 2-AG levels with JZL184 was ineffective at restoring passive avoidance behavior in the *Fmr1* KO, thus the effect is more nuanced than a simple boost to the eCB system. How these findings fit into a hippocampal model where reducing CB_1 receptor activity promotes learning and memory is unclear especially given URB597 has previously been shown to disrupt performance in wild-type mice (Busquets-Garcia et al., 2011, 2013). Notably, the passive avoidance task involves significant contributions from other brain areas and interpretation is complicated by reduced nociception in *Fmr1* KO mice, thus URB597 could be having peripheral functions in this context. Nevertheless it has been noted that propofol corrects the central enhanced protein synthesis endophenotype in both *Fmr1* KO mice and FXS individuals (Qin et al., 2013). Thus these findings add to the increasingly complex picture of the role of eCBs in FXS.

CONCLUSIONS/PERSPECTIVES

The loss of FMRP profoundly affects many neuronal hubs including the eCB system. However, a clear consensus on the precise nature of the deficit in the eCB system is still emerging (Fig. 12.2). Given the enormous therapeutic potential of the eCB system (Ligresti et al., 2009) this is a source of frustration. Likely the tailored function of eCB modulation to a specific synapse in a specific state may be responsible for some of the conflicting findings from the *Fmr1* KO mouse. However contrasting dysfunctions between different brain areas and neuronal populations appears to be a feature of FXS (Contractor, Klyachko, & Portera-Cailliau, 2015; Kalmbach, Johnston, & Brager, 2015). Thus, global pharmacological interventions in FXS are significantly complicated. Nevertheless the expanding library of drugs selectively targeting individual modules of the eCB system, coupled with a better understanding of the precise changes occurring in FXS may ultimately lead to useful eCB pharmacotherapy in FXS.

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CHAPTER

13

Glycogen Synthase Kinase-3: Abnormalities and Therapeutic Potential in Fragile X Syndrome

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INTRODUCTION

During the last few years there has been tremendous progress in understanding the pathological mechanisms underlying fragile X syndrome (FXS). This knowledge has provided several leads based on findings in animal models of potential interventions that may be therapeutic in FXS. One of these is lithium and other inhibitors of glycogen synthase kinase-3 (GSK3), findings that are discussed in this review.

FRAGILE X SYNDROME: ETIOLOGY AND ANIMAL MODELS

FXS is the most common hereditary form of intellectual disability caused by a single genetic defect, the loss of expression of the *fragile X mental retardation 1* (*FMR1*) gene (Pieretti et al., 1991; Bardoni & Mandel, 2002; Kooy, Willemsen, & Oostra, 2000). This is caused by expansion of a trinucleotide CGG repeat in the 5′ UTR of the *FMR1* gene, resulting in loss of the fragile X mental retardation protein (FMRP). FMRP plays important roles in RNA binding and translation regulation, as well as regulating extracellular transport and sodium-activated potassium channels (Brown et al., 1998, 2010; Laggerbauer, Ostareck, Keidel, Ostareck-Lederer, & Fischer, 2001; Bardoni, Mandel, & Fisch, 2000).

Mouse (Bakker et al., 1994) and *Drosophila* (Wan, Dockendorff, Jongens, & Dreyfuss, 2000) are the most common animal models used to study FXS. Bakker et al. (1994) developed the first mouse model of FXS with an inactive *Fmr1* gene (hereafter referred to as *Fmr1* knockout mice). *Fmr1* knockout mice display characteristics that have several similarities to patients

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with FXS, including macroorchidism, certain features of behavior, including hyperactivity and autistic-like behaviors, among others, and some cognitive impairments (Musumeci et al., 2000; Yan, Asafo-Adjei, Arnold, Brown, & Bauchwitz, 2004; Bernerdet and Crusio, 2006; Krueger, Osterweil, Chen, Tye, & Bear, 2011). There are also increases in dendritic spine length and number, but reduced maturation of spines, in *Fmr1* knockout mice compared to wild-type littermates (Comery et al., 1997; Irwin et al., 2001, 2002). These and other characteristics of *Fmr1* knockout mice have made them an extremely useful model to study features of FXS and to identify potential therapeutic interventions, as described in the chapter on animal models in this book (Chapter 7).

GLYCOGEN SYNTHASE KINASE-3

One potential therapeutic target in FXS is the serine/threonine kinase GSK3, based on results discussed further. GSK3 exists in two isoforms, GSK3α and GSK3β, which together are referred to as GSK3 (Woodgett, 1990). Regulation of GSK3 is primarily mediated by inhibitory serine-phosphorylation at serine21 in GSK3α and serine9 in GSK3β. The inhibitory serine-phosphorylation of GSK3 can be induced by multiple signaling pathways that converge on GSK3, such as signaling by several growth factors and neurotransmitters (Beurel, Grieco, & Jope, 2015). Impairments in these pathways can cause less serine-phosphorylated GSK3 causing inadequate inhibition of GSK3, resulting in hyperactive GSK3, which has been implicated as contributing to several prevalent diseases, such as Alzheimer's disease, diabetes, and mood disorders (Jope & Johnson, 2004; Mines, Yuskaitis, King, Beurel, & Jope, 2010). GSK3 has numerous effects on the functions of cells due to its involvement in many receptor-coupled signal transduction pathways and its capacity to phosphorylate at least 100 substrates. For example, the activity of GSK3 is regulated by intracellular signals induced by multiple neurotransmitters (such as serotonin, dopamine, acetylcholine, glutamate), hormones and growth factors (such as insulin, brain-derived neurotrophic factor, estrogen), and by the Wnt signaling pathway that regulates β-catenin (Beurel et al., 2015). GSK3, in turn, regulates a large array of cellular functions, such as microtubule dynamics, synaptic plasticity, apoptosis, autophagy, dynamic alterations of spines, dendrites and axons, and gene expression, particularly via its phosphorylation of over 25 transcription factors.

Studies of the actions of GSK3 were facilitated when it was discovered that lithium, a mood stabilizer used in the treatment for bipolar disorder, selectively inhibits GSK3 (Klein & Melton, 1996; Stambolic, Ruel, & Woodgett, 1996). Lithium directly inhibits GSK3 activity by competing with magnesium for binding to GSK3 and also after in vivo treatment increases the inhibitory serine-phosphorylation of GSK3 (Jope, 2003). Investigations of lithium's effects made possible many discoveries of actions of GSK3 and revealed that inadequately inhibited GSK3 is linked to several diseases in addition to bipolar disorder. These connections between GSK3 and pathological processes prompted the development of several small molecule selective inhibitors of GSK3 (Martinez, Castro, & Medina, 2006), such as indirubin derivatives (Leclerc et al., 2001), TDZD derivatives (Martinez, Alonso, Castro, Pérez, & Moreno, 2002), L803-mts (Plotkin, Kaidanovich, Talior, & Eldar-Finkelman, 2003), and SB216763 (Coghlan et al., 2000), as well as many others, in part because of limitations in the clinical use of lithium. These limitations include the necessity of monitoring lithium levels, because elevations above

the therapeutic concentration can be toxic. Additionally, side effects of lithium administration can include hand tremor, hypothyroidism, weight gain, polyuria, and renal damage, among others (Oruch, Elderbi, Khattab, Pryme, & Lund, 2014).

Once hyperactive GSK3 has been shown to be linked to diseases, a useful model to study is GSK3 knockin mice (McManus et al., 2005). In homozygous GSK3α/$\beta^{21A/21A/9A/9A}$ knockin mice, the regulatory serines of both GSK3 isoforms are mutated to alanines, forming S9A-GSK3β and S21A-GSK3α. These mutations maintain GSK3 unable to be inhibited by serine-phosphorylation, the predominant mechanism by which GSK3 is regulated (Jope & Johnson, 2004). GSK3 knockin mice reproduce and develop normally, and express both isoforms of GSK3 at levels identical to wild-type mice, but without GSK3 serine-phosphorylation, so they maintain maximal GSK3 activity but within the physiological range because GSK3 is not overexpressed (McManus et al., 2005; Polter et al., 2010). Thus, these mice are tremendously useful to test to what extent hyperactive GSK3 alone, in the absence of FMRP deletion or any overt pathology, can account for alterations associated with loss of FMRP.

Direct evidence that GSK3 may be involved in the pathology of FXS and be a target for the development of treatments for FXS was obtained in studies of the regulation of GSK3 in brain regions from *Fmr1* knockout mice. Brain regions of adult *Fmr1* knockout mice had lower levels of inhibitory serine-phosphorylation of both GSK3 isoforms, phospho-ser21-GSK3α and phospho-ser9-GSK3β, compared with wild-type littermates (Min et al., 2009; Yuskaitis et al., 2010b). Since the total levels of both GSK3α and GSK3β were the same in brain regions of *Fmr1* knockout and wild-type mice, these findings indicated that GSK3 expression is normal in *Fmr1* knockout mouse brain, but the inhibitory control of GSK3 is impaired. Impaired inhibitory regulation of GSK3 was found in adult *Fmr1* knockout mice on both the FVB and C57BL/6 backgrounds, demonstrating that this is a robust change that is not dependent on mouse strain. Furthermore, inhibitory serine-phosphorylation of GSK3 in whole brain extracts from *Fmr1* knockout mice was also found to be lower than in wild-type mice (Liu, Chaung, & Smith, 2011). The diminished inhibitory serine-phosphorylation of GSK3 in adult *Fmr1* knockout mice was corrected by acute or chronic treatment with lithium, raising the possibility that lithium may ameliorate impairments caused by the hyperactive GSK3 (Min et al., 2009; Yuskaitis et al., 2010b; Liu et al., 2011).

MORPHOLOGICAL AND BIOCHEMICAL EFFECTS OF GSK3 INHIBITION IN *FMR1* KNOCKOUT MICE

Treatment with lithium and other GSK3 inhibitors has been reported to alter several structural and biochemical characteristics of *Fmr1* knockout mice. FXS is commonly associated with macroorchidism (abnormally large testes), which also occurs in *Fmr1* knockout mice (Bakker et al., 1994; Comery et al., 1997). Chronic lithium treatment of adult *Fmr1* knockout mice significantly reduced testicular weight, but did not alter testicular weight in adult wild-type mice (Yuskaitis, Beurel, & Jope, 2010). Dendritic spine length increases and altered spine morphology are characteristic of FXS and also occur in *Fmr1* knockout mice (Comery et al., 1997). Liu et al. (2011) confirmed increased apical and basal dendritic spine length in *Fmr1* knockout mice compared to wild-type littermates. Furthermore, treatment with lithium normalized dendritic spines in *Fmr1* knockout mice (Liu et al., 2011). Increased protein

synthesis is thought to be a critical alteration in *Fmr1* knockout mice that contributes to abnormal behaviors and impaired cognition. Lithium treatment significantly diminished the increased cerebral protein synthesis in *Fmr1* knockout mice, indicating that this may contribute to the amelioration by lithium of behavioral abnormalities in *Fmr1* knockout mice (Liu, Huang, & Smith, 2012). Reactive astrogliosis has been observed in postmortem brains of patients with autism-spectrum disorders, and approximately 30% of patients with FXS meet the criteria for autism-spectrum disorders (Vargas, Nascimbene, Krishnan, Zimmerman, & Pardo, 2005; Laurence & Fatemi, 2005). Adult *Fmr1* knockout mice expressed increased levels of the classical marker of astrogliosis, glial fibrillary acidic protein (GFAP), in the hippocampus, striatum, and cerebral cortex, and chronic lithium administration reduced GFAP levels in both adult *Fmr1* knockout and wild-type mice (Yuskaitis et al., 2010a). The mechanism for this response to lithium was not assessed, but may involve regulation of the transcription factor signal transducer and activator of transcription-3 (STAT3). STAT3 promotes GFAP expression but is inhibited by GSK3 inhibitors, including lithium (Beurel & Jope, 2008). This suggests that inhibition of STAT3 may account for the reduced in vivo GFAP levels following lithium treatment.

Adult hippocampal neurogenesis provides a mechanism of plasticity in the hippocampus that is thought to be involved in some types of learning and memory. *Fmr1* knockout mice display impaired adult hippocampal neurogenesis, and elevated expression of GSK3β in neural precursor cells, and neurogenesis was increased by administration of the GSK3 inhibitor SB216763 (Guo et al., 2012). However, other studies in *Fmr1* knockout brain regions did not reveal changes in the expression of either GSK3 isoform (Min et al., 2009; Yuskaitis et al., 2010b), and in human neural precursor cells reduced expression of FMRP led to decreased levels of GSK3β (Telias, Mayshar, Amit, & Ben-Yosef, 2015). Thus, it remains unclear if GSK3β expression is regulated by FMRP and whether neurogenesis is altered in *Fmr1* knockout mice or FXS in a GSK3-dependent manner.

BEHAVIORAL ABNORMALITIES IN *FMR1* KNOCKOUT MICE IMPROVED BY GSK3 INHIBITOR TREATMENTS

McBride et al. (2005) first found an effect of lithium treatment related to FXS, reporting that lithium treatment ameliorated impairments in courtship behavior in the *Drosophila* model of FXS. This discovery was the first evidence that lithium may have therapeutic effects in FXS. A subsequent report confirmed the improvements by lithium treatment of FXS-associated impairments in the *Drosophila* model and showed that the effect was sustainable throughout the aging process (Choi et al., 2009). Since lithium inhibits GSK3 and also has other effects, notably modulation of phosphoinositide signaling (Jope, 1999), the key target for its effects in the *Drosophila* model of FXS remained to be established.

Several behavioral characteristics of *Fmr1* knockout mice have been found to be altered following treatment with lithium or other GSK3 inhibitors (Table 13.1). *Fmr1* knockout mice display increased susceptibility to audiogenic seizures, which frequently evolve to lethal status epilepticus (Musumeci et al., 2000). Lithium treatment dose-dependently reduced the susceptibility of *Fmr1* knockout mice to audiogenic seizures, but audiogenic seizure susceptibility was unaltered in wild-type mice (Min et al., 2009). In the same *Fmr1* knockout mice,

TABLE 13.1 Summary of Behavioral Effects of GSK3 Inhibitors in FX Mice

Behavior	FX mice	GSK3 inhibitor	References
Novel object recognition	Impaired	Normalized	Franklin et al. (2014); King and Jope (2013)
Temporal ordering	Impaired	Normalized	Franklin et al. (2014); King and Jope (2013)
Coordinate spatial processing	Impaired	Normalized	Franklin et al. (2014); King and Jope (2013)
Categorical spatial processing	Impaired	Normalized	Franklin et al. (2014); King and Jope (2013)
Passive avoidance	Impaired	Normalized	Yuskaitis et al. (2010b)
Trace conditioning	Impaired	Normalized	Guo et al. (2012)
Delayed radial arm maze	Impaired	Normalized	Guo et al. (2012)
Locomotor activity	Hyperactive	Normalized	Min et al. (2009)
Audiogenic seizures	Hypersensitive	Normalized	Min et al. (2009)
Elevated plus maze	Increased open arm time	Normalized	Yuskaitis et al. (2010b)
Elevated zero maze	Increased open arm time	Normalized	Liu et al. (2011)
Social preference	Impaired	Improved	Mines et al. (2010)

lithium also dose dependently reduced the prevalence of status epilepticus that occurred following audiogenic seizure induction (Min et al., 2009). This reduction of audiogenic seizure susceptibility in *Fmr1* knockout mice induced by administration of lithium was matched by the administration of two other GSK3 inhibitors, AR-A014418 (Bhat et al., 2003) and SB216763 (Coghlan et al., 2000). The fact that three structurally diverse GSK3 inhibitors each reduced audiogenic seizure susceptibility in *Fmr1* knockout mice, without affecting audiogenic seizure susceptibility in wild-type mice, provided strong evidence that the hyperactive GSK3 in *Fmr1* knockout mouse brain mediates the phenotypic seizure abnormalities in *Fmr1* knockout mice (Min et al., 2009).

Locomotor hyperactivity is a highly reproducible phenotype of *Fmr1* knockout mice that models hyperactivity displayed by patients with FXS (Bakker et al., 1994). Therefore, the effects of lithium were examined in *Fmr1* knockout mice to test if it was possible to diminish the locomotor hyperactivity. As in previous reports, *Fmr1* knockout mice exhibited increased locomotor activity measured in a novel open field paradigm (Min et al., 2009; Yuskaitis et al., 2010b; Liu et al., 2011). In this test, *Fmr1* knockout mice also displayed increased center square entries and in center square duration, compared with wild-type mice (Min et al., 2009). Administration of the GSK3 inhibitor SB216763 did not alter the locomotor activity of wild-type mice in the novel open field, but in *Fmr1* knockout mice it normalized locomotor activity, center square entries, and center square duration, demonstrating that inhibition of GSK3 ameliorates these behaviors in *Fmr1* knockout mice (Min et al., 2009). Chronic lithium administration at a dose relevant to that used therapeutically in human patients is also normalized to wild-type levels the total ambulatory distance traveled by *Fmr1* knockout mice in the novel open field, but did not alter the distance traveled by wild-type

mice (Yuskaitis et al., 2010b; Liu et al., 2011). The correction of locomotor hyperactivity of *Fmr1* knockout mice by two structurally distinct GSK3 inhibitors indicates that hyperactive GSK3 in *Fmr1* knockout mice makes a significant contribution to the locomotor hyperactivity phenotype.

The behavior of adult *Fmr1* knockout and wild-type mice on the elevated plus maze paradigm also was assessed after lithium treatment. The elevated plus maze is often used as an estimate of anxiety, displayed by hesitation to explore the open arms and increased time spent in the closed arms. However, interpretations of the task result remains complex, particularly with mice, such as *Fmr1* knockout mice, that display locomotor hyperactivity. *Fmr1* knockout mice spent significantly less time in the closed arms and more time in the open arms compared to wild-type mice, a response that would classically be interpreted as exhibition of less anxiety (Yuskaitis et al., 2010b; Liu et al., 2011). However, *Fmr1* knockout mice also had increased closed arm entries, classically interpreted as increased anxiety (Yuskaitis et al., 2010b). Thus, the locomotor hyperactivity of *Fmr1* knockout mice may preclude clear interpretations of the measurements using the elevated plus maze paradigm. Notwithstanding the difficulty in interpreting behaviors in the elevated plus maze, lithium administration normalized behavior in the elevated plus maze paradigm in *Fmr1* knockout mice to be equivalent to that of wild-type mice (Yuskaitis et al., 2010b; Liu et al., 2011). The effect of lithium was also tested on anxiety-like behaviors in *Fmr1* knockout mice in the elevated zero maze, containing two closed and two open quadrants (Liu et al., 2011). More time was spent by *Fmr1* knockout mice in the open quadrants than wild-type mice, and lithium administration reduced the time that *Fmr1* knockout mice spent in the open quadrants, eliminating the difference between *Fmr1* knockout and wild-type mice (Liu et al., 2011). Thus, *Fmr1* knockout mice display behavior different from wild-type mice in each of these tests, and the behaviors of *Fmr1* knockout mice are normalized by lithium treatment, but it remains difficult to relate these behaviors to that of subjects with FXS.

Autistic-like behaviors are common characteristic of patients with FXS, such as developmental delays and communication impairments (Hagerman, Ono, & Hagerman, 2005; Belmonte & Bourgeron, 2006; Hatton et al., 2006), and social behavior deficits have been extensively documented in *Fmr1* knockout mice (Mineur, Sluyter, de Wit, Oostra, & Crusio, 2002; Spencer, Alekseyenko, Serysheva, Yuva-Paylor, & Paylor, 2005; Bernerdet & Crusio, 2006; Mineur, Huynh, & Crusio, 2006; McNaughton et al., 2008; Liu & Smith, 2009; Moy et al., 2009). The two-phase social interaction behavior paradigm (McNaughton et al., 2008) was used to test the effects of lithium treatment on social behavior in *Fmr1* knockout mice. This test consists of a sociability phase 1, the introduction of one novel stimulus mouse (S1), and a social preference phase 2, the introduction of a second stimulus mouse (S2). *Fmr1* knockout mice generally behaved equivalently to wild-type mice during the sociability phase, and lithium administration increased measures of sociability in both wild-type and *Fmr1* knockout mice (Mines et al., 2010; Liu et al., 2011), increasing the time in the socializing chamber (Mines et al., 2010; Liu et al., 2011), increasing the number of nose contacts indicative of social approach (Mines et al., 2010), and the time spent sniffing the stimulus mouse (Liu et al., 2011). In the social preference phase 2, wild-type mice display preference for S2 over S1, but *Fmr1* knockout mice lacked this preference and spent equivalent times with S1 and S2 mice (Mines et al., 2010; Liu et al., 2011) and displayed a lower number of nose contacts with S2 and time sniffing S2 than wild-type mice (Mines et al., 2010;

Liu et al., 2011). These abnormal behaviors in the social preference task exhibited by *Fmr1* knockout mice were repaired toward wild-type mice behaviors by chronic lithium treatment (Mines et al., 2010; Liu et al., 2011). Lithium administration also modestly reduced markers of social anxiety in *Fmr1* knockout mice (Mines et al., 2010). To test in another manner, besides lithium treatment, if altered social behaviors exhibited by *Fmr1* knockout mice might be mediated by hyperactive GSK3, another model of hyperactive GSK3 was used, GSK3 knockin mice. As *Fmr1* knockout mice, GSK3 knockin mice did not display altered behavior in the sociability phase 1 test, but, similar to *Fmr1* knockout mice, exhibited impairments in the social preference phase 2 (Mines et al., 2010). Altogether, these results indicate that inhibition of GSK3 may be useful for reducing impairments in social behaviors and social anxiety in FXS.

COGNITIVE IMPAIRMENTS IN *FMR1* KNOCKOUT MICE RESCUED BY ADMINISTRATION OF GSK3 INHIBITORS

The effects of inhibition of GSK3 on impaired learning in *Fmr1* knockout mice was first examined using the passive avoidance task. Compared with wild-type mice, *Fmr1* knockout mice displayed a deficit in learning in the passive avoidance task and this was significantly improved following lithium treatment (Yuskaitis et al., 2010b), a finding that was subsequently confirmed (Liu et al., 2011). Administration of the GSK3 inhibitor SB216763 significantly improved impairments in two hippocampal-dependent learning tasks, the trace conditioning learning task and delayed nonmatching-to-place radial arm maze (Guo et al., 2012).

Several groups have found impaired cognition in the novel object recognition test in *Fmr1* knockout mice (Ventura, Pascucci, Catania, Musumeci, & Puglisi-Allegra, 2004 ; Pacey et al., 2011; Eadie, Cushman, Kannangara, Fanselow, & Christie, 2012; King & Jope, 2013; Franklin et al., 2014). We expanded this to also reveal impairments in *Fmr1* knockout mice in temporal ordering of objects, and coordinate and categorical spatial processing (King & Jope, 2013; Franklin et al., 2014). Identification of these robust cognitive impairments provided an important tool to test potential therapeutic interventions in cognitive measures in mice that are not based on aversive stimuli, such as foot shocks.

A visual novel object recognition task, which requires the dentate gyrus (Hunsaker & Kesner, 2008; Goodrich-Hunsaker, Hunsaker, & Kesner, 2008; Goodrich-Hunsaker, Hunsaker, & Kesner, 2008) and assesses the ability to discriminate between a familiar and novel object, was used to evaluate the potential benefits of GSK3 inhibition on learning deficits in *Fmr1* knockout mice. Wild-type littermate mice spent significantly more time exploring the novel versus familiar object. In contrast, *Fmr1* knockout mice spent equivalent times exploring the novel and familiar objects, indicating that *Fmr1* knockout mice are unable to remember the familiar object. Chronic lithium treatment proved to be remarkably effective in not only improving, but essentially normalizing; severe deficits in *Fmr1* knockout mice in novel object recognition (King & Jope, 2013). Furthermore, we found that three mechanistically different inhibitors of GSK3, lithium, TDZD-8, a highly selective ATP noncompetitive inhibitor (Martinez et al., 2002), and VP0.7, an allosteric (not competitive with ATP or substrate) selective GSK3 inhibitor (Palomo et al., 2011), each was remarkably effective in rescuing impaired novel object recognition in *Fmr1* knockout mice (King & Jope, 2013; Franklin et al., 2014). These

results demonstrate unequivocally that inhibition of GSK3 is capable of reversing a cognitive impairment in adult *Fmr1* knockout mice.

We also assessed whether *Fmr1* knockout mice have deficits in temporal ordering of visual objects, a dorsal, and ventral hippocampal CA1-dependent task in which rodents spend less time exploring the object most recently presented during a previous habituation period (Hunsaker, Kim, Willemsen, & Berman, 2012; Honey, Watt, & Good, 1998; Wallenstein, Eichenbaum, & Hasselmo, 1998; Rolls & Kesner, 2006; Hoge & Kesner, 2007; Hunsaker & Kesner, 2013). In this task, we exposed mice to a series of three pairs of objects and then measured the time spent with the initial object when it was reintroduced along with the most recent object that was explored. Successful temporal ordering is evident when more time is spent exploring the initial object. Wild-type mice displayed successful temporal ordering because more time was spent exploring the initial object, whereas *Fmr1* knockout mice spent significantly less time exploring the initial object presented, revealing a temporal order deficit. *Fmr1* knockout mice treated with each of the three tested GSK3 inhibitors, lithium, TDZD-8, and VP0.7, spent significantly more time exploring the first object compared to the most recent object presented, similarly to untreated wild-type mice. These results demonstrate that temporal ordering of visual object is impaired in *Fmr1* knockout mice and that this deficit is corrected by inhibition of GSK3.

We assessed whether *Fmr1* knockout mice displayed deficits in pattern separation, or spatial processing, using coordinate and categorical tasks, which require the dentate gyrus (Goodrich-Hunsaker, Hunsaker, & Kesner, 2005). In the coordinate spatial learning task, the distance between two identical objects is altered between the habituation and testing periods. Pattern separation is indicated when significantly more time is spent exploring objects during the testing period after repositioning the objects compared to the last 5 min of the habituation phase. Wild-type mice displayed increased object exploration time during testing compared to the last 5 min of the habituation phase, indicating successful pattern separation. In contrast, *Fmr1* knockout mice spent significantly less time than wild-type exploring the objects during the test period, indicating impaired pattern separation. Administration of all three GSK3 inhibitors, lithium, TDZD-8, and VP0.7, reversed the deficit in *Fmr1* knockout mice, as they spent significantly more time exploring the objects during testing compared to habituation after treatment with a GSK3 inhibitor. The categorical spatial learning task involves interchanging the positions of two identical objects following the habituation phase, while maintaining the same distance between them. *Fmr1* knockout mice spent significantly less time than wild-type mice exploring the objects after they had been transposed, again revealing impaired spatial pattern separation in *Fmr1* knockout mice. Administration of GSK3 inhibitors did not alter the amount of time wild-type mice spent exploring the objects after they were transposed, but significantly increased the exploration times of *Fmr1* knockout mice, demonstrating a reversal of the deficit. Thus, the results of the coordinate and categorical spatial learning tests demonstrated impaired spatial pattern separation in *Fmr1* knockout mice that is normalized by the administration of GSK3 inhibitors.

Altogether, these experiments demonstrated deficits in *Fmr1* knockout mice in novel object detection, temporal ordering for objects, and coordinate and categorical spatial processing tasks, and demonstrated that administration of a GSK3 inhibitor essentially normalized these cognitive behaviors.

We further determined to what extent hyperactive GSK3 alone is sufficient to cause the cognitive deficits in *Fmr1* knockout mice that are rescued by GSK3 inhibitor treatment. To

test if hyperactive GSK3 is sufficient to induce cognitive deficits exhibited by *Fmr1* knockout mice, we used GSK3 knockin mice (described earlier, importantly not overexpressing GSK3) with intact FMRP to test if cognitive deficits are displayed by GSK3 knockin mice similarly to *Fmr1* knockout mice. GSK3 knockin mice exhibited impairments in novel object recognition, temporal order memory, and coordinate spatial processing compared with WT mice (Pardo et al., 2015). These results demonstrate that hyperactive GSK3 is sufficient to cause these impairments, further emphasizing the importance of dysregulated GSK3 in contributing to cognitive deficits in *Fmr1* knockout mice.

In *Fmr1* knockout mouse brain regions we found that both GSK3 isoforms, GSK3α and GSK3β, are abnormally active (Min et al., 2009; Yuskaitis et al., 2010b). The two GSK3 isoforms overlap in many functions, but also are known to differ in their ability to phosphorylate certain substrates (Force & Woodgett, 2009; Soutar et al., 2010). For example, Peineau et al. (2007) reported that GSK3β is particularly important in regulating LTP and LTD. To determine if either isoform predominates in impairing performance in these cognitive tasks, we assessed individually GSK3α and GSK3β knockin mice (with the other isoform not being mutated) in each cognitive task. Novel object recognition was impaired in GSK3β, not GSK3α, knockin mice, whereas temporal order memory was not impaired in GSK3α or GSK3β knockin mice, and coordinate spatial processing was impaired in both GSK3α and GSK3β knockin mice (Pardo, Abrial, Jope, & Beurel, 2016). Thus, novel object recognition can be impaired by abnormally active GSK3β, rather than GSK3α, intact inhibitory serine-phosphorylation of either GSK3 isoform is sufficient to maintain temporal order memory, and coordinate spatial processing was the most sensitive to increased GSK3 activity of the cognitive tasks that were examined, as constitutive activation of either GSK3α or GSK3β was sufficient to impair performance.

Altogether, investigations of several tasks involving learning and memory have identified deficits in *Fmr1* knockout mice that are repaired by administration of GSK3 inhibitors. Thus, hyperactive GSK3 in *Fmr1* knockout mice appears to play a crucial role in causing cognitive deficits in *Fmr1* knockout mice, lending support to the hypothesis that GSK3 inhibitors may be efficacious in patients with FXS.

ELECTROPHYSIOLOGICAL ABNORMALITIES IN *FMR1* KNOCKOUT MICE IMPROVED BY GSK3 INHIBITORS

Impairments in synaptic plasticity have been a focus of studies of FXS ever since early reports found that FMRP is important for the normal maturation of synaptic connections (Weiler et al., 1997; Weiler & Greenough, 1999; Antar, Afroz, Dictenberg, Carroll, & Bassell, 2004). A key finding was the discovery that metabotropic glutamate receptor (mGluR)-dependent long-term depression (LTD) was enhanced in *Fmr1* knockout mice in the hippocampal CA1 region (Huber, Gallagher, Warren, & Bear, 2002). These and other reports (e.g., McBride et al., 2005; Yan, Rammal, Tranfaglia, & Bauchwitz, 2005; Dölen et al., 2007) supported the mGluR theory of FXS (Bear, Huber, & Warren, 2004), proposing that many of the protein synthesis-dependent functions of metabotropic receptors are increased in FXS and that mGluR5 antagonists are potential therapeutics for FXS (Chapter 9). Studies of *Fmr1* knockout mice often use the mGluR5 antagonist MPEP (2-methyl-6-phenylethynyl-pyridine), although this is not an entirely specific antagonist, which rescued several impairments, such as heightened

audiogenic seizure susceptibility (Yan et al., 2005). Subsequently, it was found that administration of MPEP increased in vivo the inhibitory serine-phosphorylation of GSK3 in *Fmr1* knockout mouse brain, but had little effect in wild-type mice (Min et al., 2009; Yuskaitis et al., 2010b). This finding demonstrated that intracellular signaling from mGluR5 to GSK3 is abnormal in *Fmr1* knockout mice, and demonstrated an overlap in the effect of MPEP with GSK3 inhibitors. Importantly, it was subsequently shown that lithium treatment in adolescent *Fmr1* knockout mice (from 5–6 weeks of age until 9–11 months of age) or adult *Fmr1* knockout mice (from 8 weeks of age to 4–5 months of age) normalized mGluR-dependent LTD in the hippocampus, without affecting WT mice (Choi et al., 2011). The mechanism was not identified but it may be due to inhibition of GSK3 by lithium because GSK3 promotes LTD, as well as inhibiting long-term potentiation (LTP) (Peineau et al., 2007).

We examined the possibility that impairments in cognition identified in *Fmr1* knockout mice may be due to dysregulated synaptic plasticity mediated by hyperactive GSK3. We identified in *Fmr1* knockout mice a deficit in *N*-methyl-D-aspartate receptor-dependent LTP at medial perforant path synapses onto dentate granule cells that correlated with the impairments in cognitive tasks described earlier that are dependent on normal function of the dentate gyrus (Franklin et al., 2014). Importantly, application of GSK3 inhibitors repaired the LTP deficits in *Fmr1* knockout mice. Thus, it appears that regulation by GSK3 of LTD and LTP contributes to abnormal synaptic plasticity in *Fmr1* knockout mice and contributes to their impairments in learning and memory.

CLINICAL TRIALS

The promising report that lithium corrects FX-associated abnormalities in flies and mice was corroborated in a pilot clinical trial of lithium in patients with FXS (Berry-Kravis et al., 2008). Lithium use in humans is well-established because it has been used clinically since 1950 as a mood stabilizer for the treatment of mood disorders, especially bipolar disorder (Jope, 1999). The clinical effects in FXS patients given lithium carbonate orally were assessed in a pilot open-label 2 month trial (Berry-Kravis et al., 2008). Anxiety, aggression, lethargy, stereotypy, mood swings, tantrums, and abnormal outbursts were improved in lithium-treated patients. Decreases in hyperactivity and inappropriate speech were indicated in ratings by caregivers (Berry-Kravis et al., 2008). Importantly, lithium was the first treatment found to improve cognition in FXS patients. Overall, these findings bolstered the possibility that lithium, and perhaps other GSK3 inhibition, may be therapeutic in FXS.

SUMMARY

Altogether, lithium and other inhibitors of GSK3 have proven to be beneficial for a remarkably large number of different phenotypes in *Fmr1* knockout mice and a pilot trial supported the possibility that this may translate into contributing to the treatment of patients with FXS (see Table 13.1 for overview). Most of the effects of lithium that have been reported to affect phenotypes of *Fmr1* knockout mice have been replicated with other inhibitors of GSK3. This indicates that many of lithium's therapeutic effects in *Fmr1* knockout mice result from its

inhibition of GSK3, but since lithium also has other targets (Jope, 1999), it must be kept in mind that some actions of lithium may be due to effects other than GSK3 inhibition. Abnormally active GSK3 is also implicated in many of the deficits in *Fmr1* knockout mice by findings that GSK3 knockin mice, which express hyperactive GSK3 but at physiological levels, recapitulate many of the abnormalities displayed by *Fmr1* knockout mice. It is also relevant that other treatments that have been proposed as therapeutics for FXS, such as MPEP (Min et al., 2009; Yuskaitis et al., 2010b) and lovastatin (Lee, Jaw, Tseng, Chen, & Liou, 2012), reduce GSK3 activity, raising the possibility that part of their therapeutic actions derive from this effect. With the recent development of many specific inhibitors of GSK3 (Martinez, Gil, & Perez, 2011), there is much opportunity to assess if these drugs, along with lithium, achieve beneficial effects in patients with FXS.

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CHAPTER

14

Defects in Rho GTPase Signaling to the Spine Actin Cytoskeleton in FMR1 Knockout Mice

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INTRODUCTION

Fragile X and Disturbances in Spine Morphology

Severe intellectual disability occurs in approximately 4 out of 1000 individuals (Roeleveld, Zielhuis, & Gabreëls, 1997), and with a higher prevalence in males than females (Leonard & Wen, 2002). The most common cause of inherited intellectual disability is fragile X syndrome (FXS), an X-linked disorder which recently was also identified as the most frequent monogenetic cause of autism (Gross, Hoffmann, Bassell, & Berry-Kravis, 2015). In addition to moderate to severe cognitive impairment, FXS is characterized by other behavioral and neurological problems including attention deficit, hyperactivity, stereotypy, seizures, impulsivity, sensory hyperarousal, anxiety, and autistic behaviors, as well as by distinct physical characteristics (Hagerman, Lauterborn, Au, & Berry-Kravis, 2012; Yu & Berry-Kravis, 2014). The syndrome occurs when the *FMR1* gene that encodes fragile X mental retardation protein (FMRP) is silenced (Bell et al., 1991; Verkerk et al., 1991), resulting in the loss of FMRP protein which normally binds to specific mRNAs and acts as a translational regulator (Santoro, Bray, & Warren, 2012; Willemsen, Oostra, Bassell, & Dictenberg, 2004). As FXS results from a single gene defect, a transgenic mouse model of the disorder in which the *Fmr1* gene was knocked out (Bakker et al., 1994) was successfully created and found to exhibit a range of behavioral abnormalities that parallel the human condition including hyperactivity, propensity for seizures, and deficits in learning and memory (Kazdoba, Leach, Silverman, & Crawley, 2014; Kooy, 2003). Thus, the *Fmr1* knockouts (KOs) are a useful model to study the underlying brain defects of the disorder and for testing potential therapies to improve cognitive function and behavior.

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In brains of both human FXS and murine *Fmr1* KOs, the most reliably described consequence of the loss of FMRP is probably the abnormal morphology of dendritic arbors of cortical pyramidal cells and, in particular, the presence of greater than normal numbers of spines that are long and thin, suggesting an immature phenotype (Comery et al., 1997; Irwin et al., 2002; Irwin et al., 2001; Irwin, Galvez, & Greenough, 2000; Lauterborn, Jafari, Babayan, & Gall, 2015; Pop et al., 2014; Wisniewski, Segan, Miezejeski, Sersen, & Rudelli, 1991). Studies of the FXS mouse model have shown that these spine abnormalities are more robust in neocortex (Comery et al., 1997; Galvez & Greenough, 2005; McKinney, Grossman, Elisseou, & Greenough, 2005) as compared to hippocampus (Bilousova et al., 2009; Braun & Segal, 2000; Grossman, Elisseou, McKinney, & Greenough, 2006; Lauterborn et al., 2015; Levenga et al., 2011; Segal, Kreher, Greenberger, & Braun, 2003; Su et al., 2011), suggesting that mechanisms regulating spine morphology or FMRP function could vary between forebrain fields. It is also clear that *Fmr1* KO spines do not exhibit the same responses (i.e., elaboration, shape change) as do those in wild-type rodents to stimuli, such as depolarization (Antar, Li, Zhang, Carroll, & Bassell, 2006), sensory deprivation (Pan, Aldridge, Greenough, & Gan, 2010) and environmental factors, including enriched environment (EE) rearing (Lauterborn et al., 2015; Restivo et al., 2005). For example, in studies using young *Fmr1* KO and wild-type mice expressing green fluorescent protein in hippocampal CA1 pyramidal neurons, we found that 2 months of EE rearing, initiated at 21 days of age, had differential effects on pyramidal cell dendrites between genotypes (Lauterborn et al., 2015). Although spine head volumes were ~40% smaller in *Fmr1* KOs as compared to wildtypes regardless of housing condition (EE vs. standard) (Fig. 14.1A), other genotype differences emerged with EE rearing only. In particular, enrichment lead to a left shift (toward shorter lengths) in the length–frequency distribution for spines on secondary dendrites in the wildtypes but had no effect on this profile in the KOs. In comparing spine shape, including both length and head breadth measures, EE rearing increased the proportion of short spines with broad heads in the wildtypes but not in the KOs. Moreover, although EE rearing increased the length and branching of tertiary dendrites in both genotypes, the change from the standard housing condition was greater for wild-type mice. Such findings argue that even under conditions of enrichment, which presumably would result in more synaptic drive than standard housing, mechanisms regulating both dendritic and spine elaboration, and spine morphology, are perturbed in the mutant. They further suggest that, at least for studies of fragile X, animals exposed to enriched as opposed to standard rodent housing are more likely to exhibit processes and features that underlie cognitive impairment in humans, who are routinely exposed to diverse and varied environmental factors.

Fmr1 KO Mice Exhibit Hippocampal Synaptic Plasticity Defects

Consistent with memory impairments in FXS, the *Fmr1* KO mice have discrete, but reliable, defects in long term potentiation (LTP) (Hu et al., 2008; Larson, Jessen, Kim, Fine, & du Hoffmann, 2005; Lauterborn et al., 2007; Lee et al., 2011; Li, Pelletier, Perez Velazquez, & Carlen, 2002; Meredith, Holmgren, Weidum, Burnashev, & Mansvelder, 2007; Shang et al., 2009; Yun & Trommer, 2011; Zhao et al., 2005), a form of synaptic plasticity that is thought to underlie certain forms of learning (Bliss & Lomo, 1973; Collingridge & Bliss, 1995; Granger & Nicoll, 2013; Morris, 2003). Impairments have been described for multiple fields of neocortex

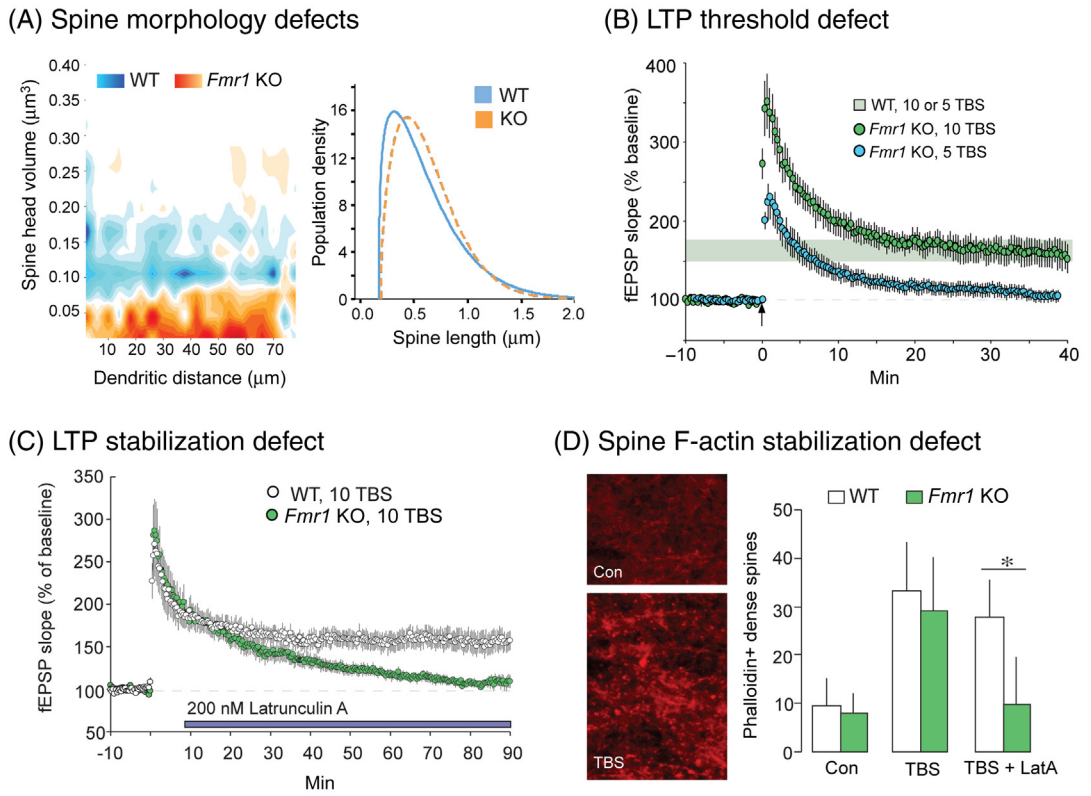

FIGURE 14.1 **Summary of spine and LTP defects in the *Fmr1* KO hippocampus.** (A) Plots show spine morphology defects for hippocampal CA1 pyramidal neurons in *Fmr1* KO mice housed in an enriched environment (EE) for 2 months following weanling; data from secondary dendrites. Left, heat map shows the average spine head volume across the length of dendrites examined for wildtype *(WT; blue)* and FMR1 KO *(orange)* spines: spine head volumes are reliably smaller in KOs than in WTs. Right, plot of spine length frequency distribution shows the KOs have longer spines than WTs. (B) *Fmr1* KOs exhibit an LTP threshold effect: 10 burst TBS elicits LTP in the KOs whereas 5 bursts does not (*TBS* applied at *upward arrow*; *horizontal bar* shows typical level of LTP in WTs with 5 or 10 burst TBS. (C) Latrunculin A, applied beginning at 10 min following 10 burst TBS, disrupts LTP in KO but not WT slices. (D) Left, photomicrographs show phalloidin labeling of F-actin in spines in CA1 stratum radiatum in a control *(con)* slice and one recently activated by TBS. Right, graph shows the effect of TBS on number of densely phalloidin-labeled spines in CA1 *Fmr1* KO and WT slices: TBS induced comparable increases in labeled spines between genotypes; latrunculin A applied at 10 min post-TBS eliminated this increases in slices from KO but not WT mice (*$P < 0.05$). Source*: Adapted in part from Lauterborn, J., Rex, C., Kramar, E., Chen, L., Pandyarajan, V., Lynch, G., & Gall, C. (2007). Brain-derived neurotrophic factor rescues synaptic plasticity in a mouse model of fragile X syndrome.* The Journal of Neuroscience, 27*(40), 10685–10694; Lauterborn, J. C., Jafari, M., Babayan, A. H., & Gall, C. M. (2015). Environmental enrichment reveals effects of genotype hippocampal spine morphologies in the mouse model of Fragile X Syndrome.* Cerebral Cortex, 25, *516–527; and Chen, L. Y., Rex, C. S., Babayan, A. H., Kramar, E. A., Lynch, G., Gall, C. M., & Lauterborn, J. C. (2010). Physiological activation of synaptic Rac>PAK (p-21 activated kinase) signaling is defective in a mouse model of fragile X syndrome.* The Journal of Neuroscience, 30*(33), 10977–10984: use of material from Lauterborn et al. (2015) Cerebral Cortex, Environmental enrichment reveals effects of genotype hippocampal spine morphologies in the mouse model of fragile X syndrome, vol 25, pp. 516-527 by permission from Oxford University Press; use of other material from Lauterborn et al. (2007) and Chen et al. (2010) by permission of The Journal of Neuroscience.*

and paleocortex but early studies found potentiation in hippocampus was normal. We re-evaluated this issue with focus on the Schaffer-commissural (S-C) projections to apical dendrites of field CA1 pyramidal cells, and found that the mutants exhibit an elevated threshold to induction of LTP and disturbances in stabilization of the effect (Lauterborn et al., 2007). Specifically, in acute hippocampal slices from adult mice, a conventional 10 burst train of theta burst stimulation (TBS, four pulses at 100 Hz spaced by 200 ms)(Kramar & Lynch, 2003; Larson, Wong, & Lynch, 1986; Rex et al., 2005) elicited robust field CA1 LTP in *Fmr1* KOs that was comparable to potentiation in slices from wild-type mice. Ten bursts is well above the threshold needed for inducing LTP in rats and mice, and produces levels of potentiation that are maximal for a single bout of TBS in CA1: adding more bursts or pulses per burst does not affect the magnitude or stability of response enhancement (Kramar et al., 2004; Larson et al., 1986). However, using five burst TBS, an amount that is near the threshold for inducing LTP (Kramar et al., 2009), initial potentiation was comparable in KOs and wildtypes but in the mutants failed to stabilize and responses slowly declined back to baseline levels (Fig. 14.1B). This basic finding has since been replicated by others (Lee et al., 2011).

These results show that the threshold for fully engaging mechanisms that stabilize the potentiated state is elevated in *Fmr1* KOs relative to wildtypes. They further show that despite disturbances in processes that stabilize LTP, the impairment can be circumvented by increases in synaptic drive; that is, normal LTP can be induced with suprathreshold (10 burst) stimulation. A similar threshold effect has been observed in frontal cortex where deficits in spike timing potentiation in *Fmr1* KOs were only evident with threshold levels of stimulation but could be overcome with stronger synaptic drive (Meredith et al., 2007).

Together these results indicate that, at least for pyramid type neurons, the KOs retain the capacity for expression of enduring synaptic plasticity despite the elevated threshold for engagement of mechanisms that stabilize the potentiated state. As described later, recent advances in understanding the mechanisms of stabilization suggest these impairments might be closely linked to the long appreciated structural abnormalities in the KOs. In particular, there is now considerable evidence that the stabilization of LTP, such as aspects of spine morphology, depends upon dynamic properties of the dendritic spine actin cytoskeleton.

In the following sections, we will discuss the key regulatory pathways that govern activity-induced reorganization of the actin cytoskeleton in dendritic spines needed for enduring synaptic plasticity in hippocampus with emphasis on the small Rho GTPases and their effectors. As described, it is now clear that there are discrete defects in signaling through these pathways in the fragile X mouse model that both contribute to disturbances in spine morphology and underlie impaired LTP and learning.

CHANGES IN THE SPINE ACTIN CYTOSKELETON SUPPORT SYNAPTIC PLASTICITY

Dendritic spines are enriched with filamentous (F-) actin (Matus, 2000), which is critical for establishing and sustaining spine morphology (Hotulainen & Hoogenraad, 2010; Lynch, Rex, & Gall, 2007), and can exhibit rapid actin-based motility that is regulated by glutamate receptor activation (Fischer, Kaech, Knutti, & Matus, 1998; Fischer, Kaech, Wagner, Brinkhaus, & Matus, 2000). Given evidence that LTP involves changes in spine shape (Chang &

Greenough, 1984; Harris, Fiala, & Ostroff, 2003; Lee, Schottler, Oliver, & Lynch, 1980) and can persist for long periods if not indefinitely (Abraham, Logan, Greenwood, & Dragunow, 2002; Staubli & Lynch, 1987) it was long considered that the spine actin cytoskeleton played an important role (Lynch & Baudry, 1984). Studies over the last few years have shown this is the case and have identified critical mechanisms involved (Harris et al., 2003; Lynch et al., 2007; Nicoll, 2003; Rudy, 2015b; Toni et al., 2001). In particular, the induction of hippocampal LTP is associated with a rapid NMDA receptor dependent increase in spine F-actin content (Fukazawa et al., 2003; Kramar, Lin, Rex, Gall, & Lynch, 2006; Lin et al., 2005) shown, in some cases, to persist for weeks (see Rudy, 2015a for review). Results of studies using pharmacological inhibitors of actin assembly, such as latrunculin A, indicate that potentiation depends upon this actin remodeling. Latrunculin A binds actin monomers thereby preventing their addition to growing (polymerizing) actin filaments and leading to the dissolution of recently formed, treadmilling polymers (Coué, Brenner, Spector, & Korn, 1987). A number of studies have shown that latrunculin infusion inhibits or decreases the magnitude of LTP (Krucker, Siggins, & Halpain, 2000). Studies using short periods of latrunculin infusion show that new F-actin is critical for the initial stabilization of potentiation but not for enduring expression of the potentiated state: specifically, latrunculin applied either before stimulation or within the first 10 min thereafter, disrupts LTP whereas later treatments have no effect (Rex et al., 2010). Moreover, early latrunculin application inhibits the spine enlargement that occurs with LTP (Matsuzaki, Honkura, Ellis-Davies, & Kasai, 2004). These results strongly support the view that new actin polymerization is essential for the structural changes that occur with synaptic plasticity and are needed for LTP itself.

Dendritic spines have a highly active cytoskeleton: it has been estimated that over 80% of the spine actin is in a dynamic state and has a turnover time of less than a minute (Star, Kwiatkowski, & Murthy, 2002). The actin cytoskeleton undergoes constant "treadmilling," a process whereby actin monomers are added to the barbed end and disassembled at the pointed end of actin filaments. This process is controlled by numerous actin regulators that can facilitate actin polymerization and promote its disassembly, or stabilize and cross-link existing filaments. Among these regulators, the signaling pathways mediated by the small Rho GTPases play a central role (Hall, 1998; Newey, Velamoor, Govek, & Van Aelst, 2005; Spence & Soderling, 2015).

The Rho GTPases are guananine nucleotide binding proteins that cycle between the inactive, GDP-bound to active, GTP-bound states. The GTPases themselves are regulated by a host of activators (guanine nucleotide exchange factors, GEFs) and inhibitors (GTPase-activating proteins, GAPs) that finely modulate GTPase activity (Duman, Mulherkar, Tu, X Cheng, & Tolias, 2015). At least 14 distinct members of the Rho GTPase family have been identified in mammalian cells (Hall, 2012), with the best-characterized being RhoA, Rac1, and Cdc42. These proteins regulate a myriad of cellular processes, including changes in cell morphology and motility via signaling to the actin cytoskeleton. In brain, manipulations of the Rho GTPases and their down stream effector proteins have been shown to have potent effects on dendritic spines. Evidence for disturbances in Rho GTPase pathway proteins in individuals with abnormal spine morphologies and different forms of congenital intellectual disability, for example, Williams syndrome or nonsyndromic X-linked intellectual disability (Chelly & Mandel, 2001; Ramakers, 2002), have given rise to the proposal that dysregulation of these signaling cascades underlies what have been termed "spine disorders" that are associated with impairments in cognitive function.

With an interest in mechanisms of spine actin regulation associated with enduring synaptic plasticity and learning in normal individuals, research by our group has investigated activities of the small Rho GTPases in association with hippocampal LTP. Studies conducted over several years, using immunohistochemical analysis of spine signaling and specific inhibitors to identify relationships between receptor systems and activity regulation of Rho GTPase signaling intermediaries, have resolved distinct functions for RhoA and Rac signaling cascades in spine F-actin remodeling with synaptic potentiation (Lynch & Gall, 2013; Lynch, Rex, Chen, & Gall, 2008; Rex et al., 2009) (Fig. 14.2). To briefly summarize, we found that TBS applied to the S-C afferents to field CA1 activates signaling through the RhoA cascade (RhoA > RhoA-associated kinase (ROCK) > Lim-kinase > cofilin) and that this signaling is necessary for activity-induced increases in spine F-actin. In hippocampal slices, inhibition of ROCK (or addition of adenosine which inhibits RhoA activation) prevents increases in spine levels of phosphorylated (p) cofilin, F-actin, and LTP (Rex et al., 2009) without disrupting TBS-induced increases

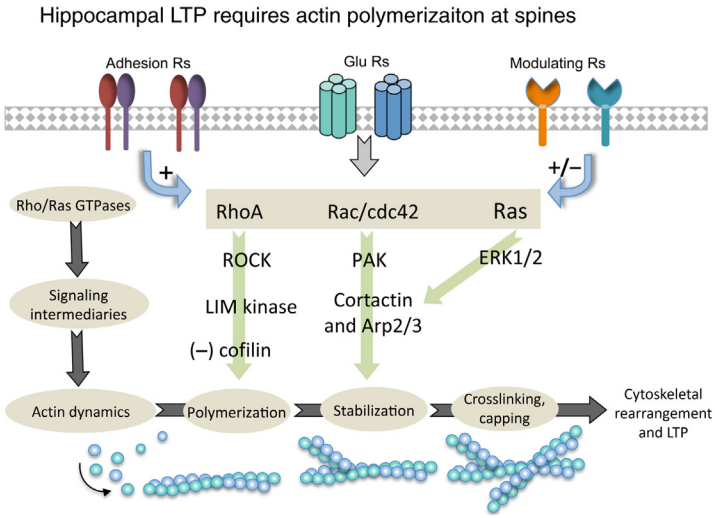

FIGURE 14.2 **Actin regulatory pathways engaged by theta burst stimulation to promote LTP.** Schematic shows signaling pathways that are engaged by TBS and necessary for cytoskeletal rearrangement and stable LTP. Different receptor groups, including glutamate receptors *(Glu Rs)*, adhesion receptors, and modulatory receptors (i.e., TrkB, adenosine), signal to the Rho GTPases RhoA and Rac/cdc42, and Ras GTPase. Each of the GTPases signals to intermediary proteins that subsequently influence aspects of actin dynamics. The RhoA-to-cofilin path regulates polymerization of new F-actin; in contrast the Rac-to-PAK pathway engages cortactin and Arp2/3 to promote the stabilization of actin filaments. The Ras-to-ERK pathway also regulates the activities of cortactin and Arp2/3 likely influencing F-actin stabilization and architecture. Source*: Summarized from Chen, L. Y., Rex, C. S., Casale, M. S., Gall, C. M., & Lynch, G. (2007). Changes in synaptic morphology accompany actin signaling during LTP.* The Journal of Neuroscience, 27*(20), 5363–5372; Rex, C. S., Chen, L. Y., Sharma, A., Liu, J., Babayan, A. H., Gall, C. M., & Lynch, G. (2009). Different Rho GTPase-dependent signaling pathways initiate sequential steps in the consolidation of long-term potentiation.* The Journal of Cell Biology, 186*(1), 85–97; Rex, C. S., Gavin, C. F., Rubio, M. D., Kramar, E. A., Chen, L. Y., Jia, Y., Huganir, R. L., Muzyczka, N., Gall, C. M., Miller, C. A., Lynch, G., & Rumbaugh, G. (2010). Myosin IIb regulates actin dynamics during synaptic plasticity and memory formation.* Neuron, 67*(4), 603–617; and Seese, R., Babayan, A., Katz, A., Cox, C., Lauterborn, J., Lynch, G., & Gall, C. (2012). LTP induction translocates cortactin at distant synapses in wild-type but not Fmr1 knock-out mice.* The Journal of Neuroscience, 32*(21), 7403–7413.*

in phosphorylated forms of p21 activated kinase (PAK). In contrast, TBS-induced activation of spine Rac and its down stream effector PAK proved critical for the stabilization of new actin filaments. Treatment of hippocampal slices with Rac or PAK inhibitors did not block activity induced-increases in p-cofilin or F-actin but did prevent stabilization of new spine F-actin and LTP such that both fairly rapidly decayed back to baseline. Thus, the RhoA and Rac signaling cascades each play critical roles in regulating different features of hippocampal LTP.

The findings by Rex et al. (2009) set the stage for evaluating whether deficits in one or more of these proteins/pathways contribute to the hippocampal LTP defects observed in the *Fmr1* KOs, as will be described later. It is important to note that our studies in these mutants have focused on evaluating the levels of proteins, and their phosphorylation state as an indicator of activity, using fluorescent deconvolution tomography (FDT), a technique developed by our group (Rex et al., 2009; Seese et al., 2013) to measure levels of immunolabeling, and the incidence of double immunolabeling, for as many as ~30,000 synapses in a single 3-dimensional (3D) sample field created from reconstructions of deconvolved image z-stacks. The FDT approach is particularly well-suited to studies of consequences of the FXS mutation, which involves defects that are primarily localized to the synaptic compartment. Moreover, given that TBS applied to the S-C projections elicits potentiation in a fraction of the synapses in the target CA1 apical dendritic field, this high throughput analytical approach has proven to be essential for discerning defects in a relatively small proportion of synapses.

FMR1 KO DEFECTS IN RHO GPTASE SIGNALING PATHWAY PROTEINS

Cofilin (ADF/Cofilin Family)

Cofilin is an essential actin regulatory protein that constitutively severs actin filaments, and thereby accelerates actin assembly dynamics by increasing the number of filament ends from which actin monomers can be added or dissociated. Phosphorylation of cofilin at Ser3 inactivates the protein allowing for actin filament elongation and the overall increases in spine F-actin that occur following LTP-inducing stimulation. Due to cofilin's critical role in regulating the actin cytoskeleton, alterations in it's spine levels or activities in association with a congenital disorder would be expected to significantly influence the morphology, dynamics, and functional properties of spines and spine synapses and, as a consequence, cognitive function. Thus, as first considered, cofilin seemed a logical candidate for contributing to synaptic defects in FXS. Moreover, studies of murine fibroblast suggested a link between FMRP and cofilin: Castets et al. (2005) showed that FMRP deficient fibroblasts had reduced levels of p-cofilin and a concomitant elevation in levels of the protein phosphatase 2A catalytic subunit, which regulates cofilin dephosphorylation (Ambach et al., 2000). These findings indicate that FMRP regulates the activity state of cofilin in nonneuronal cells, but whether this held true for neurons was unknown.

To test if the effects of synaptic activity on p-cofilin are abnormal in Fmr1 KO hippocampus, we used hippocampal slices and S-C stimulation with five burst TBS which, as noted earlier, induces normal LTP in the wild-type mice but not in *Fmr1* KOs (Lauterborn et al., 2007). Slices were processed for dual immunolabeling for cofilin and the spine synapse marker

PSD95, and FDT was used to quantify spine immunolabeling. We found that in control slices receiving low frequency stimulation only, numbers of both total cofilin immunopositive (+) and Ser3 p-cofilin+ spines in CA1 stratum radiatum were comparable between genotypes. Moreover, following TBS, numbers of p-cofilin+ spines were increased to the same extent in slices from KO and wild-type mice. In this analysis we focused on effects at 7 min post-TBS, the latency at which p-cofilin levels are maximally increased by TBS in wildtypes (Chen, Rex, Casale, Gall, & Lynch, 2007). It should be noted that the possibility of genotype-specific effects at greater latencies still needs to be evaluated before we can be certain that spine cofilin regulation is not affected by the FXS mutation.

The presence of what appears to be normal TBS-induced increases in p-cofilin in the *Fmr1* KOs indicated that activity-induced increases in spine actin polymerization were also likely to be unaffected by the mutation. This was tested using in situ phalloidin labeling and microscopic quantification of spine F-actin (Kramar et al., 2006; Lin et al., 2005; Rex et al., 2007). We found that five burst TBS of S-C afferents to field CA1 stratum radiatum elicited comparable increases in phalloidin-labeled spine F-actin in slices from *Fmr1* KO and wild-type mice (Lauterborn et al., 2007). Thus, both activity-induced signaling to cofilin and actin polymerization appear to be normal for pyramidal cell dendritic spines in the mutants. These findings supported the overall conclusion that neuronal RhoA-to-cofilin signaling is relatively unaffected by the *Fmr1* mutation, and suggest that the basis of synaptic defects may be found in the engagement of the other Rho GTPase activities specifically implicated in the stabilization of the spine actin cytoskeleton.

PAK (p21 Activated Kinase)

Prior work showed that Rac to PAK signaling is integral for stabilizing both activity-induced increases in spine F-actin and LTP in wild-type rodents (Rex et al., 2009). Thus, we considered that disturbances in this pathway, and in particular in PAK expression and management, might account for the impaired stabilization of potentiation in *Fmr1* KOs. The PAK proteins constitute a family of serine/threonine kinases (Zhao & Manser, 2012), with group 1 PAKs, PAK1 and PAK3, being abundant in brain (Burbelo, Kozak, Finegold, Hall, & Pirone, 1999). PAK activity is regulated by phosphorylation at several sites with that at Ser141/144 in the kinase inhibitory domain playing a primary role (Chong, Tan, Lim, & Manser, 2001). It is noteworthy that mutation of the PAK3 gene resulting in disruption of kinase function is associated with nonsyndromic X-linked mental retardation (Allen et al., 1998; Bienvenu et al., 2000; Gedeon, Nelson, Gécz, & Mulley, 2003), indicating that the Rac-to-PAK pathway is likely to be critical to learning and memory.

We first used western blots to evaluate the possibility that the *Fmr1* mutation disrupts basal levels PAK1 or PAK3 in hippocampus; this analysis identified no effect of genotype on total PAK protein levels. However, using the FDT approach to evaluate PAK in the spine compartment, we found that in field CA1 stratum radiatum *Fmr1* KOs had ~50% more PSD95+ synapses double-labeled for PAK3 immunoreactivity than did wildtypes (Chen et al., 2010). Nevertheless, in this same field the mutants did not differ from wildtypes regarding levels of phosphorylated PAK at synapses under control conditions: in each genotype approximately 4% of the PSD95+ synapses were enriched in p-PAK as determined using an antibody which recognizes the conserved Ser141 site of PAKs 1,2, and 3 (Chen et al., 2010). These results indicate

that although PAK protein levels are elevated at excitatory synapses in the mutant, basal levels of activated PAK are unaffected by genotype.

We next tested if effects of TBS on synaptic PAK activation are abnormal in the mutants. Using a single 10 burst train of TBS applied to S-C afferents, which induces comparable LTP in the two genotypes, we found that stimulation caused a large (~85%) and rapid increase in numbers PSD95+ synapses associated with dense concentrations of p-PAK in wild-type mice but had no effect in the KOs (Fig. 14.3); in wildtypes the increase was significant at 7 min and had dissipated by 15 min post-TBS, whereas in KOs there were no changes in spine p-PAK immunolabeling through 30 min post-TBS (Chen et al., 2010).

The most straightforward explanation for the loss of activity-induced PAK phosphorylation was an impairment in activation of the upstream regulatory GTPase. We tested this possibility using FDT and antisera for activated Rac (i.e., Rac1-GTP) in combination with

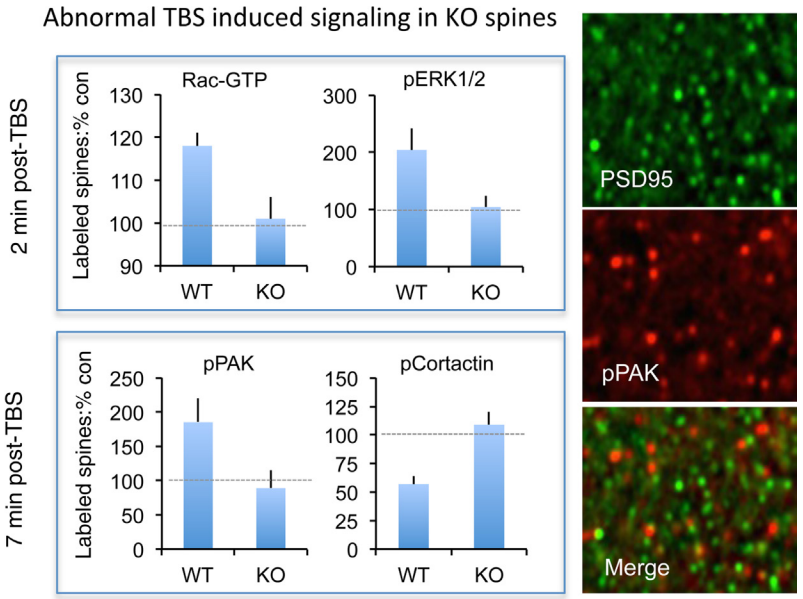

FIGURE 14.3 Defects in TBS-induced Rho/Ras GTPase signaling proteins in *Fmr1* KO spines. Left, graphs show summary of defects in TBS-induced changes in levels of activated Rac-GTP and levels of phosphorylated (p) PAK, ERK1/2, and cortactin in *Fmr1* KO spines; counts reflect the incidence of colocalization with the postsynaptic marker PSD95 expressed as a percent of the same measure from slices receiving low frequency control stimulation. As shown, within 2 min of TBS, Rac-GTP and pERK1/2 are markedly increased in WT but not *Fmr1* KO spines. Similarly, at 7 min post-TBS pPAK levels are greatly elevated in WT but not in KO spines. At the same latency, TBS effected a decrease in spine pCortactin content in WTs spines but this was not seen in the KOs. Right, representative photomicrographs showing immunolabeling for PSD95 (top; *green*), pPAK (middle; *red*), and the merged image of both proteins (bottom). For the FDT analyses, double-labeled profiles (with any overlap of red and green labeled fields as evaluated in 3D) are quantified. Source*: Summarized from Chen, L. Y., Rex, C. S., Babayan, A. H., Kramar, E. A., Lynch, G., Gall, C. M., & Lauterborn, J. C. (2010). Physiological activation of synaptic Rac>PAK (p-21 activated kinase) signaling is defective in a mouse model of fragile X syndrome.* The Journal of Neuroscience, 30*(33), 10977–10984 and Seese, R., Babayan, A., Katz, A., Cox, C., Lauterborn, J., Lynch, G., & Gall, C. (2012). LTP induction translocates cortactin at distant synapses in wild-type but not Fmr1 knock-out mice.* The Journal of Neuroscience, 32*(21), 7403–7413.*

anti-PSD95. Hippocampal slices were harvested 2 min after receiving 10-burst TBS or control stimulation applied to the S-C projections (Chen et al., 2010); the short latency was dictated by prior results showing a rapidly transient activation of GTPases with induction of LTP in this system (Rex et al., 2009). Similar to results of the PAK analysis, basal spine levels of activated Rac did not differ between genotypes but TBS increased numbers of PSD95+ spines associated activated Rac1 in slices from wild-type mice only (Fig. 14.3). These results confirm that in hippocampal neurons lacking FMRP, LTP inducing stimulation fails to activate dendritic spine Rac, resulting in failed signaling through the Rac-PAK pathway. It is noteworthy that work in *Drosophila*, as well as *Fmr1* KO fibroblasts confirm that FMRP disrupts Rac activity in other cell types (Billuart & Chelly, 2003; Castets et al., 2005; Schenck et al., 2003) and that, at least for mammalian cells, Rac-mediated changes in the actin cytoskeleton (Castets et al., 2005) are perturbed.

These results, and our prior demonstration that Rac-PAK signaling is critical for the stabilization of newly formed actin filaments (Rex et al., 2009), suggested then that in *Fmr1* KOs new F-actin formed in association with TBS may be vulnerable to disruption for prolonged periods of time. We tested this possibility in hippocampal slices using infusion of latrunculin A. In hippocampal slices from wild-type rats (Rex et al., 2009; Rex et al., 2007) and mice (Rex et al., 2010), latrunculin infusion reverses LTP and disrupts new actin filaments if applied at 2 min, but not at 10 min after stimulation (Chen et al., 2010). In line with these results, infusion of latrunculin A at 10 min post-TBS had no effect on LTP or on the nearly threefold increase in numbers of spines containing dense F-actin in slices from wild-type mice. By contrast, in paired slices from *Fmr1* KOs the same latrunculin treatment disrupted the stabilization of LTP, which then declined to baseline, and eliminated increases in spine F-actin (Fig. 14.1C,D). Both of these effects indicate that cytoskeletal mechanisms needed for the stabilization of functional synaptic plasticity, previously shown to involve the Rac-PAK cascade (Rex et al., 2009), are indeed disrupted in the mutants.

In apparent contrast to our results indicating impairments in the FXS mutants are associated with failure to activate synaptic Rac-PAK activity, Tonegawa and coworkers reported that a dominant-negative construct that reduces PAK activity reverses neocortical spine abnormalities and restores cortical LTP in *Fmr1* KOs (Hayashi et al., 2007). Thus, their findings suggest that FMRP loss leads to heightened PAK activity in neocortex; effects on hippocampal spines were not reported. It is important to note that their approach used *Fmr1* KOs that were crossed with transgenic mice expressing a dominate-negative form of PAK1 which, as this group reported earlier, reduces PAK activity beginning in the third postnatal week and reaches ~40% inhibition by the second postnatal month (Hayashi et al., 2004). Notably, although PAK activity in the dominate-negative mice is reduced, levels are still two-fold greater in hippocampus than in cortex (Hayashi et al., 2004). Unfortunately, PAK activity levels in *Fmr1* KO neocortex or other fields, with or without the double-negative PAK1 construct, were not reported by Hayashi et al. (2007), and their spine analyses focused on temporal cortex, so it is not clear if these measures differed greatly between cortex and hippocampus. It is possible that with regard to basal PAK1 levels and PAK activity there is a greater imbalance in cortex (i.e., heightened activity) than in hippocampus in the KO. This could reflect the fact that levels of FMRP and one of its interacting proteins, FXR2P, are normally greater in cortex than in hippocampus in wildtypes (Bonaccorso et al., 2015). As a consequence, the loss of FMRP in the KOs could have differential effects between these forebrain regions. As

stated earlier, we did not find an effect of genotype on spine levels of p-PAK under baseline conditions (Chen et al., 2010) suggesting that, although activity-induced increases are deficient, basal PAK activity is relatively normal in KO hippocampus. While direct evidence for elevated PAK activity in *Fmr1* KO cortex has not been reported, Tonegawa and coworkers also found that treatment with a small-molecule PAK inhibitor (FRAX486) normalized spine density and behaviors dependent upon cortical fields, although learning was not assessed (Dolan et al., 2013). Overall, there are questions that still need to be addressed regarding regional differences in the nature and compartmentalization of PAK abnormalities in the *Fmr1* KOs and their contributions to cortical and hippocampal dysfunction in FXS. Most importantly, studies are needed to access how treatments aimed at reducing PAK activity in *Fmr1* KOs, which appear to reverse some neocortical abnormalities, affect the hippocampus and the types of memory dependent upon it. Conversely, our findings from hippocampus would suggest that facilitators of actin stabilization, whether through enhancing or circumventing Rac-PAK signaling to reach downstream effectors, would be beneficial for sustaining plasticities underlying hippocampus-dependent memory encoding although consequences of these manipulations to neocortex need to be addressed.

Cortactin (Cortical Actin Binding Protein)

Cortactin is a monomeric protein that is enriched in dendritic spines, with greater concentrations found in the F-actin rich core of the structure (Racz & Weinberg, 2004). When activated by phosphorylation, cortactin recruits the Arp2/3 complex to actin microfilaments and both nucleates F-actin branch points and stabilizes the actin network (Weaver et al., 2001). Studies of hippocampal cultures have shown that alterations in spine cortactin levels have significant impact on spine morphology: overexpression of the protein results in spine elongation whereas cortactin knockdown with small-interfering RNA leads to spine loss (Hering & Sheng, 2003). Given that one of the main dendritic spine abnormalities in both human FXS cases and the mouse model is an overabundance of long thin spines (Comery et al., 1997; Galvez & Greenough, 2005; Irwin et al., 2002; Irwin et al., 2001; Irwin et al., 2000; Lauterborn et al., 2015; McKinney et al., 2005; Pop et al., 2014; Restivo et al., 2005; Wisniewski et al., 1991) it was reasonable to postulate that an underlying cause could be abnormally high spine cortactin content. Moreover, given evidence for disturbances in the stabilization of LTP in the KOs, and the involvement of the cortactin/Arp2/3 complex in the stabilization of F-actin (Weaver et al., 2001), we also considered a role for cortactin in the stabilization of LTP and its impairments in the mutants.

As little was known about links between synaptic plasticity and spine cortactin, we first examined the effects of LTP-inducing, 10 burst TBS applied to S-C afferents on spine cortactin content in field CA1 of wild-type mice. The immunofluorescence analysis showed that within 7 min of TBS the numbers of field CA1 PSDs associated with intense immunolabeling for cortactin-ir was reduced to ~65% (Seese et al., 2012); numbers remained low through 45 min post-TBS and then returned to baseline values by 90 min after stimulation. The reduction in spine cortactin content was blocked by NMDA receptor antagonism further linking cortactin trafficking to conditions supporting the expression of LTP. We next asked if levels of cortactin were similarly modulated in *Fmr1* KO spines. While there was no effect of genotype on basal levels of cortactin in spines in field CA1, TBS failed to elicit the same translocation effect in

the KOs as in wildtypes (Fig. 14.3). Specifically, in the mutants the number of PSDs associated with dense cortactin-ir was unchanged through 20 min post-TBS but declined (−22%) by 45 min and were further reduced to 65% of control levels by 90 min. Thus, in *Fmr1* KOs, spine cortactin levels are reduced in response to TBS but by a much slower time course than in wild-type mice. As in earlier work, we confirmed that the 10 burst TBS used to evaluate cortactin protein content did indeed elicit comparable levels of LTP in the two genotypes. Together, the results suggest that activity-dependent changes in spine cortactin content are disturbed in the *Fmr1* KOs, and reflect defects in cellular processes set in motion by the stimulation rather than the level of potentiation initially achieved.

The activity-dependent loss and recovery of cortactin accumulation at synapses in wildtypes suggested different scenarios for mechanisms controlling regional cortactin content: The protein could be (1) quickly degraded and replenished by new protein copies or (2) translocated between the spine and dendritic compartments as previously described to occur in culture with NMDA and BDNF treatment (Hering & Sheng, 2003). To distinguish between these possibilities we evaluated effects of blebbistatin, an inhibitor of myosin II function and thus of myosin motors. The blocker was applied to hippocampal slices at a dose that has no detectable effects on field CA1 baseline fEPSPs, paired pulse facilitation, mEPSCs, I/O curves, or theta responses, but blocks LTP (Rex et al., 2010). Blebbistatin completely eliminated the TBS-induced decrease in cortactin content at PSDs in wild-type mice indicating that actomyosin motors are involved in cortactin translocation (Seese et al., 2012). As cortactin reportedly associates with microtubules in spines (Jaworski et al., 2009), and microtubule treadmilling has been suggested to contribute to protein transport between spines and dendritic shafts (Gu & Zheng, 2009; Jaworski et al., 2009), we tested if microtubules were also involved. In wild-type slices, pharmacological blockade of microtubule polymerization with nocadozale caused a ~35% decrease in the number of synapses associated with dense concentrations of cortactin; this effect was observed under control-stimulation conditions, and when applied in combination with TBS there was no additional change (Seese et al., 2012). Together these results suggest that in wildtypes cortactin is being continuously trafficked into the spine via a microtubule-dependent process and then translocated out of the spine via activity- and actin-dependent mechanisms. Thus results for TBS effects on cortactin translocation in the *Fmr1* KOs indicate that both actomyosin- and microtubule-dependent mechanisms of trafficking are affected by the FXS mutation.

Serine phosphorylation of cortactin is known to regulate the protein's interactions with F-actin and its ability to translocate within cells (Cosen-Binker & Kapus, 2006; Iki, Inoue, Bito, & Okabe, 2005; Kruchten, Krueger, Wang, & McNiven, 2008; Martinez-Quiles, Ho, Kirschner, Ramesh, & Geha, 2004). Thus, the observed disturbances in cortactin movement following TBS in the KOs could reflect abnormal phosphorylation and thus altered association with F-actin. Pursuing this idea, we evaluated the number of PSDs containing dense levels of Ser405 phosphorylated cortactin in CA1 stratum radiatum and found that these profiles were 50% less abundant in *Fmr1* KOs as compared to wildtypes under basal conditions (Seese et al., 2012). Moreover, a single train of S-C TBS produced a reliable 45% increase in the number of PSDs associated with p-cortactin in wild-type mice, but this effect was absent in *Fmr1* KOs. These results suggest that defects in cortactin phosphorylation limits the association of the protein with F-actin in *Fmr1* KOs. This conclusion was validated using coimmunoprecipitation from

synaptoneurosomes prepared from forebrain which showed that cortactin associated with ~40% less actin in *Fmr1* KOs as compared to wild-type mice.

Cortactin's serine phosphorylation is mediated by both PAK and extracellular-regulated kinase (ERK) which target the Ser113 and Ser405/418 sites, respectively (Campbell, Sutherland, & Daly, 1999; Webb et al., 2006). Phosphorylation at both sites is thought to fine-tune cortactin's interactions with F-actin and other associated proteins (e.g., Arp2/3, WASP) and to thereby regulate the growth and architecture of the actin network. Thus, in *Fmr1* KOs, the defects in activity induced cortactin phosphorylation and actin binding is consistent with the observed impairments in the Rac-to-PAK signaling cascade and abnormalities in ERK signaling as described below.

ERK1/2 (Extracellular Signal-Regulated Kinase)

The ERK cascade transduces signals from cell receptors through Ras GTPase, and then Raf and MEK, to activate ERK1/2 (a.k.a, p44/42 mitogen-activated protein kinase, MAPK) (Roskoski, 2012 for review). ERK1/2 mediates serine/threonine phosphorylation of many proteins and regulates a range of key cellular processes including gene expression, protein translation and, as will be considered here, actin reorganization. For a discussion of the consequence of the fragile X mutation on ERK1/2's role in protein expression the reader is referred to Chapter 11. In regulating the actin cytoskeleton, ERK phosphorylates cortactin thereby promoting Arp2/3 actin nucleation and actin branching (Campbell et al., 1999; Martinez-Quiles et al., 2004). Consistent with effects on the subsynaptic actin network, ERK signaling is critical for LTP (Thomas & Huganir, 2004 for review).

ERK1/2 was one of the first proteins to be assessed in the FMR1 KOs, but findings have been mixed with regard to basal levels of total and activated (Thr202/Tyr204 phosphorylated) ERK1/2 in the mutants. Some studies found comparable levels of the protein, total and/or activated, between genotypes (Gross et al., 2010; Hu et al., 2008) whereas we and others have described elevated synaptic p-ERK in mutant hippocampus (Hou et al., 2006; Price et al., 2007; Seese et al., 2012; Seese, Wang, Yao, Lynch, & Gall, 2014). It is important to note that differences across studies may be due to the analytical method used with effects of genotype being most evident with measures of ERK1/2 activity within the synaptic compartment. Our studies of forebrain synaptoneurosomes using western blots and of PSD95+ postsynaptic elements in CA1 stratum radiatum using FDT demonstrated that although synaptic levels of total ERK1/2 are equivalent between *Fmr1* KOs and wildtypes, synaptic pERK1/2 levels are 70%–90% greater in the mutant (Seese et al., 2012, 2014). Such strikingly greater levels of the activated kinase at excitatory synapses in the mutants would be expected to significantly influence how this protein interacts with its targets. We tested this prediction using coimmunoprecipitation assays of synaptoneurosomes and found that significantly less ERK1/2 coimmunoprecipitated with cortactin in samples from KOs as compared to wildtypes. The direction of change is somewhat surprising given the greater density of activated ERK but this may reflect disturbances in convergent and interacting regulation of cortactin by the tyrosine kinase Src (Kelley, Hayes, Ammer, Martin, & Weed, 2011) which as yet to be evaluated. Regardless of specific mechanism, reductions in the association of ERK and cortactin likely contribute to the impairment in basal levels of

cortactin phosphorylation and the ability of synaptic activation to induce an increase in this phosphorylation in the mutant (Seese et al., 2012).

Next, we tested if KO and wild-type mice differ in the effects of TBS on synaptic pERK. This proved to be the case: 10 burst TBS rapidly (within 2 min) doubled the number of PSDs enriched in p-ERK in hippocampal slices from wild-type mice but had no measurable effect on this measure in slices from *Fmr1* KOs (Fig. 14.3). Thus, the fragile X mutation results in elevated ERK activity in the basal state and an absence of the synaptic activity-dependent increases in the phosphoprotein that are characteristic of responses to LTP-inducing stimuli in wildtypes. Finally, as ERK is activated by signaling through Ras, our results suggest that this upstream GTPase may be overactive in the mutants. Indeed Hu et al. (2008) reported that both basal and histamine-induced levels of Ras-GTP in hippocampal CA1 field are elevated in *Fmr1* KOs as compared to wildtypes as determined by western blots. Further work is needed to determine if these effects on Ras are present within the spine compartment and if effects of glutamatergic transmission on Ras activity are impaired in the mutant.

It is unclear as to the specific functional consequences of elevated synaptic levels of activated ERK to synaptic plasticity and memory encoding in *Fmr1* KO mice. Tests of the former point are difficult in slice experiments where inhibiting ERK activity affects numerous cellular processes beyond those involving the actin cytoskeleton and the strength of synaptic transmission. However, in a recent study we gained insight into this issue by assessing hippocampus-dependent learning and associated synaptic ERK activation in *Fmr1* KOs (Seese et al., 2014). Specifically, we used FDT to evaluate postsynaptic p-ERK levels in hippocampal field CA1 of mice recently engaged in an object location memory (OLM) task: learning in this paradigm both depends on the same CA1 field evaluated in our LTP studies and is impaired in *Fmr1* KOs. We found that wild-type mice given 5 min of massed training had rapid increases in synaptic p-ERK in a narrow span of rostral CA1 stratum radiatum; these mice also learned object location as determined in a retention trial at 24 h posttraining. Given the same 5 min massed training, *Fmr1* KOs exhibited neither response (i.e., increases in p-ERK or learning). However, they did show a marked and broadly distributed decrease in synaptic p-ERK, relative to their suprabaseline levels, suggesting that the experience of handling and training normalized synaptic levels of the activated kinase. In contrast, *Fmr1* KOs given 10 min of massed OLM training both learned object location and exhibited the focal increase in postsynaptic p-ERK associated with learning in wildtypes. In these mutants synaptic p-ERK levels outside the field of learning-induced activation were similar to measures from wildtypes. Finally, mutants given spaced training, three 1 min training sessions spaced by 1 h, also learned in the object location task and exhibited both regionally limited increases in synaptic p-ERK and decreases in spine levels of the phosphoprotein outside the zone activated with encoding. These findings indicate that handling, and perhaps environmental enrichment provided by the habituation and training protocol, was sufficient to offset otherwise abnormally elevated levels of p-ERK in the KOs but this was not sufficient to enable ERK signaling associated with memory encoding. The dissociation of normalizing basal synaptic p-ERK and local increases with training indicates that the former does not occlude the latter. However, it is still possible that normalization of synaptic p-ERK was necessary but not sufficient to OLM encoding. An important goal of future work will be to test if an enriched and varied environment reliably normalizes synaptic p-ERK levels and if this enables or in some fashion lowers the threshold for learning and long-term memory.

CONCLUSIONS AND FUTURE DIRECTIONS

The findings reviewed here lead to the general conclusion that in hippocampus the loss of Fmrp has a major impact on signaling to the spine actin cytoskeleton, with the perhaps greatest consequence being a reduction in activity-induced Rac-to-PAK signaling with consequences for the management of downstream actin regulatory proteins, such as cortactin, and for the stabilization of newly formed actin filaments and LTP (Fig. 14.4). These findings further suggest that treatments designed to facilitate signaling through this pathway could offset disturbances in spine morphology and be beneficial for hippocampus-dependent learning. Lead candidates for enhancing activity in this system include TrkB agonists and agents that promote BDNF signaling through TrkB, as this neurotrophin receptor is known to engage synaptic Rac/PAK signaling in hippocampus (Rex et al., 2007) and application of exogenous BDNF rescues normal TBS-induced LTP in *Fmr1* KO hippocampus (Lauterborn et al., 2007). In line with this suggestion, systemic treatment with and the high affinity TrkB agonist 7,8-dihydroxyflavone has been reported to improve spatial memory and ameliorate hippocampal spine defects in *Fmr1* KOs (Tian et al., 2015). However, additional tests are needed to determine if effects of genotype described here are present in other brain areas, and most particularly within neocortex, where opposite effects of Fmrp loss on PAK signaling have been described (Hayashi et al., 2007). As an alternative to targeting Rac/PaK signaling with therapeutics, one might consider a roundabout approach as signaling through ERK to cortactin (Martinez-Quiles et al., 2004) could prove sufficient to promote actin stabilization in the

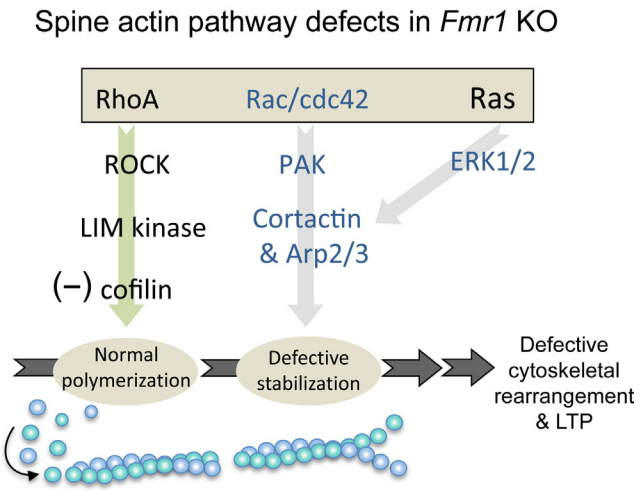

FIGURE 14.4 Summary of defects in Rho/Ras GTPase signaling pathways and actin stabilization in *Fmr1* KO spines. Schematic shows that the FMR1 mutation leaves the RhoA-to-cofilin pathway and initial activity-induced F-actin polymerization intact, but markedly affects the Rac-to-PAK pathway needed for F-actin stabilization. The latter includes abnormal TBS-induced changes in cortactin phosphorylation in the KOs, which are predicted to result in aberrant Arp2/3 recruitment (shown). These disturbances lead to deficits in F-actin stabilization in the KOs; abnormal TBS-induced activation of ERK1/2 may also contribute to this effect. We predict that the failure to stabilize F-actin leads to instability of cytoskeletal rearrangements needed to support hippocampal LTP and the observed synaptic plasticity defects in *Fmr1* KO mice.

mutants. To this end, studies are needed to determine the degree to which ERK signaling to cortactin can be engaged in *Fmr1* KOs and if this approach is sufficient to stabilize activity-induced spine actin remodeling and LTP.

Despite the defects seen in the Rac signaling pathway and the protracted time course for LTP stabilization with suprathreshold stimulation, the *Fmr1* KOs do have the capacity to express stable actin remodeling and hippocampal LTP: 30 min after 10 burst TBS, potentiation of S-C synapses in field CA1 is no longer disrupted by latrunculin infusion (Chen et al., 2010). How this stabilization is ultimately accomplished is not known. Future studies are clearly needed to understand what is happening at longer latencies following TBS to achieve stabilization of the altered cytoskeleton and potentiation in *Fmr1* KOs and perhaps also wild-type mice. Pertinent to this point, our studies of mechanisms of LTP stabilization in rat hippocampus have identified the requirement for delayed involvement of ß1 family integrins. These transmembrane receptors for extracellular matrix proteins both trigger signaling to actin (Brakebusch & Fassler, 2003; Laforest, Milanini, Parat, Thimonier, & Lehmann, 2005; Wiesner, Legate, & Fassler, 2005) and regulate the membrane expression and function of neighboring modulatory receptors (Ettinger et al., 2012; Miranti & Brugge, 2002; Miyamoto, Teramoto, Gutkind, & Yamada, 1996; Ventresca et al., 2015). We have shown that in adult rat hippocampal slices, infusion of β1 integrin neutralizing antisera initiated 30–40 min post-TBS causes reversal of S-C potentiation that becomes evident at 50 min after stimulation: similar anti-β1 infusion initiated 70 min post-TBS does not disrupt the potentiated state (Babayan et al., 2012). Together with other findings these results indicate that β1 integrins, which are enriched at glutamatergic synapses, mediate a delayed, latrunculin-insensitive phase of LTP consolidation with critical involvement during the period from 50 to 60 min after induction. It is possible that in the KOs such delayed stabilization processes are intact and, in the absence of disruption of the potentiated state in the minutes following induction, the integrin mechanisms can ultimately stabilize functional synaptic plasticity in the mutants.

A fundamental question that still needs to be addressed is how the loss of FMRP expression leads to the observed effects on the actin signaling at forebrain synapses? There are several reasonable possibilities worth considering. First, FMRP is reported to bind to mRNAs and thereby inhibit activity-dependent local protein synthesis at spines. The loss of translation regulation by FMRP alters hippocampal protein concentrations by ~15%–20% (Richter, Bassell, & Klann, 2015 for review). Thus, the loss of FMRP could alter, directly or indirectly, levels of kinases and phosphatases that play a role in regulating the specific signaling proteins described here. A second possibility involves the potential misappropriation of proteins with which FMRP directly interacts, such as cytoplasmic FMR1-interacting protein 1 (CYFIP1) (Schenck, Bardoni, Moro, Bagni, & Mandel, 2001; Schenck et al., 2003). In addition to playing a role in inhibiting protein synthesis in association with FMRP, studies have shown that CYFIP1 (a.k.a, Sra-1, PIR121) influences actin remodeling via binding with the WAVE complex (Abekhoukh & Bardoni, 2014 for review). Interestingly, treatment of cortical neurons with the trophic factor BDNF, results in Rac1 activation and a shift in the balance of CYFIP binding partners: following treatment, less CYFIP is bound with FMRP whereas greater levels are found associated with the WAVE complex (De Rubeis et al., 2013). When CYFIP is bound to the WAVE complex it is inactive (Corey & Ridley, 2002; Derivery, Lombard, Loew, & Gautreau, 2009; Eden, Rohatgi, Podtelejnikov, Matthias, & Kirschner, 2002), but upon dissociation the WAVE complex is able to activate the Arp2/3 complex to elicit nucleation of new

branch points on actin filaments (Mullins, 2000 for review). Thus, removal of FMRP would be predicted to tip the balance of CYFIP1 activities toward the actin regulatory pathways leading to abnormal actin cytoskeletal rearrangement and architecture. Future studies are needed to test this hypothesis and, if results prove positive, to further test if this displacement of CYFIP1 plays a role in the aberrant activation of Rac signaling following TBS. Finally, a number of other proteins have been shown to regulate Rac activity and defects in one or more of these could be involved. In particular, Tiam1, Kalirin7, and β-PIX are GEFs that promote Rac activation, whereas a1-chimaerin and Bcr/Abr are GAPs that inhibit Rac activity (Tolias, Duman, & Um, 2011). Future studies are needed to assess levels and activities of these upstream regulators at *Fmr1* KO synapses to determine if there are specific disturbances that accord with activities of elements of Rac-PAK signaling in the mutants.

Dysregulation of the Rac-to-PAK signaling pathway is not an exclusive feature of X-linked intellectual disability (FXS, nonsyndromic). Recent work indicates that levels and activities of PAK are altered in other cognitive disorders in adult brain. Markedly reduced levels of PAK1 and PAK3 isoforms have been reported for human Alzheimer's disease brain, including hippocampus, and in animal models of the disorder (Ma et al., 2008; Zhao et al., 2006); altered levels of phosphorylated PAK have been described as well (Arsenault, Julien, Tremblay, & Calon, 2011; Nguyen et al., 2008; Zhao et al., 2006). Work on Huntington's Disease has shown that PAK1 interacts with mutated huntingtin protein, promoting its aggregation and toxicity, and is colocalized with huntingtin aggregates in brains of persons with this disorder (Luo, Mizuta, & Rubinsztein, 2008). Taken together with the extensive results from studies of the fragile X model mouse described here, there is now considerable evidence that disturbances in the Rac-to-PAK signaling cascade represent a shared locus of impairment for at least four cognitive disorders. Whether defects in this signaling pathway represent a common feature of yet other forms of intellectual disability remains to be determined.

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CHAPTER

15

Matrix Metalloproteinases in Fragile X Syndrome

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INTRODUCTION

Fragile X syndrome (FXS) is the most prevalent single gene cause of inherited intellectual disability and the most common known cause of autism (Yu & Berry-Kravis, 2014). This disorder affects about 1 in every 4000 males and 1 in every 6000 females from all socioeconomic and ethnic backgrounds (Rogers, Wehner, & Hagerman, 2001; Kau et al., 2004; Clifford et al., 2007; Loesch et al., 2007; Hagerman et al., 2009; Clapp et al., 2010; Garber, Visootsak, & Warren, 2008; Kooy, 2003). Individuals with FXS display a range of symptoms from mild learning problems to more severe cognitive issues, including language deficits and behavioral dysfunctions. FXS subjects often display obsessive-compulsive disorder with hand-flapping, autistic behaviors, and attention deficit and hyperactivity disorder. Approximately 25% of patients suffer childhood seizures that improve with age (Clapp et al., 2010; Garber et al., 2008). Beyond cognitive and behavioral abnormalities, FXS patients have characteristic physical traits that include long faces, large protruding ears, enlarged testicles in males (macroorchidism), flat feet, hyperextensible joints, and delicate skin. These nonneural characteristics involve connective tissues comprised of extracellular matrix (ECM), including cartilage and ligaments. Within the CNS, ECM components play critical roles in development, neuronal survival, and synaptic function. The discovery that MMP9 impacts neurological and nonneural aspects of FXS provided an important link between ECM dynamics and synaptogenesis, particularly dendritic spine maturation (Bilousova, Rusakov, Ethell, & Ethell, 2006; Bilousova et al., 2009; Dansie et al., 2013; Sidhu, Dansie, Hickmott, Ethell, & Ethell, 2014).

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FXS is caused by expansion of a CGG repeat in the 5′ untranslated region of the *fragile X mental retardation* (*FMR1*) gene, which is located on the X chromosome at q27.1 (Clapp et al., 2010; Bourgeois et al., 2007; Kooy, Willemsen, & Oostra, 2000). Most of the human population has <55 CGG repeats at this locus, but 55–200 repeats are considered FXS premutations that are often found in mothers with FXS children. *FMR1* premutations are not usually associated with developmental abnormalities early in life, but in the 5th and 6th decades ~50% of male carriers (Garber et al., 2008; Garcia-Arocena & Hagerman, 2010; Jacquemont et al., 2004) and ~8% of female carriers (Coffey, Cook, & Tartaglia, 2008) develop fragile X-associated tremor-ataxia syndrome (FXTAS). Furthermore, ~20% of female premutation carriers are prone to fragile X-associated premature ovarian insufficiency (FXPOI) before the age of 40 (Garber et al., 2008). Associations between FXTAS, FXPOI, and ECM abnormalities have not been reported, with the exception of single study reporting that *Fmr1*-KO-associated macroorchidism does not occur in *Mmp9*-deficient mice (Sidhu et al., 2014). Individuals with FXS typically have >200 CGG repeats in the 5′-untranslated region of *FMR1* (Fig. 15.1) that leads to promoter hypermethylation, and transcriptional silencing of *FMR1*, with lower levels of FMR1 protein (FMRP) (Luo et al., 2010; Kooy et al., 2000; Oostra & Willemsen, 2009). As males have a single X chromosome, boys and men are more severely affected by FXS mutations than girls and women. Females that carry an *FMR1* mutation have less severe phenotypes due to mosaic patterns of FMRP expression resulting from random X chromosome inactivation (XCI) of the affected X chromosome, and it has been reported that patterns of XCI in the lymphocytes of FXS patients correlate with executive function, but patterns of XCI in the neuorns and glia of the CNS are not currently feasible (Sobesky et al., 1996). Alternate forms of mosaicism can also arise from variations in repeat size and the extent of promoter methylation in different cells that vary from tissue to tissue (Garber et al., 2008).

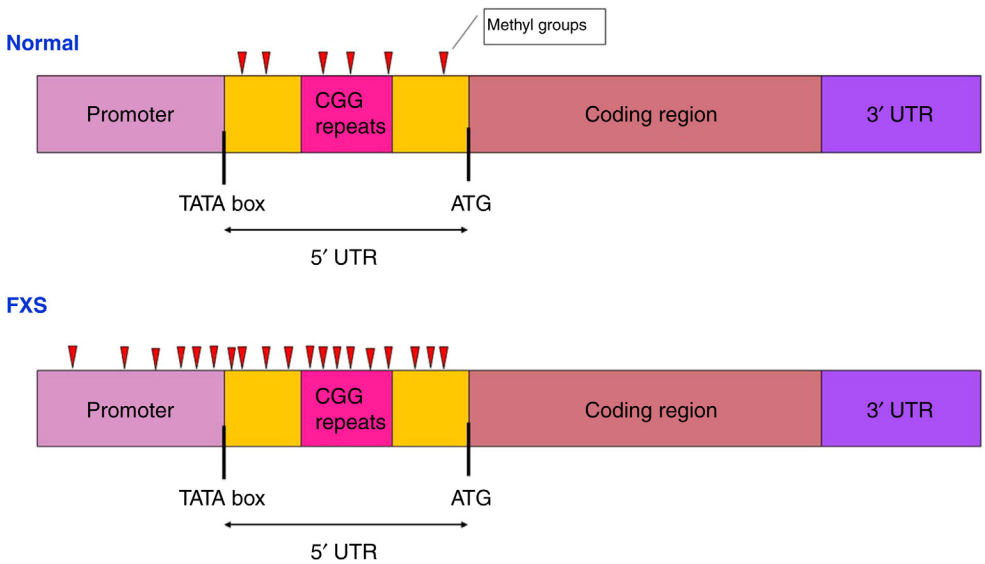

FIGURE 15.1 **Illustration of FMR1 promoter and 5'-untranslated region (UTR) methylation patterns in normal and FXS genes.**

FIGURE 15.2 **Protein domains of FMRP. NLS, nuclear localization signal; KH1 and KH2, K homology domains 1 and 2; NES, nuclear export signal; RGG, arginine-glycine-glycine rich motif.**

The protein product of *FMR1* is FMRP, which have five functional domains: (1) Two KH domains that bind RNA; (2) an RGG box that has been implicated in RNA binding specificity; (3) a nuclear localization signal (NLS); (4) a nuclear export signal (NES); and (5) two coiled coils that are mediate protein–protein interactions (Oostra & Willemsen, 2009; Kooy et al., 2000; Kooy, 2003; Blackwell, Zhang, & Ceman, 2010; Bassell & Warren, 2008) (Fig. 15.2). RNA binding sites in FMRP facilitates the formation of ribonucleoprotein complexes with target-specific mRNAs, proteins, and polyribosomes. It is unclear whether FMRP binds to microRNA. FMRP is highly expressed in the cytoplasm of neurons where it shuttles in and out of the nucleus due to NLS and NES motifs. This nucleus-to-cytoplasm circuit may be important for FMRP's role as a transporter of target mRNAs to specific neuron compartments, including dendritic spines (Luo et al., 2010; Willemsen, Oostra, Bassell, & Dictenberg, 2004). A high proportion of FMRP interacts with purine-rich G-quartet motifs or U-rich motifs in target mRNA's (Brown et al., 2001; John et al., 2004; Denman, 2003; Chen, Yun, Seto, Liu, & Toth, 2003; Darnell et al., 2011). In addition to regulating the transport of target mRNA's to appropriate cellular locations, FMRP also inhibits their translation by stalling ribosomes until intra- or extracellular signals cause FMRP to disengage (Darnell et al., 2011). *FMR1* mutations that reduce or eliminate FMRP expression cause translational changes in the targeted mRNA pool, which may contribute to the FXS phenotype. FMRP has also been shown to modulate the rapid release of neurotransmitters and short-term plasticity through protein–protein interactions with the regulatory β4 subunit of the BK potassium channels; FMRP-deficiency affects synaptic transmission with excessive action potential broadening and enhanced neurotransmitter release (Deng, Sojka, & Klyachko, 2011; Deng et al., 2013). Outside of the brain, FMRP is highly expressed in the testes of male FXS patients, who have macroorchidism (enlarged testicles).

FMR1-DEFICIENCY AND DENDRITIC SPINE MORPHOLOGY

Learning and memory deficits associated with FXS have been linked to the effects of FMRP-deficiency on dendritic spine development and maturation (Clapp et al., 2010; Luo et al., 2010; Willemsen et al., 2004). Dendritic spines are small protrusions on the surface of dendrites that serve as postsynaptic contact sites for most excitatory synapses in the brain (Harris, 1999). Studies with surgically resected human brain tissue and postmortem samples from FXS-affected individuals have established delayed dendritic spine maturation with higher than normal ratios of long thin (immature) filopodia-like spines to short stubby spines typically seen at mature synapses. *Fmr1* knockout (KO) mice-an establihsed mouse model for FXS-also show a high proportion of immature-to-mature dendritic spines (Braun & Segal, 2000; Irwin, Galvez, & Greenough, 2000). These mice are gene KO's for *Fmr1* and hence do not completely replicate the human condition of more variable FMRP expression due to

CGG-repeats. Nonetheless, *Fmr1* KO mice exhibit phenotypes that are similar to human FXS with respect anxiety, susceptibility to audiogenic seizures, macroorchidism, as well as learning and visual-spatial memory deficiencies (Braun & Segal, 2000; Kooy, 2003; Cruz-Martin, Crespo, & Portera-Cailliau, 2010; Comery et al., 1997; Bassell & Gross, 2008; Bernardet & Crusio, 2006; Levenga et al., 2011; Bilousova et al., 2009; Dansie et al., 2013; Sidhu et al., 2014).

Dendritic spines were first described by Santiago Ramon y Cajal as thorns on the surface of Purkinje cell dendrites using a silver impregnation method developed by Camillo Golgi (Ramon & Cajal, 1888, 1899). Remarkably, Golgi's own slides still show dendritic spines on those cells (Dominick Purpura, IBRO lecture 1988), but he considered them artifacts, which was a major source of friction between the two to the point that they would not even look at each other during their Nobel Prize ceremony. Since Cajal's time, improvements in imaging and visualization techniques have made it easier to study dendritic spines, establishing their importance as postsynaptic sites for most excitatory synapses (Hering & Sheng, 2001). Dendritic spines are present on many different neuronal populations the most thoroughly characterized of which are spiny pyramidal neurons in the hippocampus—a population that is strongly affected in *Fmr1* KO mice and FXS patients. Dendritic spines are classified into four major types using morphological criteria: (1) immature filopodium with no spine head; (2) thin spine with a long neck and small spine head; (3) mature stubby spine with a broad head, and (4) mushroom-shaped spine with a short neck and large spine head (Fig. 15.3) (Ethell & Pasquale, 2005). During development, spines evolve from an immature morphology to a mature spine morphology concomitant with an increase in the density of synaptic input along the dendrite. Types 3 and 4 have large synaptic contacts densities to accommodate robust neurotransmitter release from the presynaptic side and a large postsynaptic density that accommodates many neurotransmitter receptors and postsynaptic signaling (Yuste & Bonhoeffer, 2004). Dendritic spines form early in development when synaptogenesis occurs, with pruning and remodeling occurring through to adulthood. In FXS, and other neurodevelopmental disorders, the development and maturation of dendritic spines is abnormal. For example, in FXS there is a higher density of postsynaptic structures, but fewer mature dendritic spines. Dendritic spines work as semiindependent biochemical compartments with their own cell surface receptors, translational machinery and calcium stores. The cytoskeleton of a dendritic spine consists primarily of actin filaments, specifically the β- and γ-actin isoforms, and few microtubules, which are more prominent in the dendrite's shaft (Cohen, Chung, & Pfaff, 1985; Wyszynski et al., 1997). Spine heads contains an electron-dense structure referred to as the postsynaptic density (PSD), which directly opposes the active zone of the presynaptic terminal where neurotransmitter release occurs (Fig. 15.3) (Li & Sheng, 2003). PSDs can vary in size and are proportional to the area of their matching presynaptic active zone (Harris, Jensen, & Tsao, 1992; Tashiro & Yuste, 2003). Many cell surface receptors and ion channels lie on the surface of the PSD, forming complexes with intracellular signaling effector molecules and scaffolding proteins that transduce extracellular signals into intracellular signals that impact dendritic spine morphology (Kaech, Fischer, Doll, & Matus, 1997).

Actin proteins dynamically shift between a pool of monomeric globular actin (G-actin) and filamentous actin (F-actin) that provide the cytoskeletal support for dendritic spine morphology (Rao & Craig, 2000; Halpain, 2000). Actin filaments in the spine neck and core of the head are organized into longitudinal bundles in contrast to the peripheral edges of the spine head

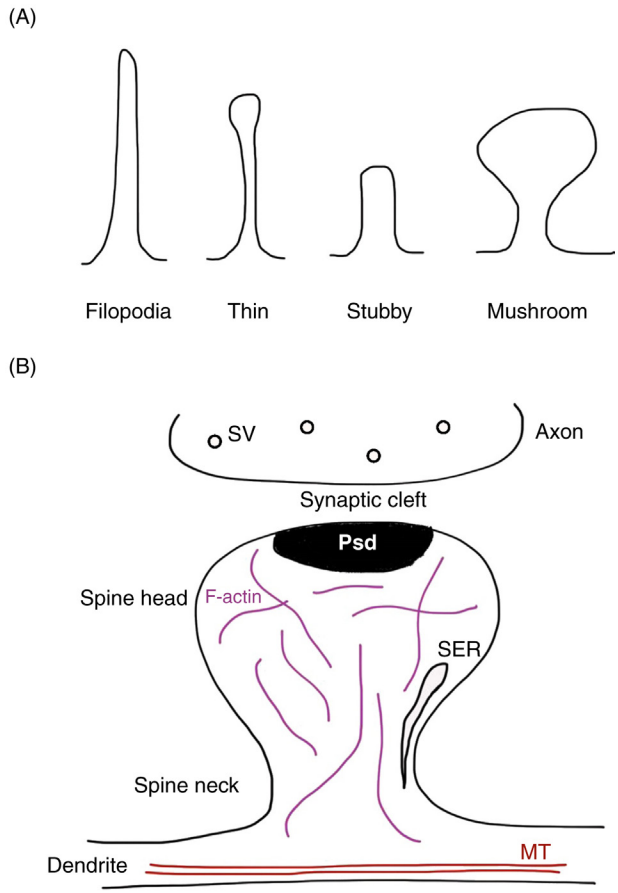

FIGURE 15.3 (A) Dendritic spine shapes. Filopodia and thin spines are indicative of developing or immature dendritic spines; whereas, stubby and mushroom shaped spines are indicative of mature and synaptically active dendritic spines. (B) Basic components of excitatory synapses. Presynaptic bouton at the terminus of an axon branch (above) contains synaptic vesicles, which are released into the synaptic cleft. Neurotransmitter receptors are clustered in the post-synaptic density (PSD) of the dendritic spine (below). Cytoskelelatal elements in the spine head include F-actin and pools of G-actin (not shown). Within the dendrite shaft, microtubules (MT) provide cytoskeleton stability. Smooth endoplasmic reticulum (SER) extends from the dendrite into the base of the dendritic spine.

where a fine meshwork of actin filament predominate (Fifkova & Delay, 1982). Rearrangements of F-actin impacts spine morphology so pathways that regulate actin can significantly affect synaptic activity (Trachtenberg et al., 2002; Pollard, 2003). For example, actin binding proteins that promote reorganization of actin or increased branching, such as neurabin I, SPAR, and arp2/3, respectively, increase spine head size while proteins that depolymerize actin, such as cofilin, reduce spine head size (Ethell & Pasquale, 2005; Shi, Pontrello, DeFea, Reichardt, & Ethell, 2009; Pontrello et al., 2012). Actin dynamics and spine morphology are regulated by synaptic activity through neurotransmitter receptors, especially glutamate receptors (Portera-Cailliau, Pan, & Yuste, 2003; Matus, Brinkhaus, & Wagner, 2000; Passafaro,

Nakagawa, Sala, & Sheng, 2003; Fiala, Allwardt, & Harris, 2002), extracellular signaling through cell surface receptors, such as EphB receptor and ephrin-B ligand interactions (Dalva et al., 2000; Ethell et al., 2001; Henkemeyer, Itkis, Ngo, Hickmott, & Ethell, 2003; Moeller, Shi, Reichardt, & Ethell, 2006; Shi et al., 2009), neuroligins and neurexins (Chih, Dean, Engelman, Isacoff, & Scheiffele, 2004), cadherins (Togashi et al., 2002), integrins (Chavis & Westbrook, 2001; Shi & Ethell, 2006), syndecans (Yamaguchi, 2002), and other receptors for ECM proteins (Mataga, Mizuguchi, & Hensch, 2004; Oray, Majewska, & Sur, 2004).

EXTRACELLULAR MATRIX

As previously mentioned, characteristic nonneural features of FXS occur in tissues that have high levels of connective tissue and extracellular matrix (ECM). For example, FXS patients have flat feet and loose joints (ligaments), soft and delicate skin (collagen), large prominent ears (cartilage), long faces (pharyngeal arches), and macroorchidism (*tunica albuginea* & *mediastinum testis*). ECM is essential for the structure of every tissue in the body and it can be comprised of a wide variety of proteins, including laminin, collagen, fibronectin, and proteoglycans, among others. The characteristic physical traits of FXS establish that *FMR1* deficiency affects the regulation of at least some ECM components. Within the CNS, approximately 20% of the brain is occupied by extracellular spaces (Ruoslahti, 1996), the volume and composition of which vary with brain region (Bruckner, Bringmann, Koppe, Hartig, & Brauer, 1996). Interestingly, these spaces are larger in regions, such as the hippocampus, striatum, and cerebellum, compared to neocortical areas (Zhang & Verkman, 2010). These intercellular spaces contain ECM that includes perineuronal nets (PNNs) that form around the soma and proximal dendrites of some neurons (Celio, 1999; Yamaguchi, 2000). PNNs are most prominent around inhibitory interneurons although they may surround parts of some excitatory neurons. ECM structures control three-dimensional (3D) organization, movement, growth, and neuron morphology, which all contribute to the structural integrity of the CNS (Celio, Spreafico, De Biasi, & Vitellaro-Zuccarello, 1998). In addition to serving important functions during neurodevelopment, ECM proteins are also critical to CNS repair after injury (Kwok, Dick, Wang, & Fawcett, 2011). Although CNS synapses do not have basal laminae, they do have ECM components within the synaptic cleft and around both pre and postsynaptic structures, including dendritic spines (Pappas, Kriho, & Pesold, 2002; Chen, Indyk, & Strickland, 2003; Lucic, Yang, Schweikert, Forster, & Baumeister, 2005).

ECM in the CNS confers elasticity, contributes to signaling pathways that impact adhesion and repulsion, or plays a crucial role in cell survival (Kleinman, Philip, & Hoffman, 2003). Macromolecules that make up the ECM in the CNS include proteoglycans, and glycosaminoglycans (heparin, heparin sulfate, keratin, keratin sulfate, dermatan, dermatan sulfate) (Rutka, Apodaca, Stern, & Rosenblum, 1988), noncollagenous glycoproteins (laminin, fibronectin, tenascin, vitronectin, entactin), and to a limited extent collagen (around blood vessels). Scaffolding proteins are prominent components of the CNS-ECM, including laminin, fibronectin, and tenascin which cross-links with other ECM components to form lattices between cells. These structures are particularly important during development as they help form physical and biochemical barriers and tracts that guide cell migration and axon growth, as well as supporting dendritic spine formation (Tian et al., 1997; Bahr et al., 1997; Bukalo, Schachner, &

Dityatey, 2001; Bernard-Trifilo et al., 2005; Shi & Ethell, 2006). The formation and breakdown of the ECM is a dynamic process that is occurring continually even in the basal state and is necessary for multiple processes, such as growth, neuroplasticity, movement, and repair. However, a strict balance between the activities of these proteolytic enzymes and their inhibitors is essential in maintaining appropriate ECM structures; excessive ECM proteolysis can impact cell survival and may lead to tissue degradation, as occurs in some neurodegenerative diseases, such as amyotrophic lateral sclerosis; whereas, reduced ECM proteolysis can reduce matrix turnover and impact cell survival and reduce the clearance of intercellular compartments by interstitial fluids (i.e., CSF), which may allow toxic metabolites to accumulate. The ECM also served as a repository for cytokines and growth factors that play important roles in mediating cell repulsion or adhesion during inflammation, growth, and/or repair, which can be activated and released from the ECM by MMPs. For example, tumor necrosis factor-α (processed by TACE/ADAM17) enhances synaptic efficiency and is important for synaptic scaling (Beattie et al., 2002; Stellwagen & Malenka, 2006), CXCL-10 attenuates axonal sprouting following spinal cord repair (Glaser, Gonzalez, Sadr, & Keirstead, 2006), and neurotrophins, such as nerve growth factorand brain-derived neurotrophic factor (BDNF) regulate neuronal survival and development. Lastly, some ECM components confer mechanical properties, including rigidity or elasticity, which serve as scaffolds that surround synapses in the brain (Kleinman et al., 2003).

Synapse formation and neuroplasticity can also be influenced by proteolytic cleavage of cell-to-cell adhesion proteins, including as N- or E-cadherins, which are known substrates for matrix metalloproteinases (MMPs). Several studies have shown that MMP-7 and MT5-MMP cleave E- and N-cadherin respectively to regulate neuronal cell adhesion (Noë et al., 2001; Monea, Jordan, Srivastava, DeSouza, & Ziff, 2006). MMP cleavage can also regulate bidirectional signaling mediated by the ephrin-EphB receptor pathway, which play key roles in axon guidance, synaptogenesis, cell migration, and neurogenesis. For instance, MMP-mediated cleavage of EphB2 receptor cleavage is involved in endothelial cell sprouting (Georgakopoulos et al., 2006) and repulsive EphB2 signaling (Lin, Sloniowski, Ethell, & Ethell, 2008).

METALLOPROTEINASES

Matrix metalloproteinases (MMPs) are a family of extracellular endopeptidases that cleave an extensive array of cell surface proteins and components of the ECM (Gururajan, Grenet, Lahti, & Kidd, 1998). As part of a superfamily of zinc-dependent proteinase known as the metzincin proteases, MMPs activity has been implicated in development, health, and disease. During development, MMPs play critical roles in regulating morphogenesis and morphogenesis. Some MMP's expression is maintained in the adult CNS where they play important roles in learning and memory, synaptic plasticity, the maintenance of normal physiology, and repair. However, dysregulated MMP expression can contribute to pathology and high levels of some MMPs have been implicated in cancer and neurological disorders, such as multiple sclerosis (Yong, 2005; Ethell & Ethell, 2007). Other members of the metzincin superfamily include the A-disintegrin-and-metalloproteinase (ADAM), serralysins, and astacins, all of which share the highly conserved metalloproteinase catalytic domain consisting of three conserved histidine residues in a zinc-binding domain arranged as HExxHxxGxxH

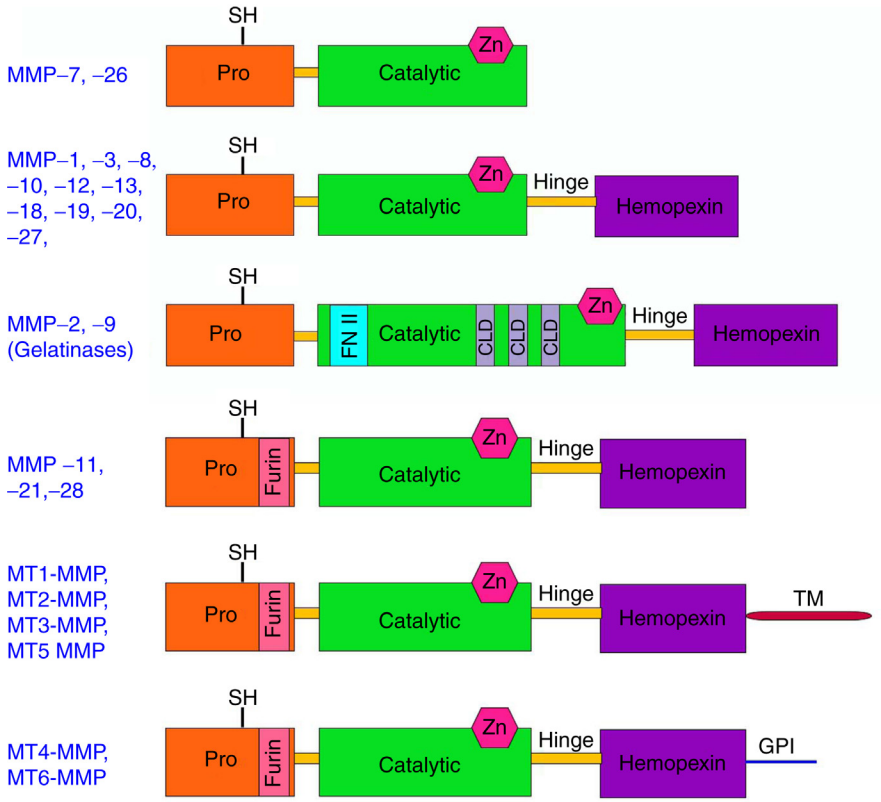

FIGURE 15.4 **Schematic representation of the most abundant matrix metalloproteinases, indicating key domains and elements.** Pro, Prodomain; SH, thiol; Furin cleavage site; catalytic domain with Zn binding site; FNII, fibronectin homology domain type II; CLD, collagen-like domain; hinge region, hemopexin domain; TM, transmembrane domain; GPI, glycosylphosphatidylinositol anchor.

(Fernandez-Catalan et al., 1998). Currently, 24 mammalian MMPs have been identified, each with different substrate preferences, although many substrates are susceptible to cleavage by more than one MMP. MMPs are generally divided into collagenases, gelatinases, stromelysins, matrilysins and 'other' MMPs based on major substrate preference. All but 6 MMPs are secreted into extracellular spaces, with the remainder being tethered to the plasma membrane by a transmembrane domain (Fig. 15.4) (MMP-14, -15, -16, and -24 or the MT1-, MT2-, MT3-, and MT5-MMPs) or a glycophosphatidylinositol (GPI) link (MMP-17 and -25 or MT4-, and MT6 MMPs) (Agrawal, Lau, & Yong, 2008; Ethell & Ethell, 2007; Milward, Fitzsimmons, Szklarczyk, & Conant, 2007; Flannery, 2005; McCawley & Matrisian, 2001).

ECM proteins and other signaling ligands that are cleaved by MMPs can lose contact with their receptors and binding targets, which increases the mobility of cleavage fragments. Diffusion of those cleavage products allows them to interact with cell surface receptors at nearby sites and more distant sites, which may affect dendritic spine morphology (Dityatev & Schachner, 2003; Shi & Ethell, 2006; Ethell & Ethell, 2007; Dziembowska & Wlodarczyk, 2012; Szepesi, Bijata, Ruszczycki, Kaczmarek, & Wlodarczyk, 2013). Laminin is a critical ECM component, and MMP

substrate, that plays key roles in early embryogenesis, neuronal survival, and synapse morphology. A cross-shaped glycoprotein, laminin is made up of three polypeptide chains (α, β, and γ) and each chain has domains with characteristic sequences and tertiary structures associated with specific functions. For example, globular domains of the laminin α-chain bind to integrins, while the β-chain binds to collagen and cell surface proteins, and the γ-chain interacts with entactin. Sixteen mammalian laminin isoforms a (Timpl & Dziadek, 1986; Beck, Hunter, & Engel, 1990; Luckenbill-Edds, 1997; Tzu & Marinkovich, 2008) are assembled in a two-step process; first α, β, and γ chains assemble to form triple stranded coiled-coil structures, which then polymerize with entactin and other ECM components to form networks-including basal lamina-that associate with proteoglycans. In the CNS, laminin is commonly found around the soma and axonal tracts of neurons and glia and in the PNS laminin is enriched in the basement membrane of schwann cells (Powell & Kleinman, 1997). Laminin is secreted by cells into extracellular spaces where it interacts with receptors on the surface of neighboring cells to induce changes in their behavior that may cause cell attachment to substrate orcell.

Laminin can interact with integrin receptors, nonintegrin receptors, and other carbohydrate moieties in the ECM (Reichardt & Tomaselli, 1991; Tzu & Marinkovich, 2008).

Integrins are heterodimeric transmembrane receptors composed of α and β subunits, which are found on plasma membranes of many cell types. Laminin interacts with at least eight different integrin receptors, including α1β1, α2β1, α3β1, α6β1, α6β4, α7β1, αvβ3, αvβ5, and α5v1 (Tzu, Li, & Marinkovich, 2005; Tzu & Marinkovich, 2008). Each of these integrin dimers recognize and bind to RGD-containing-motifs in the globular domains of laminin α-chains. This interaction causes activation of the integrin cytoplasmic domains that trigger intracellular signaling cascades with such mediators as focal adhesion kinase (FAK), small rho GFPases and mitogen-activated protein kinases (Givant-Horwitz, Davidson, & Reich, 2005). Those intracellular signaling cascades mediate a broad spectrum of cellular responses involved in such functions as neural crest cell migration, neurite outgrowth, nerve regeneration after injury, dendritic spine maturation, and synapse remodeling (Malinda & Kleinman, 1996; Luckenbill-Edds, 1997; Shi & Ethell, 2006).

Importantly, laminin is a substrate for MMP-9 cleavage (Doucet & Overall, 2010); MMP-9 is a gelatinase with substrate preference for VV(P/V)(L/Y)SXXX sequences (Turk, Huang, Piro, & Cantley, 2001), two of which are found in the C terminus of α1, and an additional site is in the C terminus of the β1 (Doucet & Overall, 2010). MMP-9 has been shown to affect synaptic plasticity through integrin signaling changes that are mediated by laminin cleavage; cleavage of a laminin chain releases the RGD peptide that can bind to integrin receptors and activate intracellular signaling (Nagy et al., 2006; Meighan et al., 2006). Therefore, MMP-9 cleavage of laminin can allow the diffusion of RGD-containing peptides to nearby structures where they can activate integrin. Other MMPs, such as MMP-7, have also been found to dramatically impact dendritic spine morphology by promoting the rapid transformation of mature spines into immature-filopodial spines (Bilousova et al., 2006; Wlodarczyk, Mukhina, Kaczmarek, & Dityatev, 2011). Although MMP-7 is not expressed in the CNS under physiological conditions, in pathological conditions infiltrating monocytes express and release MMP-7 within the CNS (Ethell & Buhler, 2003). For example, MMP-7 expression was found in perivascular cuffs during experimental autoimmune encephalomyelitis (EAE), which is an experimental model for multiple sclerosis (Buhler et al., 2009). Interestingly, MMP-7 KO mice are resistant to myelin-oligodendrcyte-glycoprotein-induced EAE.

Developmental expression of MMPs happens throughout the body, including MMP-2, -9, -11, -12, and in the adult there is tissue-specific expression of some MMPs. For example, MMP-2, -3, and -9 are expressed in the CNS in different subgroups of cells. MMP-9 is expressed only by neurons and astrocytes, microglia expresses MMP-3, and vascular endothelial cells of blood vessels express MMP-2. MMP expression can also change in response to neurological diseases and neuroinflammatory diseases, such as cerebral ischemia, traumatic brain injury, and stroke (Brkic, Balusu, Libert, & Vandenbroucke, 2015; Ethell & Ethell, 2007). For instance, increased expression of both MMP-2 and -9 have been reported in macrophages of postmortem brain samples from patients with Multiple sclerosis (Maeda & Sobel, 1996). Further, MMP-7 expression increases in arthritic joints (Gjertsson, Innocenti, Matrisian, & Tarkowski, 2005) and although it is not usually expressed in the CNS, infiltrating monocytes infiltrate the CNS and express MMP-7 during EAE (Buhler et al., 2009), and perhaps in MS as well. High levels of MMP-9 expression have been seen in the skin and cerebrospinal fluid of patients with amylotrophic lateral sclerosis (Fang et al., 2009) and in postmortem brain tissue of Huntington's disease patients (Silvestroni, Faull, Strand, & Moller, 2009). At physiological levels MMPs are involved in maintaining the integrity of the blood brain barrier (BBB), but higher MMP activity, making the CNS more susceptibe to infiltrating monocytes and other immune cells. Compromised BBB integrity combined with the ability of MMPs to activate proinflammatory cytokines, make MMPs well-positioned to impact an array of pathological conditions in the CNS (Candelario-Jalil, Yang, & Rosenberg, 2009; Baeten & Akassoglou, 2011). Higher levels MMP-9 are crucial for the development of FXS-associated effects in the CNS and for the development of nonneural characteristics that occur in *Fmr1* KO mice (Sidhu et al., 2014). *Fmr1* KO mice exhibit enhanced mGluR dependent LTD, a deficiency in LTP, increased anxiety and hyperactivity, reduced social interaction behavior or ability to distinguish between social novelty, macroorchidism, increased basal protein synthesis, and increase in the phosphorylation and activation of intracellular signaling effector molecules including mTOR, eIF4e, and Akt compared to WT animals. Many physiological, cellular, and behavioral aspects of the *Fmr1*-deficient phenotype require the presence of high levels of MMP-9, as they do not occur in *Fmr1/mmp9* KO mice. Unlike *Fmr1* KO mice, double KO mice have normal (WT) dendritic spine profiles (in vivo and in vitro), normal levels of MMP-2 in the hippocampus, they show LTD comparable to WT mice in response to DHPG treatment (although they remain deficient in LTP), display reduced anxiety, do not display socialization deficits, males don't have macroorchidism, and they have levels of phosphorylated mTOR, eIF4e, and Akt that are not significantly different than WT mice (Sidhu et al., 2014).

MMP-9 IN FXS

Mammals have 2 gelatinases that are also known as MMPs, gelatinase A (MMP-2) and gelatinase B (MMP-9) that can be distinguished using gelatin zymography; MMP-2 is 72 kDa and MMP-9 is 92-kDa gelatinase. Human *MMP-9* is located on chromosome 20 at q11.2–13.1. A major ECM substrate of MMP-9 is gelatin, but it also degrades native collagen types I, IV, V, VII (in decreasing order of specificity), elastin, fibronectin, laminin, aggrecans, link proteins, and vitronectin (Visse & Nagase, 2003; McQuibban et al., 2001; Van den Steen, Proost, Wuyts, Van Damme, & Opdenakker, 2000). Other substrates include stromal cell derived factor which

it inactivates, connective tissue activating peptide (CTAP)-III/ neutrophil activating peptide (NAP)-2, platelet factor (PF)-4, GROa which are all degraded, it activates pro-IL-8, pro TNF-α, pro-TGF-β1, and pro-IL-1β, it releases the cell surface bound IL-2Rα, causes shedding of FGFR1, cleavage of plasminogen, galectin-3, α2-macroglobulin, a1-proteinase inhibitor, and vascular endothelial growth factor (Ochieng et al., 1994; Patterson & Sang, 1997; Schönbeck, Mach, & Libby, 1998; Yu & Stamenkovic, 2000; Van den Steen, Proost, Wuyts, Van Damme, & Opdenakker, 2000; McQuibban et al., 2001; Visse & Nagase, 2003; Peixoto et al., 2012). MMP-9 also cleaves and activates pro-MMPs, including MMP-2, -9, and -13, and at high enough levels it can inactivate some mature MMPs through proteolysis (Visse & Nagase, 2003). In addition to auto-activation, pro-MMP-9 can also be cleaved and activated by MMP-3, -7, -26, and MT1-MMP. Active MMP-9 is involved in a number of processes including embryonic development, neuronal development, myelination (through processing of IGF-BPs), reproduction, and tissue remodeling (Lau, Cua, Keough, Haylock-Jacobs, & Yong, 2013; Yong, Zabad, Agrawal, Goncalves-Dasilva, & Metz, 2007; McCawley & Matrisian, 2001). MMP-9 is also involved in LTP and in the surface trafficking of the NR1 subunit of NMDA receptors—through an integrin-β1 dependent mechanism (Szklarczyk et al., 2008; Michaluk et al., 2009). Elevations in MMP-9 activity have been associated with pathological conditions, such as arthritis, cancer, intracerebral hemorrhage, ischemia, and some cardiac disorders (Sheu et al., 2001; Patterson & Sang, 1997; Bergers et al., 2000; Gu et al., 2005; Nagy et al., 2006; Dziembowska & Wlodarczyk, 2012). *Fmr1* KO mice exhibit a more immature dendritic spine profile, in vivo and in vitro, and higher levels of MMP-9 and MMP-2 activity in the hippocampus, even though there is no difference in mRNA from either gene, compared to WT (Sidhu et al., 2014) discern social novelty. *Fmr1*-deficiency also causes higher basal protein synthesis and increased phosphorylation/activation of key signaling effectors, such as mTOR, eIF4e, and Akt. Importantly, those signaling effectors were normalized in double KO mice (Sidhu et al., 2014). In agreement with those findings, Gkogkas et al. (2014) showed pharmacogenetic inhibition of eIF4e dependent MMP-9 mRNA translation reversed the *Fmr1* KO phenotypes (Gkogkas et al., 2014). Moreover, others reported that FMRP regulates the translation of the *mmp-9* and *mmp2* mRNAs, at least in the hippocampus (Janusz et al., 2013).

Fmr1/Mmp9 double KO mice do not develop behavioral and anatomical changes that typically occur in single *Fmr1* KO mice. The stranger mouse paradigm tests whether a mouse will recognize a novel mouse compared to one it has previously encountered. *Fmr1* KO mice spend an equal amount of time interacting with a novel (stranger) mouse that with one it has seen before, indicating they cannot or will not distinguish between the two. In contrast, double KO mice spent more time investigating stranger mice, just like WT mice. One of the more remarkable findings in the *Mmp9/Fmr1* double KO mouse study had to do with the enlarged testicles (marcoorchidism) seen in male *Fmr1* KO mice and human males with FXS. The length, width, and volume of *Fmr1* KO mice is significantly larger that WT mice, but there was no significant difference between WT and double KO males. That is, MMP-9-deficiency prevented all signs of macroorchidism. The *tunical albuginea* and mediastinum testis are tough connective tissue coverings on the testicles and FXS-associated macroorchidism likely results from deficiencies in those structures that allow the testicles to enlarge at puberty. The fact that macroorchidism does not occur in *Mmp9/Fmr1* double KO mice indicates that MMP-9 activity is necessary for this defect to occur. This finding provides conclusive evidence for the hypothesis that many FXS-associated defects are related to ECM-connective tissue defects. In human FXS patients

a loosening of connective tissues likely contributes to unstable joints, gastrointestinal issues (basement membrane), and distended aortas. Interestingly, the flat feet (excessive foot pronation) seen in FXS patients are related to stretching of the spring ligament and tendon of the tibialis posterior muscle, both connective tissue structures; notably, the parents of FXS patients on minocycline (an MMP-9 inhibitor) have reported an increase in foot arch formation leading to more stable foot positioning and less wearing on the instep of their shoes.

It has been proposed that higher sensitivity of class I mGluRs (metabotropic glutamate receptors) underlie the deficits seen in FXS, both in human subjects and in *Fmr1* KO mice (Bear, Huber, & Warren, 2004), and that reducing mGluR5 activity, or the activity of downstream signaling pathways associated with mGluRs, will ameliorate cognitive and behavioral aspects of the FXS phenotype. Although mGluR signaling might play a role in this disorder, it is not the sole cause of all the symptoms as alterations in mGluR signaling are unlikely to impact macroorchidism or other nonneural connective tissue features of FXS (Bear et al., 2004; Bassell & Gross, 2008; Michalon et al., 2012). Metabotropic GluRs may act downstream of other molecules to increase MMP-9 expression or activity MMP-9. In addition, effector molecules activated in the *Fmr1* KO mice, such as mTOR and eIF4e or Akt can also be regulated by downstream signaling cascades of other receptors and by different pathways. For instance, increased mTOR phosphorylation could be due to BDNF signaling through its receptor, TrkB, which shares similar intracellular signaling pathways with the mGluRs. BDNF protein levels are known to be higher in the hippocampus of *Fmr1* KO mice and the TrkB mRNA is a known target of FMRP (Castren & Castren, 2014; Louhivuori et al., 2011; Uutela et al., 2012). Several MMPs cleave and activate neurotrophins, and MMP-9 in particular has been shown to cleave pro-BDNF (Hwang, Park, Choi, & Koh, 2005; Yang et al., 2009; Mizoguchi, Yamada, & Nabeshima, 2011). Higher levels of MMP-9 may function in a positive feedback loop in the KO mice, cleaving more pro-BDNF into mature BDNF that can then bind to TrkB and activate signaling cascades that promote the translation of even more MMP-9 (Santos, Comprido, & Duarte, 2010). Moreover, a FMRP binding protein, cytoplasmic FMRP interacting protein 1 (CYFIP1), binds directly to eIF4e, and BC1 increases the binding affinity of FMRP for this complex (Napoli et al., 2008; De Rubeis et al., 2013). Together FMRP and CYFIP1 form a translation inhibitory complex in dendritic spines and this repression is regulated by BDNF in an activity-dependent manner (De Rubeis et al., 2013; Schenck, Bardoni, Moro, Bagni, & Mandel, 2001; Napoli et al., 2008). BDNF signaling induces conformational changes in CYFIP1 through Rac1 which causes CYFIP1 to dissociate from eIF4e and allows the synthesis of specific target proteins including Arc that promote AMPAR internalization. Conformational changes of CYFIP1 also allow it to affect changes in actin polymerization and hence dendritic spine morphology (De Rubeis et al., 2013).

Since many MMP-9 substrates occur in and around CNS synapses (Ethell & Ethell, 2007), integrins and Eph receptors are the most likely candidates to mediate MMP-9 effects in *Fmr1* KO neurons. Both integrins and EphB receptors are known to signal through the recruitment and activation of FAK, the Src family of nonreceptor tyrosine kinases, and the PI3K/Akt cascade (Chen, Haegeli, Yu, & Strickland, 2009; Guo & Giancotti, 2004; Legate, Wickstrom, & Fassler, 2009; Moeller et al., 2006; Maddigan et al., 2011). Moreover, β1 integrin, another MMP substrate, has been shown to negatively regulate the activity of protein pyrophosphatase2a (PP2A), which de-phosphorylates and inactivates Akt (Fornaro, Steger, Bennett, Wu, & Languino, 2000; Pankov, 2003). The PI3K-Akt-mTOR pathway was been implicated in

FXS through its regulation of protein synthesis via Elongation factor 1α (Hou & Klann, 2004; Ronesi & Huber, 2008).

Reductions in MMP-9 activity improve both the behavioral deficits and physical traits associated with FXS (Bilousova et al., 2009; Paribello et al., 2010; Utari et al., 2010; Leigh et al., 2013; Schneider et al., 2013; Sidhu et al., 2014), making MMP-9 a promising target for therapeutic development. One compound successfully used to treat FXS subjects is minocycline, an FDA-approved antibiotic that also inhibits MMP-9 (Seukeran & Eady, 1997; Goulden, Glass, & Cunliffe, 1996). In three different clinical trials minocycline was shown to improve a range of FXS-associated symptoms, including major improvements in language use, increased speech coherency, improved attention spans, less irritability, decreased anxiety, increased social communication and a general improvement in global behavioral scores (Paribello et al., 2010; Utari et al., 2010; Leigh et al., 2013; Schneider et al., 2013). Clinical studies of minocycline treatment for FXS reported only minor side effects, such as occasional mild gastrointestinal problems, headaches, and diarrhea (Paribello et al., 2010; Utari et al., 2010)—although it is well tolerated by a majority of subjects; however, a small minority of children may be susceptible to minocycline-induced autoimmunity, particularly lupus (Farver, 1997; El-Hallack & Giani, 2008). Taking into account minocycline's ability to effectively reduce MMP-9 activity and off-target effects of this broadly acting antibiotic, the long-term treatment of FXS patients would benefit from new drugs that block MMP-9 without antimicrobial activity.

CONCLUSIONS

Many different signaling pathways and receptors are dysregulated in FXS with effects inside and outside the CNS. Among the most notable features of FXS are defects in connective tissues that lead to such things as loose joints, prominent ears, long faces, delicate skin, flat feet, distended aortas, and macroorchidism. Clearly, loss of *FMR1* transcription impacts ECM protein production and/or turnover. MMPs are the most prominent family of enzymes to modify ECM, and MMP-9 activity is consistently higher in *Fmr1* KO mice and FXS subjects. Transgenic mice that lack both *Fmr1* and *Mmp-9* show few features of the FXS phenotype seen in *Fmr1* KO mice, indicating that high levels of MMP-9 are required for many FXS-associated features to occur. The *Fmr1*/*Mmp9* double KO mice also showed improvements in anxiety and socialization over *Fmr1* KO mice. Furthermore, FXS subjects treated with minocycline, a crude yet effective MMP-9 inhibitor, show improvements in socialization, attention, and other behaviors, as well as clear improvements in nonneurological features of this disorder. Cultures of mature hippocampal neurons treated with MMP-9 protein quickly change their dendritic spines from short stubby (mature) structures into long, immature filopodial like spines that mimick the immature spine phenotype seen in *Fmr1* KO hippocampal cultures (Bilousova et al., 2009). This same relationship between *Fmr1* and MMP-9 has also been reported in the *Drosophila* model of FXS where overexpression of TIMP1 (the endogenous regulator of MMPs) or a *mmp* null mutation rescued all nervous system defects seen in the *dfmr* null flies, suggesting a genetic interaction between these two genes and/or their protein products (Siller & Broadie, 2011). However, it should be mentioned that the neuroanatomy and neurobiology of flies and mammals is very different, so conclusions drawn from insect work must be reproduced in mammalian models. Other groups have also shown MMP-9

involvement in synaptic plasticity and regulation of NMDAR currents mediated through integrin activity (Nagy et al., 2006; Meighan et al., 2006) suggesting all these components that are defective in FXS may be linked. The ECM plays a critical role in the development, maintenance and structure of most tissues in the body, so including the brain, it should not be surprising that a disorder that affects connective tissues all over the body has ECM aberrations in the CNS. MMPs are principal regulators of the ECM all over the body. Within the brain MMP-9 plays an important role in synaptic strength, which is disrupted in FXS. MMP-9 is an ideal target for the development of a next generation of therapeutics to treat this FXS, alone or in combination with other treatments under development.

ABBREVIATIONS

CNS Central nervous system
ECM Extracellular matrix
Fmr1 geneFragile X mental retardation 1 gene
FMRP Fragile X mental retardation protein
FXS Fragile X syndrome
KO Knockout
MMP Matrix metalloproteinase

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Further Readings

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CHAPTER

16

Ion Channel Dysfunction and FXS

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INTRODUCTION

Voltage-dependent ion channels are transmembrane proteins responsible for sensing changes in the membrane potential, and mediating an appropriate physiological response. In the nervous system, they perform a crucial role in determining the intrinsic excitability of neurons, both globally and locally within specific subneuronal compartments, such as the axon initial segment or individual dendritic branches. In addition to shaping the computational properties of these compartments and their rules for plasticity, certain ion channels participate in the regulation of neurotransmitter release, modulation of intracellular signaling pathways, or gene regulation. Voltage-gated ion channels are typically composed of a pore-forming domain, with a selectivity for certain ions (e.g., Na^+, Ca^{2+}, K^+, or Cl^-), as well as a voltage-sensing domain that serves to set the gating dynamics of the channel. Certain channels may also contain additional sensor domains, responsive to intracellular ion levels (e.g., the Ca^{2+}/Na^+ gated potassium channel family, described in greater detail later) or intracellular signaling molecules (e.g., HCN, GIRK). For a more extensive review of the structure, regulation, and function role of voltage-gated ion channels in the nervous system, the reader is referred to (Hille, 2001; Levitan & Kaczmarek, 2015; Stuart, Spruston, & Hausse, 2016)

At the molecular level, voltage-gated ion channels are composed of a complex comprising the pore-forming alpha subunits, as well as a range of auxiliary subunits and other regulatory molecules [e.g., calmodulin kinase II binding to the L-type Ca^{2+} subunit Cav1.2 (Hudmon et al., 2005)]. These accessory factors serve to modulate their properties or subcellular location [reviewed in (Gutman et al., 2005; Lai & Jan, 2006; Vacher, Mohapatra, & Trimmer, 2008; Leterrier, Brachet, Fache, & Dargent, 2010; Jensen, Rasmussen, & Misonou, 2011; Zamponi & Currie, 2013)]. A range of cellular processes, operating at the transcriptional, posttranscriptional, translational, or posttranslational levels, further increase the diversity of ion channel properties [reviewed in

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(Vacher et al., 2008; Wahl-Schott & Biel, 2009; Cerda & Trimmer, 2010; Jan & Jan, 2012; Lipscombe, Andrade, & Allen, 2013; Zamponi & Currie, 2013). This fine-tuning of channel function—throughout development and in response to neuronal activity may be essential for establishing and adapting the precise role of a given ion channel within a specific neuron type and neuronal subcompartment (e.g., Frick, Magee, Koester, Migliore, & Johnston, 2003).

Over recent years, converging evidence suggests that an alteration in the expression or biophysical properties of voltage gated ion channels may be an important feature of the pathophysiology of fragile X syndrome (FXS) [for reviews see (Brager, Akhavan, & Johnston, 2012; Szlapczynska, Bonnan, & Ginger, 2014; Johnston, Frick, & Poolos, 2016)]. Indeed, numerous mRNA targets of FMRP encode either alpha-subunits of voltage-gated ion channels or regulatory beta-subunits (Brown et al., 2001; Darnell et al., 2001; Chen, Yun, Seto, Liu, & Toth, 2003; Darnell et al., 2011). In addition, a novel noncanonical role for FMRP in the regulation of ion channel properties via direct binding to specific ion channel subunits or their accessory proteins has also been elucidated (Brown et al., 2010; Deng et al., 2013; Ferron, Nieto-Rostro, Cassidy, & Dolphin, 2014; Myrick et al., 2015). The recognition that changes in ion channels (in the absence of FMRP) can contribute to alterations in the intrinsic properties of neurons, and ultimately to the pathophysiology of FXS has led to a growing appreciation of ion channel dysfunction as one of the underlying features of FXS [reviewed in (Brager et al., 2012; Szlapczynska et al., 2014; Johnston et al., 2016)]. The following sections illustrate the current knowledge of ion channel defects and their potential pathophysiological roles in FXS.

VOLTAGE-DEPENDENT POTASSIUM CHANNELS

FMRP Regulates Kv3.1 Voltage-Dependent Channels That are Required for High Frequency Firing

Messenger RNA for the voltage-dependent K^+ channel, Kv3.1, was one of the first mRNAs found to bind FMRP (Darnell et al., 2001), a finding that has been repeated using several different approaches (Strumbos, Brown, Kronengold, Polley, & Kaczmarek, 2010; Darnell et al., 2011). There are four subunits in the Kv3 family of channels (Kv3.1, Kv3.2, Kv3.3, and Kv3.4) and recent evidence indicates that mRNA for Kv3.3 is also an FMRP target (Darnell et al., 2011). The Kv3.1 channel itself can exist as one of two splice variants named Kv3.1*a* and Kv3.1*b* (Luneau et al., 1991). Both Kv3.1b and Kv3.3 are expressed throughout the nervous system in neurons that are capable of firing at high rates, but particularly high levels are found in neurons of the auditory brainstem (Perney & Kaczmarek, 1997; Li, Kaczmarek, & Perney, 2001), which are capable of responding to sound stimuli by firing at rates of 600 Hz or more.

The Kv3.1 and Kv3.3 channels are sometimes termed "high-threshold" channels because they begin to activate only when the membrane potential is more positive than about −10 mV, as occurs on the downstroke of an action potential (Kanemasa, Gan, Perney, Wang, & Kaczmarek, 1995). Their rapid activation and deactivation with changes in voltage accounts for the ability of neurons expressing these channels to fire at high rates (Rudy et al., 1999; Rudy & McBain, 2001; Shevchenko, Teruyama, & Armstrong, 2004; Sacco, De Luca, & Tempia, 2006). The role of FMRP in establishing the relative levels of Kv3.1 in different neurons has been established most clearly for neurons in the medial nucleus of the trapezoid body (MNTB), which

are part of the brainstem circuitry that establishes localization of sounds in space (Brown & Kaczmarek, 2011). MNTB neurons in mice can normally fire repetitively in response to stimulation at rates of up to 600 Hz. In mice in which the Kv3.1 gene has been deleted, however, neurons are incapable of responding to stimulation at rates greater than 200 Hz (Macica et al., 2003).

Levels of Kv3.1 channels are not uniform throughout the MNTB. In wild-type animals, Kv3.1 is expressed along a gradient in the MNTB, with highest levels in the neurons at the medial, high-frequency end of the nucleus (Li et al., 2001; von Hehn, Bhattacharjee, & Kaczmarek, 2004). This tonotopic gradient can be measured both by immuocytochemistry and by direct patch clamp recordings of Kv3.1 current in neurons from the lateral and medial aspects of the MNTB (Fig. 16.1A) (Strumbos et al., 2010a). In mice lacking FMRP (*Fmr1*$^{-/-}$ mice), however, no such gradient of Kv3.1 can be detected along the tonotopic axis (Fig. 16.1B). Moreover, MNTB neurons in *Fmr1*$^{-/-}$ mice have significantly higher overall levels of Kv3.1b protein (Strumbos, Polley, & Kaczmarek, 2010). Thus FMRP is required for the normal gradient of Kv3.1 expression in MNTB neurons, presumably by suppressing translation of Kv3.1 mRNA, particularly at the lateral low frequency end of this nucleus.

Further evidence for the regulation of Kv3.1 levels by FMRP has come from studies of changes in levels of Kv3.1 protein in response to changes in the auditory environment. Sustained exposure of intact rats or mice to physiological levels of sound for periods of 20–30 min produces an increase in total levels of the Kv3.1 channel protein in MNTB (Strumbos et al., 2010a; Strumbos et al., 2010b). The effect of stimulation depends on characteristics of the sound. Specifically, total levels of Kv3.1 protein increased following exposure of the animals to sounds that are modulated at 400 Hz (Strumbos et al., 2010b), a rate that MNTB neurons can follow provided they have sufficiently high levels of Kv3.1 channels (Macica et al., 2003; Kaczmarek, 2012). The ability of auditory stimulation to increase levels of total Kv3.1 protein, however, requires FMRP. Auditory stimulation fails to produce any change in levels of Kv3.1 channels in *Fmr1*$^{-/-}$ mice (Fig. 16.1C) (Strumbos et al., 2010a). These results indicate that FMRP is necessary for maintenance of the tonotopic gradient and for activity-driven increases in Kv3.1, and are consistent with a role for FMRP as a repressor of protein translation.

Changes in levels of Kv3.1 in auditory brainstem neurons mediated though FMRP are likely to adjust the intrinsic excitability of these neurons in response to different levels of sound exposure. Overexpression of Kv3.1 channels increases excitability by allowing neurons to fire at higher rates (Kaczmarek et al., 2005). Because loss of FMRP leads to elevated channel expression throughout the MNTB, these high levels of Kv3.1 may contribute to the hypersensitivity of FXS patients to auditory stimuli. Moreover, because Kv3.1 channels are widely expressed in rapidly firing neurons throughout the nervous system (Massengill, Smith, Son, & O'Dowd, 1997; Perney & Kaczmarek, 1997; Rudy et al., 1999; Rudy & McBain, 2001; Lien & Jonas, 2003), it is likely that loss of FMRP also alters their excitability in a similar way. Nevertheless, it is possible that, during the course of development to the adult, some compensation for changes in excitability produced by the loss of FMRP may occur (Wang, de Kok, Willemsen, Elgersma, & Borst, 2015).

Kv1 Family Channels

Kv1 potassium channels regulate the firing threshold, the temporal integration of synaptic inputs, and the intrinsic excitability of many neurons, including those of the MNTB (Brew &

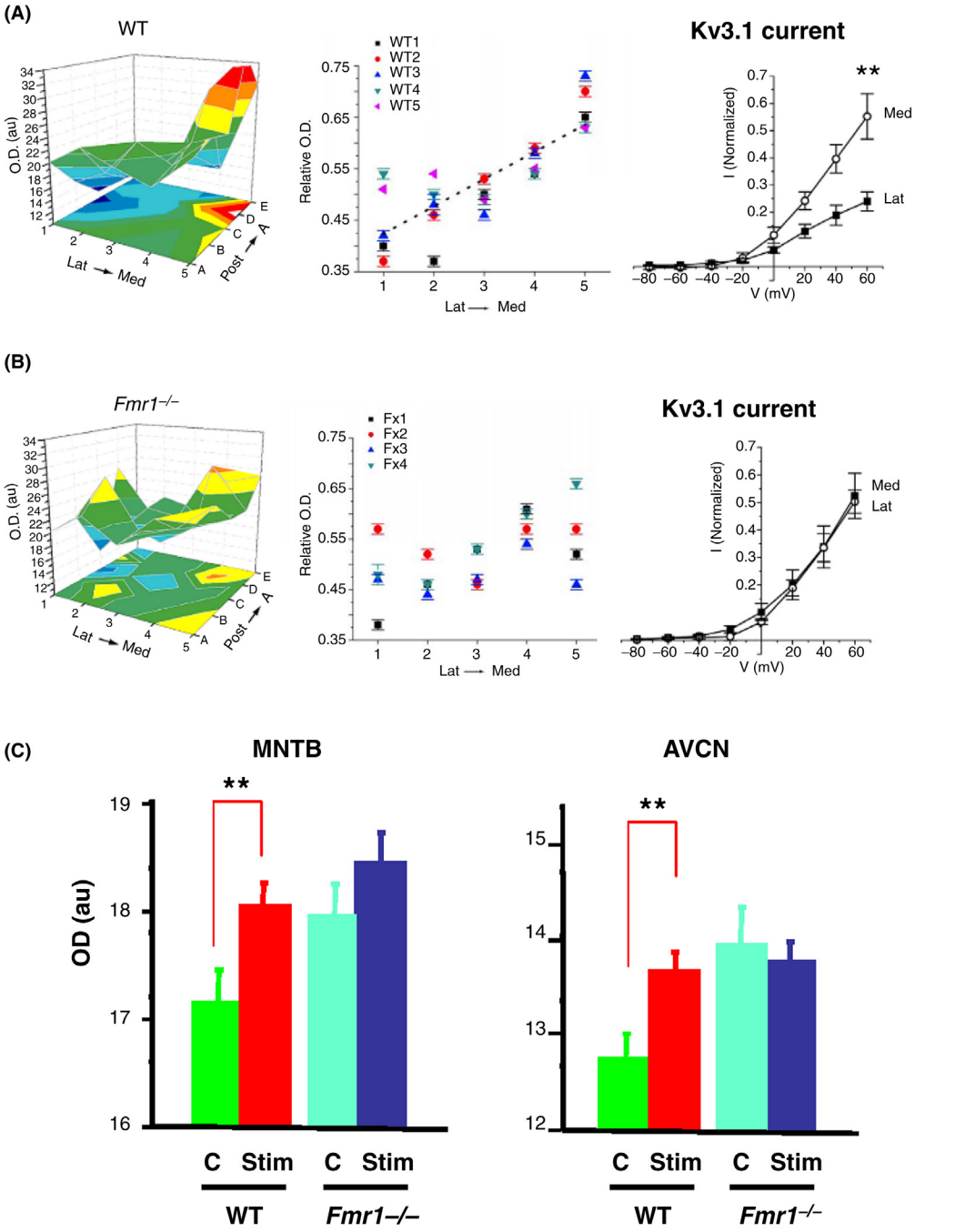

FIGURE 16.1 **Loss of FMRP eliminates gradients of Kv3.1 channel expression and activity-dependent increase in levels of Kv3.1. A.** (A) Gradient of Kv3.1 along the lateral–medial axis of the medial nucleus of the trapezoid body (*MNTB*) of wild type mice. Left, A representative three-dimensional curve-fit of the level of immunlabeling in serial sections of the MNTB. Center Levels of Kv3.1 in five zones from lateral to medial in five wild type animals. Right Levels of Kv3.1 current in lateral and medial MNTB neurons of wild-type mice. (B) Lack of a gradient of Kv3.1 in *Fmr1*$^{-/y}$. All measurements as in (A). (C) Auditory stimulation fails to increase Kv3.1 levels in MNTBs of *Fmr1*$^{-/-}$ mice. Bar graphs show total levels of MNTB Kv3.1b immunoreactivity in MNTB, and the anteroventral cochlear nucleus (*AVCN*) of wild-type and *Fmr1*$^{-/-}$ mice kept in a soundproof room (C) or given 30 min of exposure to a physiological level of sound (Stim). *Source: Figures modified from Strumbos, J. G., Brown, M. R., Kronengold, J., Polley, D. B., Kaczmarek, L. K. (2010). Fragile X mental retardation protein is required for rapid experience-dependent regulation of the potassium channel Kv3.1b.* Journal of Neuroscience, 30, *10263–10271.*

Forsythe, 1995; Trussell, 1999; Dodson, Barker, & Forsythe, 2002; Dodson et al., 2003; Mathews, Jercog, Rinzel, Scott, & Golding, 2010). Messenger RNA for the Kv1.2 subunit, which is localized in all cell compartments (axons, terminals, cell body, and dendrites (Sheng, Tsaur, Jan, & Jan, 1994), also binds FMRP (Darnell et al., 2011). This finding suggests that changes in the expression and function of Kv1.2 are involved in FXS. Consistent with this assumption, recent findings reported that Kv1-mediated current is downregulated in the medial prefrontal cortex (mPFC) of *Fmr1*$^{-/y}$ mice, resulting in increased excitability of layer 5 tract projecting pyramidal neurons, but not in neighboring intraencephalic projecting neurons located within same layer 5 of mPFC (Kalmbach, Johnston, & Brager, 2015). This finding emphasizes the notion that FXS-related ion channel dysfunction is brain region and neuron-type specific (Zhang et al., 2014). Moreover, alterations in the excitability of discrete populations of projection neurons of the mPFC are likely to have wider consequences for the excitability of specific neuronal circuits. This result is also consistent with recent findings supporting the idea that FXS is associated with deficits in connectivity between different neocortical regions, which can be characterized as "connectopathies" (Haberl et al., 2015).

Kv4.2 Channels

Kv4.2 is one of the major subunits contributing to a rapidly activating- and inactivating form of A-type K^+ current described in neocortical and hippocampal pyramidal neurons [e.g., (Hoffman, Magee, Colbert, & Johnston, 1997; Korngreen & Sakmann, 2000)]. Messenger RNA encoding Kv4.2 (*Kcnd2*) has been identified as a high confidence FMRP target mRNA (Darnell et al., 2011). Confirmatory evidence, demonstrating the interaction of FMRP with *Kcnd2* mRNA was independently provided by two groups (Gross, Yao, Pong, Jeromin, & Bassell, 2011; Lee et al., 2011). However, the functional consequences of these findings were different. Gross et al. (2011) reported that FMRP is a positive regulator of Kv4.2 translation, and that Kv4.2 expression is reduced in the dendrites of CA1 pyramidal neurons and dentate gyrus granule cells from *Fmr1*$^{-/y}$ mice. Kv4.2 expression was shown to be globally reduced in whole-cell extracts from the neocortex and surface expression of Kv4.2 was reduced in cultured cortical neurons and hippocampal slices. In contrast, Lee et al. (2011) concluded that FMRP is a translational repressor of Kv4.2 in the hippocampus, based on a combination of biochemical, molecular, and live cell imaging approaches.

A full explanation for the aforementioned differences has not yet been elucidated, but may lie in the different genetic background of the *Fmr1*$^{-/y}$ model used in both cases (Routh, Johnston, & Brager, 2013). Routh et al. (2013) demonstrated that A-type potassium current is reduced at the dendritic level, but not in the somata of CA1 pyramidal neurons from *Fmr1*$^{-/y}$ mice. Based on the kinetics of recovery from inactivation (as well as the fact that Kv4.2 is the only Kv4 subunit expressed in CA1 pyramidal neurons (Chen et al., 2006), these changes are likely due to a reduction in functional Kv4.2 containing channels in the dendrites of *Fmr1*$^{-/y}$ pyramidal neurons. However, a partially compensating change due to a hyperpolarized shift in the activation curve of the channel (compared with those of wild-type neurons) in the dendrites of *Fmr1*$^{-/y}$ neurons was also observed (Routh et al., 2013). The combined effect of these changes was an overall alteration in dendritic properties. In particular a reduction in the attenuation of AP backpropagation [which is strongly regulated by A-type K^+ channels (Hoffman et al., 1997; Migliore, Hoffman, Magee, & Johnston, 1999; Frick et al., 2003; Martina, Yao, & Bean, 2003)] was observed, as well as a resultant increase in calcium influx in the distal dendrites of *Fmr1*$^{-/y}$

neurons. Furthermore, CA1 pyramidal neurons from *Fmr1*$^{-/y}$ mice had a lower threshold for LTP induction, as would be expected from a reduction in Kv4.2 (Chen et al., 2006).

Taken together, the findings of Routh et al. (2013) do not entirely resolve the opposing findings of Lee et al. (2011) and Gross et al. (2011). Indeed it must be noted that immunohistological analysis of channel density in CA1 dendrites (Kerti, Lorincz, & Nusser, 2012) does not always correspond to the pattern of expression observed by physiological measurements [e.g., (Hoffman et al., 1997); reviewed in (Brager & Johnston, 2014)]. This is likely due to the intricate, activity-dependent regulation of I_A by activity paradigms and intracellular signaling pathways (Schrader et al., 2006). The complex role of Kv4.2 in the pathophysiology of FXS is further complicated by the finding that currents derived from Kv4-containing channels are increased in a specific population of projection neurons in the prefrontal cortex of *Fmr1*$^{-/y}$ mice (Kalmbach et al., 2015). Conversely, no change in Kv4-dependent currents was observed in adjacent, but functionally distinct neurons from the same brain area. These findings raise compelling questions about the underlying cause of these changes, notably whether they arise through a mechanism that is unrelated to translation, but rather an adaptive consequence of differing patterns of circuit level activity throughout development (Kalmbach et al., 2015).

BK_{Ca} Channels

Recent evidence suggests that big conductance Ca^{2+} and voltage-activated K^+ channels (BK_{Ca}; also known as MaxiK, slo1, $K_{Ca}1.1$) play an important role in the pathophysiology of FXS. BK_{Ca} channels are expressed in the dendritic compartment of neurons, as well as their somata, axons, and axon terminals (Benhassine & Berger, 2005; Misonou et al., 2006; Sailer et al., 2006). Thus, any changes in the expression or biophysical properties of BK_{Ca} channels are likely to have multiple consequences for the neuron. In the axo-somatic compartment, BK_{Ca} channels contribute to the fast after-hyperpolarization during AP trains by repolarizing the membrane potential, thus regulating AP width and firing rate, and consequently neurotransmitter release (Faber & Sah, 2003; Salkoff, Butler, Ferreira, Santi, & Wei, 2006). In the dendrites, BK_{Ca} channels dampen excitability by reducing the efficacy of AP back-propagation and raising the threshold for dendritic calcium spikes (Benhassine & Berger, 2009). BK_{Ca} channels have previously been shown to play a role in neurological disorders, such as epilepsy in which a change in the excitability of neurons has been implicated (Shruti, Clem, & Barth, 2008).

The first evidence for changes in BK_{Ca} channel function in FXS derive from the finding that the expression of the pore-forming α-subunit was reduced in cortical extracts from *Fmr1*$^{-/y}$ mice (Liao, Park, Xu, Vanderklish, & Yates, 2008). These findings suggested a translation-dependent role for FMRP in the regulation of BK_{Ca} function. However, more recently it was shown that FMRP binds directly to the regulatory β4 accessory subunit of these channels (Deng et al., 2013; Myrick et al., 2015) in addition to the α- subunit (Myrick et al., 2015). Importantly, the interaction with the β4 accessory subunit alters the Ca^{2+} sensitivity and therefore gating properties of the channel (Deng et al., 2013; Deng & Klyachko, 2016). One of the consequences of this altered channel function was a widening of action potentials leading to an enhancement of neurotransmitter release at presynaptic sites in cortical pyramidal neurons (Fig. 16.2A–C) (Deng et al., 2013; Zhang et al., 2014). At the dendritic level, BK_{Ca} channel dysfunction resulted in exaggerated calcium influx accompanying backpropagating action potentials, a lower critical frequency for AP trains to trigger dendritic calcium spikes, and a

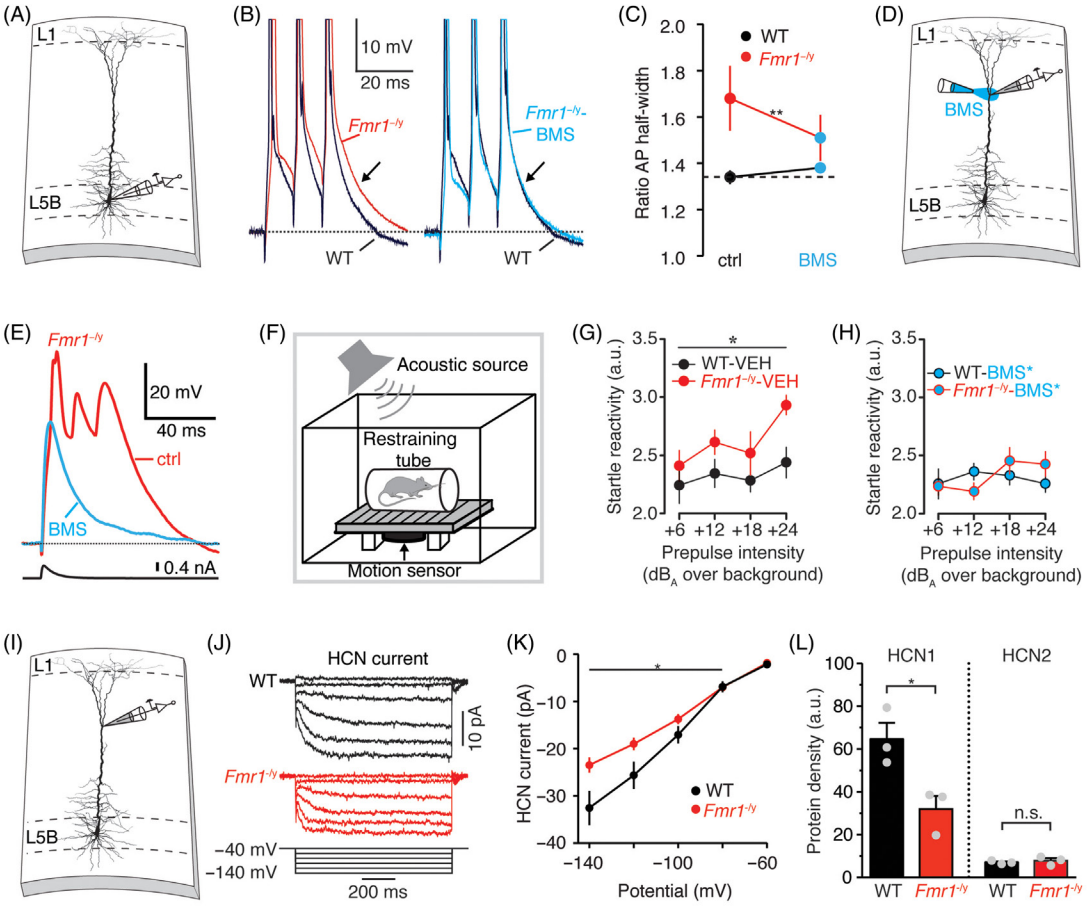

FIGURE 16.2 Dysfunction of BK_{Ca} and HCN channels in layer 5 pyramidal neurons of the somatosensory cortex of *Fmr1*$^{-/y}$ mice. (A–C) Somatic whole-cell recordings (A) demonstrate an increase in the half-width of action potentials (APs) during, and in the after-depolarization following an AP train (B) in *Fmr1*$^{-/y}$ neurons, which is rescued by the specific BK_{Ca} channels opener BMS-191011 (C, BMS). (D–E) Dendritic whole-cell recording (D) in an *Fmr1*$^{-/y}$ neuron demonstrates strong suppression of dendritic calcium spikes (evoked by current wave injections) following local puff-application of BMS-191011 (E, BMS). (F–H) Whole-body startle response to brief auditory stimuli (F) is increased in *Fmr1*$^{-/y}$ mice (G, vehicle) and rescued following the application (i.p.) of the BK_{Ca} channel opener BMS-204352 (H, BMS*). (I–L) Dendritic cell-attached voltage-clamp recordings of HCN currents (I–J) demonstrate a reduction in the peak amplitude in *Fmr1*$^{-/y}$ compared to wild-type neurons (J-K). (L) Western blots from *Fmr1*$^{-/y}$ and WT somatosensory cortex extracts for the membrane-bound fraction of HCN1 and HCN2 show a reduced relative amount of HCN1 (normalized to GAPDH) but not of HCN2 in *Fmr1*$^{-/y}$ mice. *Source: Figures modified from Zhang, Y., Bonnan, A., Bony, G., Ferezou, I., Pietropaolo, S., Ginger, M., Sans, N., Rossier, J., Oostra, B., LeMasson, G., Frick, A. (2014). Dendritic channelopathies contribute to neocortical and sensory hyperexcitability in Fmr1(-/y) mice.* Nature Neuroscience, 17, *1701–1709).*

reduced dendritic spike threshold in neocortical pyramidal neurons (Fig. 16.2D–E) (Zhang et al., 2014). Taken together, the consequences of these phenomena were an overall increase in neuronal excitability, likely explaining the circuit level hyperexcitability reported for the somatosensory cortex of *Fmr1*$^{-/y}$ mice (Goncalves, Anstey, Golshani, & Portera-Cailliau, 2013; Zhang et al., 2014). At the systems level, BK_{Ca}-mediated circuit hyperexcitability may explain a number of behavioral and neurological phenotypes associated with FXS (Deng et al., 2013; Zhang et al., 2014) [reviewed in (Contractor, Klyachko, & Portera-Cailliau, 2015)]. Indeed, administration of a pharmacological BK_{Ca} channel opener corrected a number of behavioral phenotypes in *Fmr1*$^{-/y}$ mice, including hypersensitivity to sensory stimuli (Fig. 16.2F–H) (Zhang et al., 2014), social defects, as well as anxiety, and impaired spatial memory (Hebert et al., 2014). Likewise, manipulating BK_{Ca} channel activity by genetic ablation of the β4 accessory subunit ameliorated a number synaptic and circuit level defects, such as AP broading, defects in synaptic transmission and epileptiform activity characteristic of *Fmr1*$^{-/y}$ neurons (Deng & Klyachko, 2016).

FMRP Directly Binds Slack $K_{Na}1.1$ Channels

There are two genes that encode channels that are directly activated by elevations of cytoplasmic Na^+ concentrations. These are $K_{Na}1.1$, commonly termed Slack (for *s*equence *l*ike *a* *c*alcium-activated K^+ channel, -also termed KCNT1 or Slo2.2) and $K_{Na}1.2$ (Slick- also termed KCNT1 or Slo2.1) (Kaczmarek, 2013). Messenger RNA for the Slack channel, but not for Slick, has been shown to bind FMRP (Darnell et al., 2011). The Slack protein has an unusually long cytoplasmic C-terminal domain and the distal C-terminus also interacts directly with the FMRP protein (Brown et al., 2010; Zhang et al., 2012). Demonstration of this direct interaction was first obtained using a yeast two hybrid assay under stringent conditions and was confirmed by coimmunoprecipitation experiments (Brown et al., 2010). Moreover, FMRP that is bound to Slack channels remains complexed to its target mRNAs, such as those encoding the proteins Map1b and Arc. In *Fmr1*$^{-/y}$ animals, however, no mRNAs can be coimmunoprecipitated with Slack channels (Brown et al., 2010).

The interaction between FMRP and Slack rapidly and reversibly stimulates channel activity (Brown et al., 2010; Zhang et al., 2012). These experiments used FMRP(1-298), a truncated form that contains the majority of known sites for protein–protein interactions of FMRP (Ramos et al., 2006). In neurons, intracellular injection of FMRP(1-298), rapidly increases the native Slack potassium current, which was identified using siRNA treatment, and produces narrowing of action potentials (Zhang et al., 2012). The stimulatory effect of FMRP is also evident in single channel recordings. Slack channels are large conductance channels (~180 pS), and undergo numerous openings to substates, in addition to openings to the fully-open 180 pS conductance state (Dryer, 1994; Bhattacharjee et al., 2003). In excised patches either from neurons or from oocytes expressing Slack channels, addition of FMRP(1-298) produced near-complete elimination of the substates, as well as a two- and three-fold increase in channel activity (Brown et al., 2010; Zhang et al., 2012). No effects of FMRP could, however, be observed for functional Slack channels that were missing the distal C-terminus that is required for interactions with FMRP (Brown et al., 2010).

Consistent with the direct stimulatory effect of FMRP on Slack channels, K_{Na} currents in neurons are significantly reduced in neurons of FMRP knockout mice. This has been

demonstrated using MNTB neurons in the auditory brainstem, in which K_{Na} channels account for a major component of the total K^+ current (Yang, Desai, & Kaczmarek, 2007). The K_{Na} component of current in these neurons is reduced to 50%–65% of that in controls with no change in levels of channel protein (Brown et al., 2010). The effects of reduction of K_{Na} current in neurons is expected to increase excitability and to reduce the temporal accuracy with which action potentials can lock to synaptic stimuli at high rates (Yang et al., 2007). In combination with the changes in levels of Kv3.1 channels in auditory brainstem neurons described earlier, the changes in K_{Na} currents are likely to contribute to the auditory hypersensitivity of FXS patients.

Slack channels are very widely expressed in the nervous system (Joiner et al., 1998; Bhattacharjee, Gan, & Kaczmarek, 2002; Brown et al., 2010; Nuwer, Picchione, & Bhattacharjee, 2010; Rizzi, Knaus, & Schwarzer, 2016). Moreover, a variety of human mutations have been found in *KCNT1*, the gene that encodes Slack channels, which produce functional channels that have altered levels of activity from the wild type channels. These mutations are associated with very severe intellectual disability and with early onset epilepsy (Barcia et al., 2012; Ishii et al., 2013; McTague et al., 2013; Kim & Kaczmarek, 2014; Martin et al., 2014; Vanderver et al., 2014; Rizzo et al., 2016). Thus it is highly likely that loss of FMRP/Slack channel interactions in FXS influences the same cellular pathways that are affected by the human Slack mutations, and that this contributes to the resultant developmental delay.

NONSELECTIVE CATION CHANNELS

HCN Channels

Hyperpolarization–activated and cyclic nucleotide-gated channels (HCN) are the molecular correlate of I_h (the current mediated by HCN channels). Primarily expressed in the somatodendritic compartment (at least in cortical neurons), these channels contribute to a range of neuronal and dendritic properties, such as the resting potential and resistance of the membrane, or their synaptic integration (Shah, 2014; Magee, 2016). In the dendrites, specifically, they act to limit temporal and spatial summation of synaptic inputs, and to decrease the efficacy of backpropagating action potentials (Williams & Stuart, 2000; Berger, Larkum, & Luscher, 2001; Chevaleyre & Castillo, 2002; Angelo, London, Christensen, & Hausser, 2007; Kole, Brauer, & Stuart, 2007; Tsay, Dudman, & Siegelbaum, 2007; Zemankovics, Kali, Paulsen, Freund, & Hajos, 2010). Their overall function is to reduce dendritic excitability. They also act as a resonator conductance (Narayanan & Johnston, 2007; Marcelin et al., 2012). In addition to their somatodendritic function, HCN channels are also present in the axons and axon terminals of certain neurons [reviewed in (Shah, 2014)], where they are implicated in the control of synaptic release (Aponte, Lien, Reisinger, & Jonas, 2006; Huang et al., 2011; Huang et al., 2012).

HCN channels are composed of four pore-forming "alpha"-like subunits encoded by the genes *HCN1*, *HCN2*, *HCN3*, and *HCN4*. Only HCN2 has been identified as an mRNA target of FMRP (Brown et al., 2001; Darnell et al., 2011). Both HCN1 and HCN2 are the predominant transcripts present in the rodent cortex (Lorincz, Notomi, Tamas, Shigemoto, & Nusser, 2002; Notomi & Shigemoto, 2004). These pore-forming subunits interact with

auxiliary subunits known as TPR-containing Rab8b interacting protein [TRIP8b; (Santoro, Wainger, & Siegelbaum, 2004)], which in part determines their localization and active properties [reviewed in (Biel, Wahl-Schott, Michalakis, & Zong, 2009; Lewis et al., 2009; Wahl-Schott & Biel, 2009)]. At the present time there is no evidence that FMRP regulates the expression or properties of TRIP8b. However, it has recently been shown that HCN1, HCN2, and HCN3 interact with the scaffolding protein Shank3 (Yi et al., 2016), whose mRNA is a validated target of FMRP (Darnell et al., 2011) and that this interaction alters channel function, as well as the intrinsic properties of neurons (Yi et al., 2016).

In *Fmr1*$^{-/y}$ mice, HCN1, but not HCN2, expression is upregulated in the CA1 region of the hippocampus (Brager et al., 2012). In contrast, HCN1 expression is downregulated at the protein level in the somatosensory cortex (Zhang et al., 2014). In both cases, these alterations at the protein level result in corresponding changes in the intrinsic properties of these neurons—specifically in their dendritic compartments. In the case of CA1 pyramidal neurons dendrites, an *increase* in HCN channels lowered their membrane resistance, increased the sag and rebound response and the resonance frequency, and reduced the temporal integration of synaptic potentials. Furthermore, a persistent increase in I_h following LTP induction was absent in these neurons (Brager et al., 2012). Consistent with a *decrease* in HCN channels in the dendrites of layer 5B neurons of the somatosensory cortex (Fig. 16.2I–L), the membrane properties were altered in an inverse manner (compared to CA1 pyramidal neurons) and synaptic summation was, as a consequence, enhanced. Additionally, the efficacy of action potential backpropagation was boosted (Zhang et al., 2014). These findings provide further support to the notion that FXS-related ion channel dysfunction is complex and that the pathophysiological changes may be brain area-, or even neuronal type specific (Zhang et al., 2014). Indeed Kalmbach et al. (2015) recently demonstrated a reduction in I_h, exclusively in one population of projection neurons in the dorsal medial prefrontal cortex of *Fmr1*$^{-/y}$ mice, but not in another population with different projection features. However, the underlying mechanism for these changes, and how they occur in a cell-identity specific manner remains to be elucidated. One regulating factor may be the level of activity, as it has previously been shown that both the distribution and functional properties HCN-containing channels may be altered by activity (Fan et al., 2005; Shin & Chetkovich, 2007). Thus, alterations in the network activity, operating at different time scales and in different brain regions, could lead to the pleiotropic changes in I_h function in a cell identity dependant manner. Since I_h is known to contribute to a range of physiolological processes, such as learning [e.g., (Nolan et al., 2003)] and the maintenance of oscillatory activity and its relation with cellular properties [e.g., (Narayanan & Johnston, 2007)], these alterations are likely to have complex consequences for individuals with FXS.

CALCIUM CHANNELS

L-Type Ca^{2+} Channels

L-type Ca^{2+} channels comprise a family of voltage-gated calcium channels distinguished by their high threshold for activation, large conductance, slow inactivation kinetics, sensitivity to the agonist dihydropyridine, and discrete somatodendritic distribution (Catterall, 2000;

Snutch, Sutton, & Zamponi, 2001; Vacher et al., 2008). Neuronal L-type Ca^{2+} channels exist as a macromolecular complex containing either the Cav1.2 or Cav1.3 pore-forming (α1)-subunit (encoded by the genes *cacna1c* and *cacna1d*, respectively), together with an α2- and a β-subunit, as well as several auxiliary subunits and regulatory molecules [reviewed in (Vacher et al., 2008)].

L-type Ca^{2+} channels may play a role in synaptic plasticity at synapses (Magee & Johnston, 1997; Yasuda, Sabatini, & Svoboda, 2003). In addition, they have been implicated in the regulation of neuronal gene expression [e.g., (Li, Tadross, & Tsien, 2016)]. Mutations in both *CACNA1C* and *CACNA1D* have been associated with a range of neuro-psychiatric disorders [reviewed in (Zamponi, Striessnig, Koschak, & Dolphin, 2015)]. Notably, mutations in *CACNA1C*, which lead to a form of Cav1.2 with altered inactivation properties, have been implicated in Timothy Syndrome, a rare syndromic form of autism spectrum disorder (Splawski et al., 2004).

The mRNA encoding Cav1.3 is a target for FMRP and Cav1.3 is downregulated in the frontal cortex and cerebellum of *Fmr1*$^{-/y}$ mice (Chen et al., 2003). In addition, defects in calcium signaling, as well as spike timing dependent plasticity [a form of dendritic plasticity involving L-type Ca^{2+} channels; (Bi & Poo, 1998)] were reported in layer 2/3 neurons from the prefontal cortices of *Fmr1*$^{-/y}$ mice (Meredith, Holmgren, Weidum, Burnashev, & Mansvelder, 2007). Pharmacological blockade of different Ca^{2+} channel types, suggested that these defects were related to reduced expression of L-type Ca^{2+} channels in the dendritic spines of *Fmr1*$^{-/y}$ neurons. Cav1.3 has been suggested to account for approximately 10%–20% of the L-type Ca^{2+} channel subunits in the brain (Hell et al., 1993; Sinnegger-Brauns et al., 2009). However, some evidence suggests that Cav1.3 containing channels open at more negative potentials than those containing Cav1.2 (Zhang et al., 2006). This notion may explain the observation that spike timing-dependent plasticity defects were rescued by stronger stimulation paradigms (Meredith et al., 2007).

N-Type Ca^{2+} Channels

The *$Ca_V2.2$* gene encodes N-type calcium channels, which are located primarily in nerve terminals in both the central and peripheral nervous systems. Ca^{2+} entry through these channels during action potentials are directly responsible for evoked transmitter release at many synapses (Turner, 1998). Messenger RNA for $Ca_V2.2$ is an FMRP target (Darnell et al., 2011). Like the BK_{Ca} and Slack K^+ channels, however, FMRP also forms a direct protein–protein complex with the $Ca_V2.2$ channel (Ferron et al., 2014). This interaction occurs between the C-terminus of FMRP and both the linker between the II and III domains and the cytoplasmic C-terminus of the channel. Both of these regions of the channel are known to couple the channel to presynaptic proteins that regulate neurotransmitter release.

Knockdown of FMRP produces a two-fold increase in Ca^{2+} current in neurons of the dorsal root ganglion, and enhances neurotransmitter release from these cells (Ferron et al., 2014). Conversely, coexpression of FMRP with $Ca_V2.2$ channels in a mammalian cell line suppresses current amplitude. The findings are most consistent with a model in which the direct interaction of FMRP with the $Ca_V2.2$ channel targets the channel complex for proteosomal degradation, and thus determines the fraction of channels that are inserted into the plasma membrane (Ferron et al., 2014).

CONCLUSIONS

Loss of FMRP, as occurs in FXS, has been found to alter both intrinsic excitability and synaptic transmission in many different brain regions. The nature of these changes is specific to different types of neurons in those areas and, to a large extent, depend on the particular mix of ion channels expressed by the neurons. For at least three types of channels, the Na^+-activated K^+ channel Slack ($K_{Na}1.1$), the large conductance Ca^{2+}-activated K^+ channel (BK, $K_{Ca}1.1$) and the N-type Ca^{2+} channel ($Ca_V2.2$), FMRP directly binds the channel complex, and deficits result from the loss of the protein–protein interaction between FMRP and the channel. For other channels, whose mRNAs bind FMRP but for which there is no known protein–protein interaction, loss of FMRP alters the rate of translation of the channels and/or impairs the ability of activity to stimulate changes in channel levels. These channels include Kv3.1 and Kv4.2 voltage-dependent K^+ channels, nonselective HCN1 channels and L-type $Ca_V1.3$ Ca^{2+} channels. Thus changes in excitability and firing patterns result from two distinct mechanisms: loss of protein-protein interactions between FMRP and ion channels and/or changes in ion channel levels due to altered mRNA translation. Both mechanisms contribute to the pathophysiology of FXS.

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CHAPTER

17

Reactivation of the *FMR1* Gene

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INTRODUCTION

The disease-causing mutation in practically all FXS cases is the expansion over 200 repeats of the CGG tract located in the 5′UTR of the *FMR1* gene (Verkerk et al., 1991). Its product, FMRP, is an RNA-binding protein important for regulating the translation of dendritic mRNAs in response to synaptic activation (Bagni & Greenough, 2005; Santos, Kanellopoulos, & Bagni, 2014). Based on the CGG size two different pathological *FMR1* alleles can be distinguished: premutation alleles (PM) with 55–200 repeats and full mutation alleles (FM) with over 200 CGGs. These two size classes are distinguished because they have different molecular and clinical consequences. Carriers of premutation alleles are at risk of an adult-onset neurodegenerative disease known as fragile X-associated Tremor/Ataxia Syndrome (FXTAS, OMIM #300623) (Hagerman & Hagerman, 2015). Female carriers are also at risk of fragile X-associated primary ovarian insufficiency (FXPOI, OMIM #300624), a condition that is associated with reduced fertility and early menopause (Allingham-Hawkins et al., 1999). In contrast, full mutation alleles are associated with the cytogenetic expression of a rare folate-sentsitive fragile site (FRAXA) and with the clinical condition known as fragile X syndrome (FXS, OMIM #300624), possibly the major monogenic cause of intellectual disability and autism (Pirozzi, Tabolacci, & Neri, 2011).

Premutation and full mutation alleles lead to different phenotypes, because the repeat expansion has different effects on *FMR1* gene expression. Premutation alleles associate with increased transcription of the gene (and slight reduction of FMRP) and have a gain-of-function pathogenetic mechanism; disease symptoms result from the deleterious consequences of high levels of mRNA containing the long CGG expansion (Tassone et al., 2000; Primerano et al., 2002). On the contrary, methylated full mutation alleles have a loss-of-function pathogenetic mechanism since extreme CGG expansion results in local heterochromatin formation and transcriptional silencing of the *FMR1* gene (Pieretti et al., 1991). Finally, a unique situation has been described in rare individuals of normal intelligence that carry an unmethylated full mutation (UFM), that is, although the CGG tract is expanded beyond 200 repeats, the *FMR1* promoter remains active and mRNA is overtranscribed like in premutation carriers (Smeets

Fragile X Syndrome. http://dx.doi.org/10.1016/B978-0-12-804461-2.00017-2

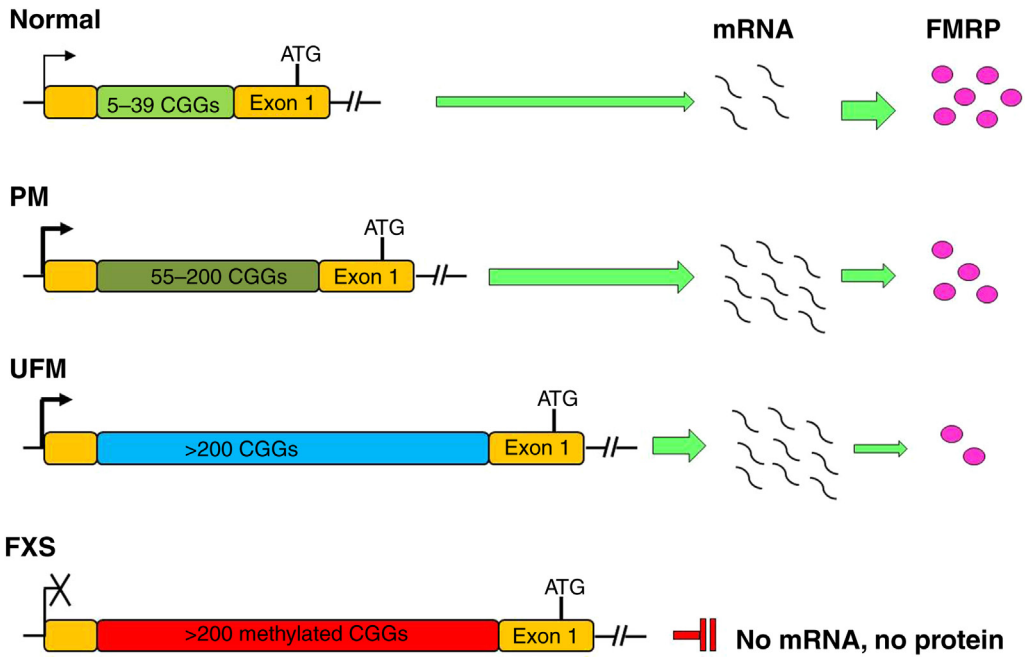

FIGURE 17.1 **The four classes of *FMR1* alleles: normal (5–39 CGG), premutation (PM, 55–200 CGG), unmethylated full mutation (UFM, >200 CGG without methylation), and full mutation (FM, >200 CGG with cytosine methylation)** . The *arrows* indicate the transcriptional start site. The polymorphic CGG repeat is in the untranslated part of exon 1.

et al., 1995; Pietrobono et al., 2005; Tabolacci et al., 2008b). An overview of the transcriptional activity at the *FMR1* locus in the different classes of alleles is given in Fig. 17.1.

In any case, FXS is basically caused by the absence of FMRP, either because of transcriptional silencing or, more rarely, because of gene deletion (Meijer et al., 1994; Collins et al., 2010; Luo et al., 2014) or point mutation (De Boulle et al., 1993; Lugenbeel, Peier, Carson, Chudley, & Nelson, 1995; Grønskov, Brøndum-Nielsen, Dedic, & Hjalgrim, 2011).

How the expanded CGG repeat can cause overexpression of the neighboring *FMR1* gene in the premutated alleles and silencing in the full mutated ones is only partly understood (Usdin & Kumari, 2015). However, it is important to remember that the same mechanisms are apparently shared by other folate-sensitive fragile sites (Sutherland & Richards, 1999; Debacker & Kooy, 2007). Infact, after FRAXA (Lubs, 1969) was reported and its molecular basis was elucidated (Oberlé et al., 1991; Verkerk et al., 1991; Kremer et al., 1991), a CGG expansion has been detected in nine other folate-sensitive fragile sites (FRA2A, FRA7A, FRA10A, FRA11A, FRA11B, FRA12A, FRA16A, FRAXE, and FRAXF). The expanded CGG alleles, as in FXS, become hypermethylated and five neighboring genes (*FMR2*/*AFF2*, *CBL2*, *DIP2B*, *AFF3*, *ZNF713*) undergo transcriptional silencing, just as *FMR1*. Interestingly, an unmethylated allele at the FRA12A locus correlates with increased transcription of the downstream *DIP2B*, as described in FXS premutation alleles (Winnepenninckx et al., 2007) and mitotically unstable (unmethylated) premutations have been reported in the *ZNF713* gene at the FRA7A locus (Metsu et al., 2014).

As just mentioned, the behavior of the CGG repeat in the *FMR1* promoter is not exceptional and is shared by a number of genomic loci characterized by folate-sensitive fragile sites and large CGG expansions that become methylated (Debacker & Kooy, 2007). Unlike other repeat-expansion disorders (RED) that are characterized by small expansions in coding sequences (with <100 repeats) and have a direct effect on the sequence (and structure) of a protein, large expanded repeats (>200 repeats) in noncoding regions eventually result in local chromatin changes that silence the neighboring gene(s) (Kumari & Usdin, 2009) and delay DNA replication so much that chromosome fragility results (Yudkin, Hayward, Aladjem, Kumari, & Usdin, 2014). The detailed mechanism and the timing of *FMR1* transcriptional silencing in fragile X patients are still not completely clear but our understanding has progressed over the years (Usdin et al., 2014; Usdin & Kumari, 2015). In this chapter we shall review the epigenetic status of *FMR1* premutations and full mutations and discuss the possibility of an epigenetic therapy for FXS.

EPIGENETIC STATUS OF PREMUTATED ALLELES

Before reaching the size of a full mutation (>200 CGG repeats), when above the normal range (5–55 CGGs), *FMR1* alleles are considered "premutations" that are still transcriptionally active. Actually, premutation alleles transcribe *FMR1* mRNA at higher than normal levels (Tassone et al., 2000), apparently because of increased transcription initiation (Tassone et al., 2007). Similar increases in other expanded CGG [e.g., in the DIP2B gene (Winnepenninckx et al., 2007)] have been reported, suggesting a common mechanism. In accordance to the increased transcription, histones H3 and H4 in the *FMR1* promoter of premutated alleles are 1.5–2 times more acetylated than normal alleles (Todd et al., 2010). Although the epigenetic status of premutated alleles is associated with a more open chromatin structure, the length of the CGG repeat allows the formation of hairpins on the 5'UTR of the mRNA and these structures probably account for the stalling of the 40S ribosomal subunit, which may explain the translation deficit in fragile X premutation alleles (Handa, Saha, & Usdin, 2003).

Furthermore, at the DNA level, premutations favor the use of additional promoters (Beilina, Tassone, Schwartz, Sahota, & Hagerman, 2004) and the *FMR1* promoter, such as other CpG rich promoters, may act as transcription-independent nucleation site for the zinc finger CxxC domain-containing chromatin modifying proteins, such as CFP1 (Thomson et al., 2010). CFP1 is a component of the SET1A/B-containing methyltransferase complex that induces H3K4 trimethylation, a histone mark typical of transcriptionally permissive chromatin (Clouaire et al., 2012). In this scenario, high transcription of premutated alleles would be related to the high density of CpGs in the repeat that recruits factors that inhibit gene silencing in a self-reinforcing loop which protects the gene from DNA methylation. A side effect of increased *FMR1* transcription is unfortunately FXTAS and FXPOI, two conditions affecting adult carriers of premutation alleles, apparently caused by the gain-of-function toxicity of *FMR1* mRNAs containing an expanded CGG repeat (Tassone et al., 2000; Tassone et al., 2007; Hagerman & Hagerman, 2015).

Finally, the role of R-loops formed by RNA:DNA hybrids in the CGG repeat itself by the *FMR1* mRNA itself and the nontemplate strand of the *FMR1* gene has been investigated

(Loomis, Sanz, Chédin, & Hagerman, 2014). Apparently, cotranscriptional formation of R-loops is characteristic of unmethylated CpG island promoters (Ginno, Lott, Christensen, Korf, & Chédin, 2012) and contributes to maintain these promoters active. In fact, R-loops protect from de novo methylation by DNMT3B1, the primary de novo DNA methyltransferase active during early development (Ginno et al., 2012) and the single stranded region of the R-loop is a preferential binding site for positive epigenetic regulators of transcription, including members of the H3K4 methyltransferase family and the AID cytosine deaminase (Usdin & Kumari, 2015).

EPIGENETIC SILENCING OF *FMR1* FULL MUTATION

DNA methylation was the first epigenetic modification to be associated with transcriptional silencing of *FMR1* full mutations (Oberlé et al., 1991; Sutcliffe et al., 1992) though it later became clear that it is not the first step in the molecular pathway leading to heterochromatin formation (Chiurazzi & Neri, 2003; Pietrobono et al., 2005). However, in FXS full mutation alleles, cytosine methylation affects the entire *FMR1* promoter which includes the CGG repeat in the 5′UTR of the gene (Sutcliffe et al., 1992). Naumann, Hochstein, Weber, Fanning, & Doerfler (2009) described a methylation boundary, located around 650–800 nucleotides 5′ of the CGG repeat, that separates an upstream region where CpGs are invariably methylated from a downstream region (including the entire *FMR1* promoter and CpG island) which is completely unmethylated in normal and premutation alleles, that are actively transcribed. This methylation boundary appears to be lost in full mutation alleles, which are entirely methylated throughout this region (Naumann et al., 2009). The boundary is also conserved in the mouse genome and contains binding sites for various nuclear proteins, including CTCF (CCCTC-binding factor), which is the only known insulator present in the region. This binding has been proposed to prevent methylation from spreading towards the *FMR1* promoter (Naumann et al., 2009; Lanni et al., 2013).

DNA methylation has a direct effect on the transcriptional output of the *FMR1* gene since it drastically reduces binding of the NRF1 transcription factor to the promoter (Kumari & Usdin, 2001). DNA methylation of the *FMR1* promoter was then associated with hypoacetylation of histones 3 and 4 (H3 and H4) and with hypomethylation of lysine 4 on histone 3 (H3K4). Deacetylation of lysine residues in histones 3 and 4 has long been known to be a downstream effect of DNA methylation, since the binding of methylated DNA by proteins, such as MeCP2 has been shown to recruit a histone deacetylase complex (Nan et al., 1998; Jones et al., 1998; Eden, Hashimshony, Keshet, Cedar, & Thorne, 1998). Histone H3 and H4 deacetylation is associated with chromatin compaction, preventing also the binding of the USF1/USF2 and Sp1 transcription factors to the promoter and completely silencing the *FMR1* gene (Kumari & Usdin, 2001; Kumari, Gabrielian, Wheeler, & Usdin, 2005). However, as shown in Fig. 17.2, DNA methylation is not the first event in the molecular cascade of gene silencing but is actually intertwined in a complex code of histone changes typical of facultative heterochromatin, such as dimethylation of lysine 9 on histone 3 (H3K9me2) and trimethylation of lysine 27 on histone 3 (H3K27me3), as well as histone modifications found in constitutive heterochromatin, such as trimethylation of lysine 20 on histone 4 (H4K20me3) and trimethylation of lysine 9 on histone 3 (H3K9me3) (Coffee, Zhang, Warren, & Reines, 1999; Tabolacci et al., 2005; Kumari & Usdin, 2010). These latter two modifications (H3K9me3 and H4K20me3) are found only in the vicinity of the repeat

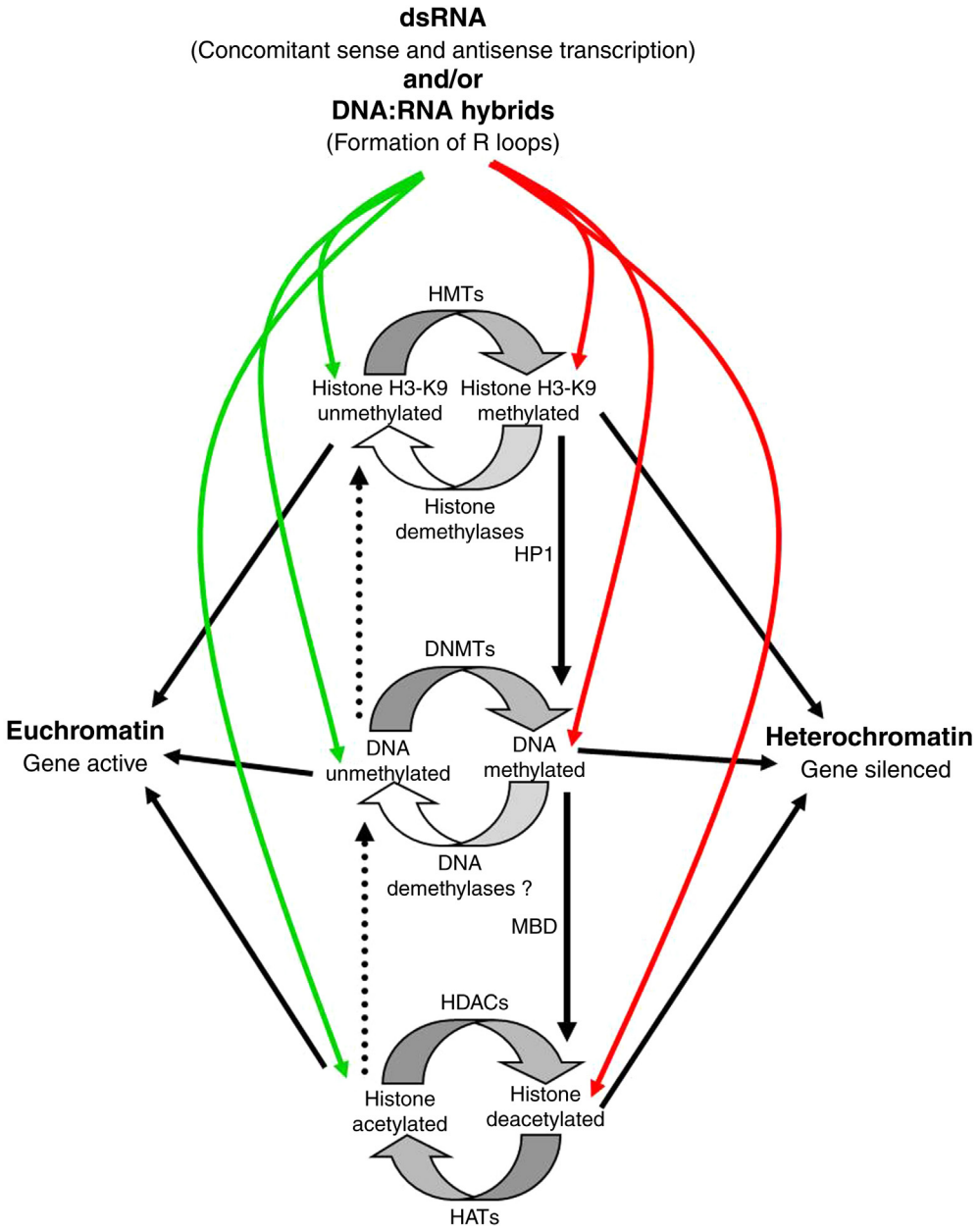

FIGURE 17.2 **Flowchart of epigenetic interactions.** Histone acetylation and methylation, and DNA methylation may be imposed on chromatin in a sequence-specific manner by double strand RNAs (dsRNAs) or by DNA:RNA hybrids (R loops formation). The resulting epigenotype is then read by chromatin-activating or chromatin-silencing complexes, which mediate local remodeling and formation of euchromatin or heterochromatin, respectively. Abbreviations: *DNMTs*, DNA methyltransferases; *HATs*, histone acetyltransferases; *HDACs*, histone deacetylases; *HMTs*,histone methyltransferases; *HP1*, heterochromatin protein 1; *MBD*, methyl-binding domain proteins.

and are likely to be imposed on chromatin before DNA methylation (Kumari & Usdin, 2010). H3K9 trimethylation has been shown to precede DNA methylation (Russo et al., 2013) and its effect is mediated by binding to the chromodomain of heterochromatin protein 1 (HP1) that, in turn, recruits DNA methyltransferases (Fuks, Hurd, Deplus, & Kouzarides, 2003) or even directly histone deacetylases (Honda et al., 2012). Eventually, all these epigenetic changes result in heterochromatin-mediated gene silencing, which is the ultimate cause of FXS.

A partial overview of the epigenetic changes observed at the *FMR1* locus is reported in Table 17.1. It is important to remember that epigenetic changes modulate, as well as reflect transcription and have been reported in several other RED: for example, H3K9 dimethylation (H3K9me2) has been reported in Myotonic dystrophy type 1 (Cho et al., 2005), H3K9 trimethylation (H3K9me3) has been described in Friedreich ataxia (Herman et al., 2006), and H3K9 trimethylation (H3K9me3), H3K27 trimethylation (H3K27me3), and H4K20 trimethylation (H4K20me3) were found in *C9orf72* expansions in ALS (Belzil et al., 2013).

All the epigenetic modifications listed earlier are limited to the *FMR1* promoter region of full mutation alleles and are apparently induced locally (in *cis*) by the primary genetic alteration, that is, the CGG repeat expansion. But how and when the expanded repeat induces the local heterochromatin formation (e.g., H3K9 methylation) is still debated.

Two main inactivation models can be envisaged: one is DNA-driven and the other RNA-driven (Usdin et al., 2014). In the first scenario, secondary structures formed by the DNA repeats [stem-loop/hairpins and G-tetraplexes (Patel, Bhavesh, & Hosur, 2000)] directly attract specific DNA-binding proteins that recruit, for example, histone H3 K9 methylase. One attractive candidate could be the CGG-binding protein CGGBP1 (Deissler, Behn-Krappa, & Doerfler, 1996) that binds only the unmethylated CGG repeats in a length-dependent fashion (Goracci et al., 2016). Although its absence does not appear to change *FMR1* transcription levels (Goracci et al., 2016), we cannot rule out that its increased binding to longer CGG repeat tracts may direct local H3K9 methylation.

However, the second scenario that proposes the involvement of locus-specific RNAs directing local heterochromatin formation is now considered more likely (Grewal, 2010; Usdin

TABLE 17.1 Major Epigenetic Modifications at the *FMR1* Promoter in Transcriptionally Active Alleles (Wild-Type and UFM) and Inactive Ones (FXS)

	Wild-type	**UFM**	**FXS**
Transcription	Normal	Increased	None
Protein levels	100%	20%–40%	0%
DNA methylation	Absent	Absent	Present
H3 and H4 acetylation	+	+	−
H3K4 methylation	+	+	−
H3K27 dimethylation	+	+	−
H3K27 trimethylation	−	−	+
H3K9 methylation	−	+/−	+
H4K20 trimethylation	−	?	+

Modifications typical for heterochromatin are indicated in bold.

et al., 2014): double-stranded RNA formed by hairpins of the *FMR1* mRNA (Handa et al., 2003) or duplex RNA generated by the *FMR1* mRNA and the antisense transcript *FMR1-AS1* (Ladd et al., 2007) could be cut by Dicer and trigger RNA interference (RNAi). However, knockdown of *Dicer*, *Ago1* and *Ago2*, which play a key role in RNAi, did not prevent *FMR1* gene silencing in neurons differentiated from FXS-hESC, ruling out the involvement of RNAi in silencing full mutation alleles (Colak et al., 2014). At the same time, *FMR1* mRNA has been proven capable of forming RNA:DNA hybrids (R-loops) during transcription when the CGG tract reaches at least the premutation size (Loomis et al., 2014): these structures are commonly formed at expanded repeat loci by the persistent pairing of the nascent mRNA with the DNA template strand, leaving the nontemplate DNA strand unpaired (Fig. 17.3). Furthermore, R-loop formation may be facilitated by hairpin formation on the nontemplate (CGG-containing) strand that would reduce the likelihood of reannealing of the two DNA strands (Usdin & Kumari, 2015). It has also been proven that even more stable R-loops are formed when UFM alleles are transcribed, as in human FXS embryonic stem cells (Colak et al., 2014) and after pharmacological *FMR1* reactivation in a FXS lymphoblastoid cell line (Groh, Lufino, Wade-Martins, & Gromak, 2014; Kumari & Usdin, 2016).

As convincingly discussed by Usdin and Kumari (2015), the different stability of the R-loop (transient in premutations and more prolonged in full mutations) and the probably different conformation of the unpaired CGG-containing sense strand (more linearized in premutations, probably rich in hairpins in full mutations) results in opposite results: the more stable R-loop with longer full mutation alleles blocks transcription initiation (and elongation) eventually silencing *FMR1* full mutations (Colak et al., 2014; Groh et al., 2014) while the unpaired nontemplate strand of premutations would actually recruit transcription activators, such as SET-containing H3K4 methyltransferases (Krajewski, Nakamura, Mazo, & Canaani, 2005) and the activation-induced cytidine deaminase (AID) (Chaudhuri et al., 2003). Therefore, repeat-induced R-loop formation would have opposing effects depending on its total length: premutation-sized alleles (Fig. 17.3**,** top panel) would result in more active local chromatin with increased *FMR1* transcription while longer full mutation alleles (Fig. 17.3, lower panel) would rather block transcription and effectively induce local heterochromatin formation (Usdin & Kumari, 2015). An important confirmation that R-loop formation is critical for silencing full mutation alleles was obtained by pharmacologically disrupting the interaction of the *FMR1*-mRNA with its template strand at the CGG-repeat and showing that this prevents promoter silencing in human ESCs (Colak et al., 2014). Kumari and Usdin (2016) recently also investigated the epigenetic changes induced by 5-azadC treatment in normal, methylated, as well as UFM, confirming the role of the R-loop reported by Loomis et al. (2014) and Colak et al. (2014) and showing the important role of EZH2, the polycomb repressive complex 2 (PRC2) component responsible for H3K27 trimethylation.

Therefore, as suggested in Fig. 17.2, the top player controlling chromatin conformation would be the specific RNA:DNA hybrid formed at the CGG repeat by the *FMR1* mRNA and the template CCG-containing DNA strand. The increased stability due to the long tract of perfect paring would block transcription, as well as recruit chromatin modifiers, such as the PRC1 (Yap et al., 2010), the PRC2, that has been found associated with the *Fmr1* transcript in mouse embryonic stem cells (Zhao et al., 2010), the LSD1-CoREST complexes responsible for H3K4me2 demethylation (Tsai et al., 2010), or the H3K9 methylase G9a (Pandey et al., 2008). Finally, we cannot rule out a role for the *FMR1-AS1* and the other noncoding transcripts of the

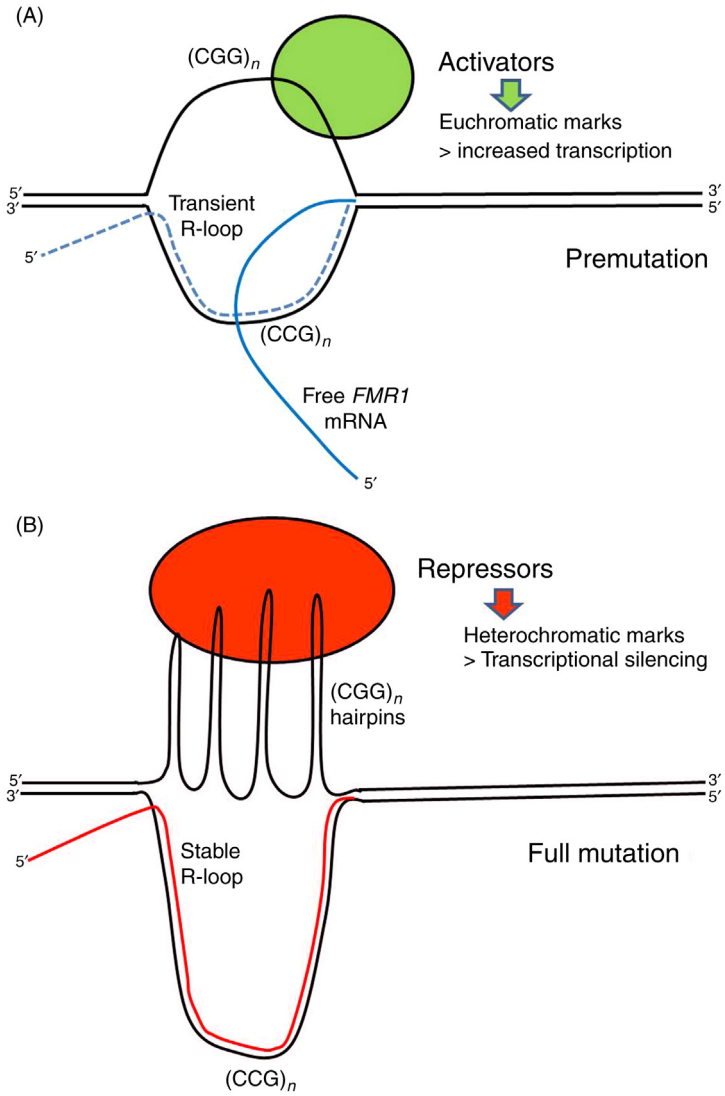

FIGURE 17.3 **RNA:DNA hybrids (R-loops) formed at the CGG repeat tract during transcription.** (A) When the length of the repeat reaches the premutation size (55–200 CGG), transient R-loops form that do not block transcription, but allow the nontemplate strand to remain single-stranded enough time to recruit transcriptional activators; (B): when the length of the repeat reaches the full mutation range (>200 CGG) R-loops become more stable and block transcription, furthermore the (longer) nontemplate strand forms double-stranded secondary structures that recruit silencing complexes that eventually compact local chromatin (heterochromatinization).

FMR1 locus, such as *FMR4* (Khalil, Faghihi, Modarresi, Brothers, & Wahlestedt, 2008), *FMR5*, and *FMR6* (Pastori et al., 2014), in regulating the chromatin status of premutation and/or full mutation alleles. Long noncoding RNAs are also involved in recruiting the H4K20 trimethylase Suv4-20h (Bierhoff et al., 2014).

When these structural and epigenetic events occur (in the germline, during embryonic development or both) is still unknown. Timing of the expansion is discussed elsewhere in this book and but it is important to remember that only full mutation alleles can be detected in oocytes (but in the unmethylated state) (Malter et al., 1997) and full mutation alleles are often unmethylated in human embryonic stem cells (hESC) before differentiation (Eiges et al., 2007; Avitzour et al., 2014). Upon differentiation FXS-hESC undergo transcriptional silencing and one of the first repressive epigenetic marks to be detected in the *FMR1* promoter is H3K9 dimethylation, clearly before the occurrence of DNA methylation (Eiges et al., 2007), underlining the fact that DNA methylation is a relatively late event in chromatin inactivation. Using immunohistochemistry to detect FMRP in chorionic villi samples of FXS male fetuses at different gestational ages showed the presence of FMRP until approximately 10 weeks but was undetectable at 12.5 weeks (Willemsen, Bontekoe, Severijnen, & Oostra, 2002), again suggesting that the epigenetic inactivation of *FMR1* full mutations is a relatively late event in development. Finally, inactive full mutation alleles do not usually revert to an active status in induced pluripotent stem (iPS) cells derived from FXS fibroblasts, which retain DNA methylation (Sheridan et al., 2011) and histone modifications typical of inactive heterochromatin, especially the H3K9 dimethylation (Urbach, Bar-Nur, Daley, & Benvenisty, 2010; de Esch et al., 2014).

RARE INDIVIDUALS WITH UNMETHYLATED FULL MUTATION

As explained earlier, silenced alleles have a heterochromatic, "compact" configuration, while transcribed alleles are characterized by a more "open," permissive, euchromatic status. Switching from active transcription to transcriptional silencing is a direct consequence of CGG expansion over 200 repeats and its consequent epigenetic modifications. However, rare individuals with apparently normal intelligence have been identified in FXS families that harbor UFM alleles (Smeets et al., 1995; Wang, Taylor, & Bridge, 1996; Pietrobono et al., 2005; Tabolacci et al., 2008b). These individuals carry CGG expansions of more than 200 repeats, completely devoid of cytosine methylation, and UFM cells express higher than normal levels of *FMR1* transcript in a similar fashion to premutation alleles (Pietrobono et al., 2005). The CpG island of the *FMR1* promoter is likewise unmethylated, while the histone marks are similar to those of a normal allele (histones 3 and 4 are acetylated, H3-K4 is methylated and H3-K27 is dimethylated) with the exception of H3-K9 is usually partially methylated (Pietrobono et al., 2005). For still unknown reasons, UFM carriers cannot complete the silencing process, thus maintaining the *FMR1* locus active. To further confirm this statement, the DNA methylation boundary in UFM cell lines is preserved like in transcriptionally active (normal and PM) alleles, while is lost in FXS (Naumann et al., 2009; Lanni et al., 2013). These rare UFM alleles could represent the "frozen" status of FXS cells before full mutations are silenced, during embryogenesis. It has been noted that methylated FM alleles are more stable than unmethylated ones (Wöhrle, Salat, Hameister, Vogel, & Steinbach, 2001), probably because the more "open" chromatin conformation facilitates further mitotic instability of the CGG repeat (Burman, Popovich, Jacky, & Turker, 1999). Recently, iPS cells have been derived from fibroblasts of a UFM individual and apparently became methylated after reprogramming (de Esch et al., 2014). The corresponding lymphoblastoid cell line was previously characterized by Pietrobono et al. (2005) and a euchromatic configuration of the *FMR1* locus had been found: it is possible that this "reversion"

from unmethylated to a methylated status was due to the technical procedure employed in the staminal induction protocol or the UFM "block" was somehow overcome.

In these rare cell lines the existence of R loops has not yet been explored. Anyhow, the mechanism proposed for FXS based on *FMR1*-mRNA inducing its silencing (Colak et al., 2014) does not explain the existence of UFM individuals, who preserve an open chromatin configuration of the *FMR1* locus and transcription in spite of the CGG expansion. It is reasonable to imagine that a block in the inactivation cascade took place (Pietrobono et al., 2005), sparing these individuals from having FXS. Binding sites for CTCF within the *FMR1* locus have been identified (Ladd et al., 2007) and a role for CTCF in controlling the chromatin conformation and gene transcription was investigated. Lanni et al. (2013) confirmed the presence of four CTCF binding sites in the upstream methylation boundary, promoter, exon 1 and intron 2 of *FMR1*, respectively. The latter site coincides with one of the transcriptional start site of *FMR1-AS1*, the long antisense transcript described by Ladd et al. (2007). For the first time it was shown that these four sites bind CTCF in UFM cells, both lymphoblasts and fibroblasts, with a binding level similar to that of normal control cells, while CTCF binding is absent in methylated full mutation alleles (Lanni et al., 2013). Notably, pharmacological demethylation with 5-aza-2-deoxycytidine (5-azadC) of FXS cells does not restore CTCF binding to the *FMR1* gene. CTCF depletion with siRNAs causes a reduction of both *FMR1* and *FMR1-AS1* transcription, which however does not appear to be caused by remethylation of the *FMR1* promoter, in both normal and UFM cell lines (Lanni et al., 2013). Therefore, CTCF appears to regulate *FMR1* and *FMR1-AS1* transcription in a coordinated fashion, possibly through the organization of chromatin loops. Finally, the antisense transcript *FMR1-AS1* in UFM cell lines was found to be expressed at higher levels compared to normal controls (Lanni et al., 2013), similar to what happens with the sense and antisense transcripts in premutation carriers (Ladd et al., 2007).

In order to identify structure-specific proteins that could recruit components of the silencing machinery, the role of the CGG binding protein 1 (CGGBP1) in *FMR1* gene was investigated (Goracci et al., 2016). CGGBP1 is a highly conserved protein that binds specifically to unmethylated CGG tracts. ChIP assays clearly demonstrated that CGGBP1 binds to unmethylated CGG triplets of the *FMR1* gene (even more strongly to premutated, as well as UFM alleles), but not to methylated CGGs. *CGGBP1* silencing with shRNAs did not affect *FMR1* transcription and CGG expansion stability in expanded alleles.

In summary, it is still not clear what prevents *FMR1* inactivation in these rare UFM individuals, who may eventually develop FXTAS because of the elevated *FMR1* mRNA levels with an expanded CGG repeat (Loesch et al., 2012) but are definitely spared from developing FXS.

TREATMENT OPTIONS FOR FXS

Fragile X syndrome is essentially caused by the absence of FMRP, the RNA-binding protein encoded by the *FMR1* gene (Bagni & Greenough, 2005; Santos et al., 2014). Since FMRP interacts with and regulates translation at synapses of multiple mRNA targets, 25 years after the identification of the gene, researchers are still unraveling the multiple pathways regulated by this protein and multiple targets for pharmacological treatments have been identified (Levenga, de Vrij, Oostra, & Willemsen, 2010; Bagni & Oostra, 2013; Schaefer, Davenport, & Erickson, 2015). On the other hand, considering that the silenced *FMR1* full mutation alleles have an intact open

reading frame (ORF), any epigenetic intervention leading to the reactivation of the endogenous *FMR1* gene in FXS patients could potentially normalize synaptic transmission and improve their mental functions (Chiurazzi & Neri, 2003; Tabolacci & Chiurazzi, 2013).

Thus, two different approaches could in principle be employed to treat FXS: (1) to normalize the defective functions due to the lack of FMRP, acting on the pathways in which it is involved; (2) to restore *FMR1* expression acting on the epigenetic mechanisms involved in the transcriptional inactivation. Both approaches were tested in vitro (mainly on FXS mouse brain slices and FXS patient cells) and in vivo (in animal models and in clinical trials). All clinical trials so far were based on evidence obtained on animal models, but it would be of utmost importance to test new therapies on human cellular models. For this reason, FXS-iPS cells were prepared by different groups, and neurons derived from FXS-iPS cells represent a useful cellular model to recapitulate the spine dysmorphogenesis of FXS (see also Chapter 6) (Eiges et al., 2007; Urbach et al., 2010; Sheridan et al., 2011; de Esch et al., 2014).

Promising results from cellular and animal studies have led to several clinical trials, all aimed at correcting the synaptic defect in FXS individuals [reviewed by Schaefer et al. (2015)]. Many of these stemmed from the discovery of excessive metabotropic glutamate receptor (mGluR) signaling at synapses lacking FMRP (Bear, Huber, & Warren, 2004), but other trials are addressing the role GABAergic receptors (Lozano, Hare, & Hagerman, 2014; Braat & Kooy, 2015), as well as other pathways dysregulated in FXS. Unfortunately, most of these clinical trials have been inconclusive until now, possibly because of compensatory mechanisms but most probably due to the pleiotropic functions of FMRP (Schaefer et al., 2015; Zeidler, Hukema, & Willemsen, 2015).

REACTIVATION OF THE *FMR1* GENE

The alternative therapeutic approach to cure FXS, that is, the possibility to revert the epigenetic marks and mechanisms that silence(d) *FMR1* full mutations, remains therefore an attractive possibility since it goes directly to the cause of the transcriptional silencing (Tabolacci & Chiurazzi, 2013). An important in vivo experiment performed by Guo et al. (2011) supporting the feasibility of this approach, showed that restoration of FMRP expression in adult neural stem cells rescues learning deficits in *Fmr1* knockout mice. These results are extremely important since they support the notion that mice who were congenitally deficient for Fmrp could be "rescued" by a postnatal restoration of the protein. More recently an elegant genetic rescue was performed by Park et al. (2015), who used CRISPR/Cas9 for a targeted deletion of the expanded CGG repeat in both embryonic stem cells and iPS cells derived from FXS patients and observed sustained *FMR1* expression in neural precursor cells and mature neurons, accompanied by reversal of all epigenetic changes. Finally, the living proof of the potential for in vivo reactivation of the *FMR1* full mutations is represented by those UFM individuals, who for some yet unknown reasons are unable to (completely) silence a fully expanded CGG tract.

As mentioned earlier, in FXS cells, DNA methylation represents the "dominant" epigenetic mark that switches off the expanded gene. Passive DNA demethylation can be obtained with 5-azacytidine (5-azaC) or, more efficiently, with 5-aza-2-deoxycytidine (5-azadC) that is incorporated into DNA as an analog of deoxycitidine during cell replication and irreversibly blocks DNA methyltransferases (Jackson-Grusby, Laird, Magge, Moeller, & Jaenisch, 1997).

In 1998 we first achieved in vitro reactivation of the *FMR1* full mutations by treating fragile X lymphoblastoid cells with 5-azadC (Chiurazzi, Pomponi, Willemsen, Oostra, & Neri, 1998),

detecting the presence of *FMR1* mRNA, as well as FMRP in a fraction of treated cells. The lower efficiency of mRNA translation, due to the CGG expansion (Feng et al., 1995), probably accounts for the observed discrepancy between mRNA and protein levels. One year later, Coffee et al. (1999) reported similar results and investigated also the acetylation status of histones after 5-azadC treatment. We then proved that also other loci silenced by expansion and methylation of a CGG repeat can be reactivated with 5-azadC, namely when we treated FRAXF lymphoblastoid cell lines and observed reexpression of the neighboring *FAM11A* gene (Shaw, Chiurazzi, Romain, Neri, & Gécz, 2002).

Subsequent studies investigating histone modifications by chromatin immunoprecipitation (ChIP) showed that treatment with 5-azadC of FXS cells induces both histone acetylation and increased methylation of H3K4, while only partly reducing H3K9 methylation (Coffee, Zhang, Ceman, Warren, & Reines, 2002; Tabolacci et al., 2005). These epigenetic changes appeared to restore a euchromatic configuration of the *FMR1* promoter, transforming a methylated into an UFM. Bar-Nur, Caspi, & Benvenisty (2012) treated also FXS-iPS cells and their derived neurons with 5-azaC and observed a significant *FMR1* reactivation after treatment. Taken together, all these studies point to the potential of epigenetic therapy in postmitotic neurons (Gavin, Chase, & Sharma, 2013).

An obvious concern that arises when considering the clinical use of 5-azadC is its toxicity. In fact, while 5-azaC and 5-azadC are generally well tolerated by patients affected with hematological malignancies (Gnyszka, Jastrzebski, & Flis, 2013), the effects of a long-term treatment are unknown. A second obstacle is the apparent requirement for cell division for 5-azadC to be effective. Interestingly, at least two reports suggest that 5-azadC may require minimal or no incorporation into DNA to effectively reduce the activity of the maintenance DNA methyltransferase DNMT1 (Ghoshal et al., 2005; Patel et al., 2010). Finally, a third objection to using drugs like 5-azadC is that their action may be unspecific and genome-wide, even though a microarray screening of 10,814 genes by Suzuki et al. (2002) showed that a very limited set of genes are actually transcriptionally upregulated by treatment with 5-azadC (51 genes) and/or trichostatin A (23 genes). In fact, a recent study suggests that 5-azadC may induce DNA demethylation only in selected genomic regions, including the *FMR1* gene promoter, leaving most others unaffected (Tabolacci et al., 2016), for example, those containing imprinted genes, such as the Prader–Willi region in chromosome 15q11 and the Beckwith–Wiedemann region in chromosome 11p13 whose epigenetic status was unchanged after 5-azadC treatment. Furthermore this study demonstrated that the demethylating effect of 5-azadC on the *FMR1* locus is not random, but rather restricted to the promoter region of *FMR1*, while the heterochromatic region upstream of the methylation boundary was not affected by treatment (Tabolacci et al., 2016).

Pomponi and Neri (1994) had shown that treatment with butyrate (a HDAC inhibitor) and acetylcarnitine (a donor of acetyl groups) could inhibit the cytogenetic expression of the FRAXA fragile site, suggesting that, as depicted in Fig. 17.2, treatments aimed at increasing histone acetylation could also reactivate the gene. However, although we observed that combined treatment with 5-azadC and various histone deacetylase (HDAC) inhibitors (butyrate, phenylbutyrate and trichostatin A) resulted in a synergistic effect on *FMR1* reactivation, HDAC inhibitors alone were unable to induce reactivation (Chiurazzi et al., 1999). Even long-term treatments (up to 3 months) of FXS cells with carnitine and acetylcarnitine (ALC) did not reactivate the *FMR1* full mutation and resulted in barely detectable promoter demethylation. All these results suggest that DNA methylation is dominant over histone hypoacetylation at

the *FMR1* locus (Pietrobono et al., 2002; Pascale et al., 2003), as also reported for other heavily methylated genes (Cameron, Bachman, Myöhänen, Herman, & Baylin, 1999). A better reactivating effect of a Class III HDAC inhibitor, splitomicin, was reported by Biacsi, Kumari, & Usdin (2008), however, it has been difficult to replicate their results. Valproic acid, which acts as histone deacetylases inhibitor but not as DNA demethylator, was shown to have a modest reactivating effect of mutant *FMR1* in vitro (Tabolacci et al., 2008a). However, in a preliminary safety clinical trial, 10 FXS subjects were treated with valproic acid for 6 months, showing a decrease in the hyperactivity phenotype (Torrioli et al., 2010). Similar findings had been previously obtained in a clinical trial with L-acetylcarnitine (ALC) (Torrioli et al., 2008), a natural compound that can efficiently increase histone acetylation, but is not sufficient to cause *FMR1* reactivation when used alone in vitro (Tabolacci et al., 2005). A schematic overview of the major epigenetic effects induced by some of the aforementioned compounds is given in Fig. 17.4.

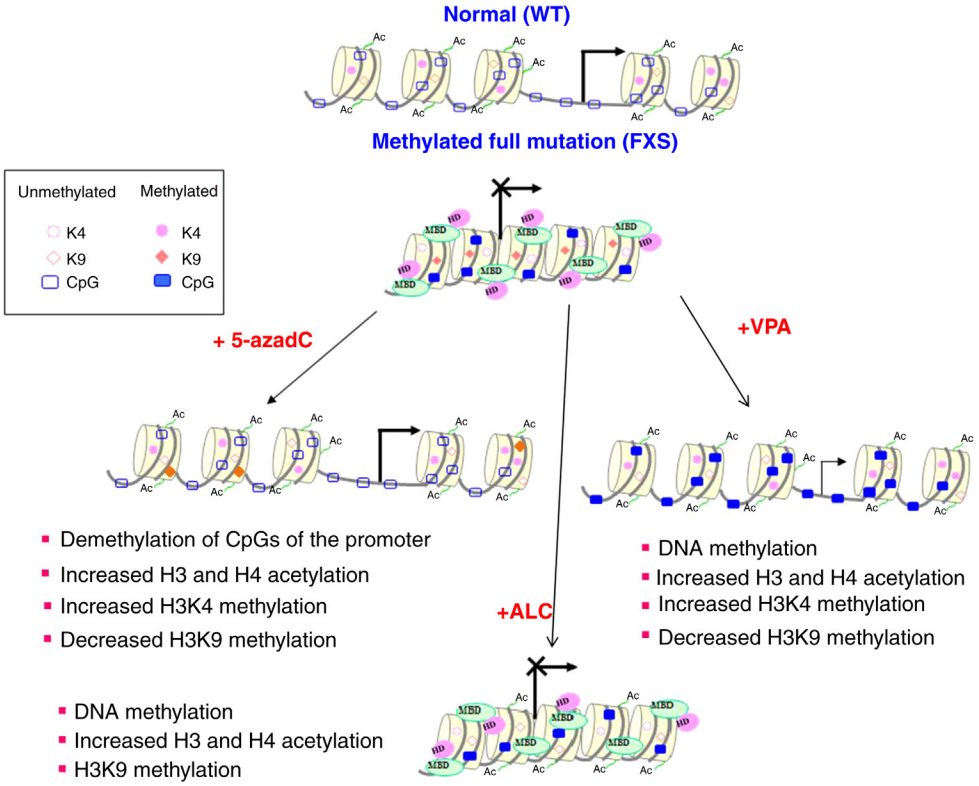

FIGURE 17.4 **Major epigenetic modifications at the *FMR1* locus after demethylating and hyperacetylating treatments.** In normal (WT) alleles a permissive euchromatic configuration is present (top), while in methylated full mutations (FXS) the heterochromatinic configuration does not allow transcription (center). The use of 5-azadC (bottom left) induces: DNA demethylation, increased H3 and H4 acetylation and H3K4 methylation and partial decrease of H3K9 methylation. ALC treatment (bottom center) produces an increase of H3 and H4 acetylation without DNA demethylation and transcriptional reactivation. Valproic acid (VPA) treatment (bottom right) induces a minimal transcriptional reactivation with hyperacetylation of H3 and H4 and increased H3K4 methylation, while H3K9 methylation remains unchanged. *Ac*, Acetyl groups bound to histones; *HD*, histone deacetylases; *MBD*, methyl-binding domain protein.

Nonetheless, there are good reasons for trying to identify other reactivating compounds with limited or no toxicity and the unspecific (re)activation of multiple genes might be minimized by optimizing dosages and combining drugs. Just as we did with HDAC inhibitors and 5-azadC for the *FMR1* gene (Chiurazzi et al., 1999) others are proposing new cancer therapies based on the synergic action of DNMT, HDAC, and HMT inhibitors (Zahnow et al., 2016).

FUTURE PERSPECTIVES

Among rare genetic conditions, FXS appears to be more suited than others for an effective pharmacological intervention. FXS is strictly monogenic, the phenotype does not normally include structural defects, practically all patients have the same genetic mutation (i.e., the expansion of the CGG repeat), the mutation does not affect the coding sequence of the gene but rather its reversible epigenetic status and the pathogenic mechanism is relatively well elucidated. However, as testified by several clinical trials, the effective correction of a genetic defect continues to be a tremendous challenge, requiring still wider basic knowledge of the disease pathophysiology. For example, considering the flowchart of events presented in Fig. 17.2, it is clear that any intervention aimed at reversing DNA methylation and histone deacetylation will not entirely remove H3K9 methylation or the eventual creation of R-loops after transcriptional reactivation, therefore will not last long after treatment discontinuation (Tabolacci et al., 2016). In fact, Kumari and Usdin (2014) documented the recruitment of Polycomb Group (PcG) repressive complexes on the *FMR1* promoter after gene reactivation with either 5-azadC or splitomicin, underlining the temporary nature of reactivating treatments tried until now. The same authors also studied the dynamics of resilencing of full mutations after 5-azadC reactivation, identifying the important role of EZH2-dependent H3K27 trimethylation for *FMR1* silencing and showing that EZH2 inhibitors can prolong the effect of 5-azadC, notwithstanding DNA remethylation (Kumari & Usdin, 2016). The use of drugs capable of destabilizing RNA:DNA hybrids (R-loops), such as compound 1a employed by Colak et al. (2014), if active in vivo, may effectively maintain *FMR1* transcription after reactivation of a full mutation allele. Kumari and Usdin (2016) actually proved that compound 1a can prolong the effect of 5-azadC treatment in vitro.

Finally, a new scenario was recently opened when Di Ruscio et al. (2013) discovered long noncoding RNAs (lncRNA) that bind to DNA methyltransferase 1 (DNMT1); these RNAs represent a new class of transcripts, that is, DNMT1-interacting RNAs. DNMT1 is considered the methyltransferase involved in the maintenance of cytosine methylation at every cell cycle. The *CEBPA* gene (implicated in hematological malignancies) was used as a model and DNA methylation levels of the locus were shown to be inversely correlated with the levels of a lncRNA of *CEBPA*. The interaction between this locus-specific lncRNA and DNMT1 prevents *CEBPA* methylation and results in robust *CEPBA* mRNA production. By RIP-sequencing Di Ruscio et al. (2013) demonstrated that such functional DNMT1-RNA association occurs at numerous gene loci, including *FMR1*. This could be a starting point to explore the mechanism of such interaction in FXS cells in view of a targeted-therapeutic approach for FXS.

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Further Reading

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CHAPTER

18

Drug Discovery for Targeted Pharmacotherapy of Fragile X Syndrome

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INTRODUCTION

Fragile X syndrome (FXS) is a monogenic condition that causes a range of developmental problems, including learning disabilities and cognitive impairment. It has a prevalence of approximately 1 in 7000 males and 1 in 11000 females (Chapter 4) (Hagerman & Hagerman, 2013). The X-linked inherited nature of the syndrome was established in 1943 (Martin & Bell, 1943), whereas the development of molecular cytogenetics techniques allowed the identification of a fragile site at the interphase X-chromosome from affected subjects (Howard-Peebles & Pryor, 1979) and the localization to the band Xq27.3 (Krawczun, Jenkins, & Brown, 1985). Despite these advances, the diagnostic was mainly based on the clinical symptoms and the family history with a large degree of inaccuracy. The discovery of the fragile X mental retardation 1 (*FMR1*) gene and resulting absence of its protein product, the fragile X mental retardation protein (FMRP) (Verkerk et al., 1991) were important discoveries that triggered the search for a therapeutic intervention based on a genetically defined pathology with the hope to modulate the course of the disease rather than only addressing the symptoms.

The identification of the mutation in the promoter region of the *FMR1* gene allowed the modeling of the pathology using transgenic mouse models as shown by the *Fmr1* knockout (KO) mice (The Dutch-Belgian Fragile X Consortium et al., 1994). The use of these models provided invaluable insights on the pathophysiology of the mutation and the role of FMRP at the biochemical, cellular, and behavioral level (Chapter 7).

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The well-defined genetic mutation in combination with the availability and characterization of a transgenic mouse model were determinant for the intense research conducted by numerous laboratories, which was strongly supported by a very dynamic fragile X community. The joined forces and the use of the mouse *Fmr1* KO model allowed the identification of several molecular pathways involved in the pathophysiology of FXS. Among the numerous identified targets, the metabotropic glutamate receptor 5 (mGlu5) and the type B gamma-amino butyric acid receptor ($GABA_B$) attracted the attention of pharmaceutical companies. Based on the findings demonstrating efficacy of mGlu5-negative allosteric modulators (NAM, subsequently also interchangeably called mGlu5 inhibitors) and $GABA_B$ receptor agonists in *Fmr1* KO mice, two different mGlu5 NAMs and one $GABA_B$ agonist were assessed clinically in FXS subjects. Unfortunately, these investigational drugs could not demonstrate therapeutic efficacy in FXS patients, despite appropriately powered and designed clinical trials.

The failure to translate the positive preclinical results into a clinical benefit raises a number of questions, such as the predictivity of the mouse transgenic model for a pharmacological intervention, the choice of the clinical outcome measures, the patient selection, and the clinical trial design.

MOLECULAR PATHOPHYSIOLOGY OF FRAGILE X SYNDROME

The genetic underpinning of FXS was discovered when variations in the length of the *FMR1* gene were associated with the human disease in 1991 (Verkerk et al., 1991). The *FMR1* gene is located on the X chromosome, contains a 4.4-kb coding region in 17 exons, and an unstable CGG region in its 5′ untranslated region (5′-UTR) that is prone to repeat expansion (Loesch & Hagerman, 2012). In the normal population about 5–39 repeats are commonly found (Fig. 18.1A), with considerable variation between individuals (Mailick et al., 2014). The genetic diagnostic criterion for FXS is a repeat expansion of ≥200 repeats, which is also referred to as full mutation (Jacquemont, Hagerman, Hagerman, & Leehey, 2007). The occurrence of ≥200 repeats causes a hypermethylation of the promoter region of the *FMR1* gene (Stoger, Kajimura, Brown, & Laird, 1997), leading to its transcriptional shutdown and the absence of FMRP protein (Moore, Le, & Fan, 2013; Sutcliffe et al., 1992) (Fig. 18.1B–C). It has been reported that the shutdown of the *FMR1* gene often is not complete in male and female subjects with ≥200 repeats (Tassone, Hagerman, Taylor, & Hagerman, 2001), and a correlation between repeat size and the degree of FMRP and symptom severity has been reported (Loesch, Huggins, & Hagerman, 2004; Loesch et al., 2007).

An intermediate repeat size of 55–200, also referred to as premutation, causes an up to several fold increase in *FMR1* transcript abundance with an only modest reduction of FMRP levels (Loesch & Hagerman, 2012) (Fig. 18.1D). The molecular pathophysiology and clinical phenotypes of FXTAS and FXPOI are subject of intense research, which have been addressed in excellent recent reviews (Hagerman & Hagerman, 2015; Hagerman, 2013; Hall et al., 2014) and thus will not be covered here.

Soon after the discovery of the link between the *FMR1* mutation and FXS, it was reported that its gene product, FMRP, functions as a RNA-binding protein and attenuator of protein synthesis for its target transcripts (Verheij et al., 1993). The resulting increased protein

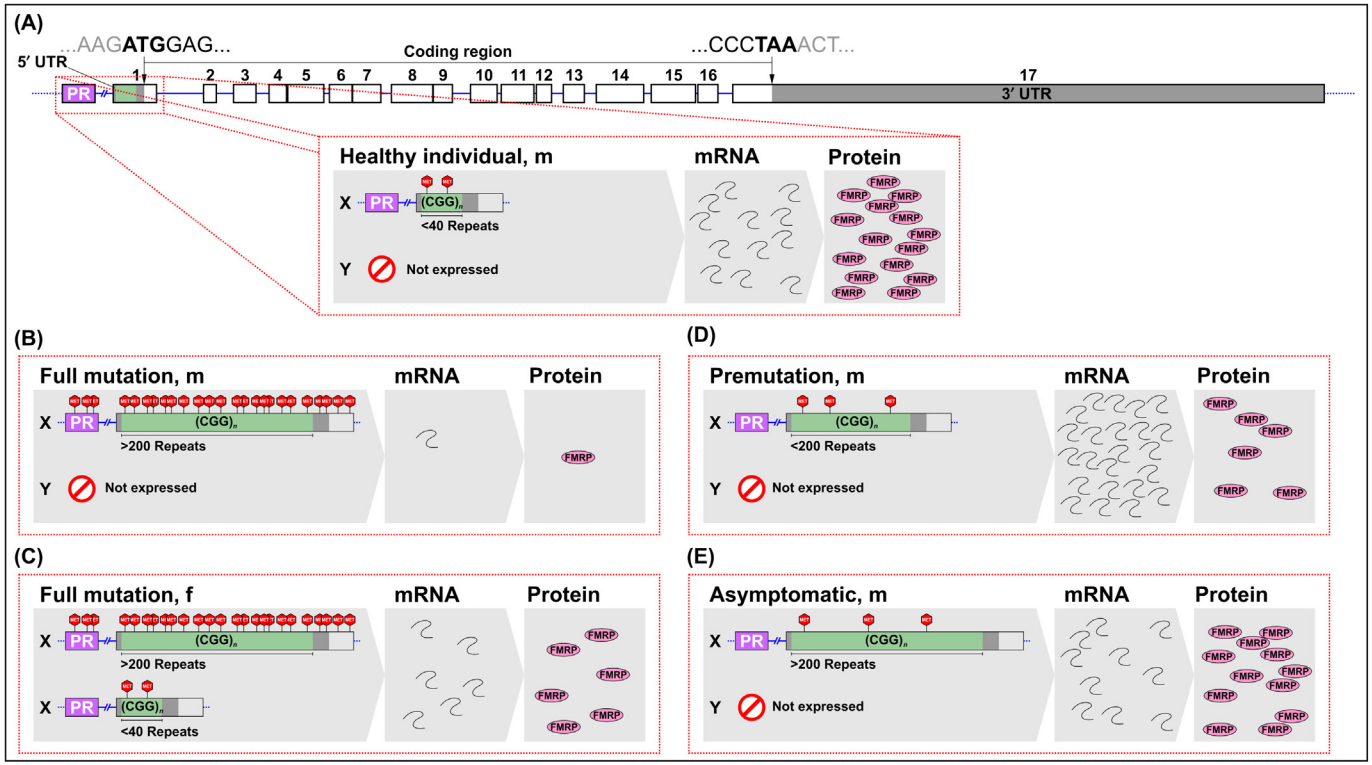

FIGURE 18.1 **Fragile X mental retardation 1 (*FMR1*) gene structure and example phenotypes of fragile X syndrome (FXS).** (A) The *FMR1* gene spans 17 exons with a coding region of about 4.4 kb. The 5′-UTR *(shown in dark gray)* contains a CGG-rich region of variable length *(shown in green)*. In healthy individuals, this region contains about 5–39 repeats accompanied by a low level of DNA methylation, resulting in normal mRNA and fragile X mental retardation protein (FMRP) protein expression. (B) In FXS males *(m)*, often more than 200 repeats are found in this CGG region together with hypermethylation of the region, which can spread to distal elements, such as the promoter region. The resulting inaccessibility of the gene to the transcriptional machinery leads to a severe reduction of mRNA and protein levels. (C) In female *(f)* carriers of the disease, the presence of a mostly nonexpanded *Fmr1* allele on the other X chromosome introduces a high amount of variability, as random inactivation of either X chromosome strongly influences the final expression of mRNA and protein in any given cell. (D) Premutation carriers, with repeat lengths of 55–200 repeats, show increased levels of *Fmr1* mRNA. However, probably due to cytotoxic mechanisms, these elevated levels of mRNA cause a reduction in FMRP protein levels. (E) In rare cases, expanded repeat regions stay unmethylated, resulting in almost normal expression of mRNA and protein levels. More details on the mechanisms at play for all these options are described in the main text. CGG_n, CGG-rich region of variable length; *PR*, promoter region; *5′/3′-UTR*, 5′/3′ untranslated region.

synthesis rate in FXS shown in *Fmr1* KO mice (Dölen et al., 2007; Michalon et al., 2012; Qin, Kang, Burlin, Jiang, & Smith, 2005) and FXS patients (Qin et al., 2013) is one of the key factors underlying the complex molecular and neuropsychiatric phenotypes in FXS. The variability of symptoms and the wide range of symptom severity in human are caused by several layers of genetic complexities, including variable CGG repeat length, variable degrees of DNA methylation, as well as somatic and X-chromosomal mosaicism (Fig. 18.1).

As discussed further below, one of the biggest challenges is that most of the information on the molecular pathophysiology of FXS has been generated in mice and in vitro systems, and by comparison, sparse information is available from human subjects for the same molecular readouts. Furthermore, in human almost all information about the *FMR1* mutation and FMRP expression levels is derived from peripheral cells, while there is very limited and partially conflicting information about repeat length, methylation status, mosaicism, and FMRP expression available from human brain tissue (Reyniers et al., 1999; Tassone, Hagerman, Gane, & Taylor, 1999; Dobkin et al., 1996; Taylor et al., 1999).

In the next chapter we will provide a succinct discussion of the FXS pathophysiology and disease-related phenotypes mainly based on work with *Fmr1* KO mice and how these data relate to findings in FXS patients.

FRAGILE X DISEASE MODELS

The availability of disease models for FXS with good construct and face validity is essential for research aiming to understand the molecular pathophysiology of the disease and for the testing of possible therapeutic interventions. The discovery of the link between the single gene mutation in the *FMR1* gene to the disease paved the way for the generation of the *Fmr1* KO mouse line (The Dutch-Belgian Fragile X Consortium et al., 1994), which rapidly developed into the most thoroughly characterized and most widely used FXS disease model.

In general, mammalian systems are preferred in drug discovery and the species of choice for genetically modified mammalian organisms was traditionally the mouse. This was based on multiple factors, including the high homology of the mouse genome to the human genome [~80%–90% amino acid sequence homology; 99% of genes have homologs (Waterston et al., 2002)], availability of inbred lines, and an extensive genetic toolbox, short generation times, simple husbandry, and well-developed protocols for testing behavior and neurophysiology. The advent of the iPS and CRISPR/CAS technologies broadened the spectrum of mammalian model systems with the addition of transgenic rats and patient-derived cell culture systems, which will be described in more detail later on.

Nonmammalian fragile X model systems, for example, *Drosophila* (Dockendorff et al., 2002) and zebrafish (den Broeder et al., 2009) have been described. These models have their strengths, such as easy access and easy manipulation of molecular pathways, but have disadvantages in other areas. Most notably, the behavioral repertoire, for example, in the areas of social behavior and cognition is much less complex in *Drosophila* and zebrafish compared to rodents. In addition, the sequence homology of the *FMR1* gene and some of the molecular targets investigated for pharmacological intervention have only modest sequence homology to the human orthologs [the *Drosophila* homolog *dfmr* has 35% overall sequence identity to human *FMR1* (Zhang et al., 2001)]. While significant work was done in *Drosophila* models

of FXS (McBride et al., 2005), the role of the signaling cascades relevant to pharmacotherapy (e.g., metabotropic glutamate signaling) is still not fully understood. On this background we decided to focus on mammalian disease models.

Fmr1 Knockout Mice

The by far most widely used disease model in FXS research is the *Fmr1* KO mouse. This mouse line, created by the Dutch-Belgian Fragile X Consortium (1994), is a "classical" KO in which the *Fmr1* gene is rendered nonfunctional by insertion of a neomycin cassette into exon 5 of the *Fmr1* gene. Over the years, a vast number of studies examined the phenotype of this model from many different angles: we have compiled the main phenotypes of the *Fmr1* KO mouse line with an emphasis on observations that were reported at least in two independent studies (Table 18.1). An important factor in this context is the fact that most of the phenotypes of *Fmr1* KO mice are very mild, that is, the amplitude for most phenotypes is very small (e.g., 10%–20% difference in dendritic spine density, protein synthesis rate, or biochemical measures, such as the ribosomal protein tyrosine kinase S6 (S6K) or ERK activity between WT and KO animals); only few phenotypes, such as audiogenic seizures or conditional avoidance

TABLE 18.1 Main Phenotypes in *Fmr1* Knockout (KO) Mice

Phenotype	Experimental evidence	Comments
Molecular phenotypes		
Elevated protein synthesis rate in brain tissue	• Increased rCPS in multiple brain regions (Qin et al., 2005, 2015) • Increased protein synthesis rate in ^{35}S-methionine pulse chase experiments (bath application) with acute hippocampal slices (Dölen et al., 2007; Guo et al., 2016; Osterweil, Krueger, Reinhold, & Bear, 2010; Osterweil et al., 2013) • Brain region–specific protein synthesis rate increase in ^{35}S-methionine in vivo labeling followed by autoradiography of brain sections (Michalon et al., 2012) • Increased puromycin incorporation rates in hippocampal slices using SUnSET (Bhattacharya et al., 2012) • Increased de novo protein synthesis in hippocampal slices using FUNCAT and cortical lysates using BONCAT (Bhattacharya et al., 2016)	—
Increased AMPA receptor internalization	• Primary rat hippocampal cultures showed decreased levels of surface GluR1 after siRNA knockdown of *Fmr1* (Nakamoto et al., 2007) • Decreased surface expression of GluR1 or GluR2 in hippocampal primary neurons from *Fmr1* KO mice (Gross et al., 2010; Henderson et al., 2012) • Decreased surface expression of GluR2 in hippocampal slices from *Fmr1* KO mice via biotinylation (Costa et al., 2012) • Decreased surface expression in LA slices via biotin labeling (Suvrathan, Hoeffer, Wong, Klann, & Chattarji, 2010) • Impaired pharmacologically induced internalization of AMPAR in cultured PFC neurons (Wang et al., 2008)	AMPA receptor internalization seems to be generally increased, regardless if LTD or LTP is affected

(Continued)

TABLE 18.1 Main Phenotypes in *Fmr1* Knockout (KO) Mice (*cont.*)

Phenotype	Experimental evidence	Comments
Elevated ERK/mTOR/PI3K activity	• Higher levels of p-mTOR in hippocampal lysates from KO mice (no change in normal mTOR levels) (western blot) (Guo et al., 2016; Liu, Huang, & Smith, 2012) • Increased levels of p-mTOR in hippocampal extracts from KO mice, but only in the synaptosome fraction (Qin et al., 2005) • Higher levels of p-ERK in hippocampal lysates from KO mice (western blot) (Guo et al., 2016; Dansie et al., 2013) • Increased pERK immunostaining in the subiculum of KO mice (Curia, Gualtieri, Bartolomeo, Vezzali, & Biagini, 2013) • Excess PI3K activity in cortical neurons (Gross et al., 2010) • Increased PI3K activity in cortical synaptic fractions (Gross et al., 2015b)	Results depend on the investigated region and the experimental setup. Studies with no changes have been described (Osterweil et al., 2010; Liu et al., 2012)
Spine phenotypes		
Increased dendritic spine density	• Increased number of Golgi-stained dendrites in hippocampal CA1 (Bhattacharya et al., 2012; Gross et al., 2015b; Busquets-Garcia et al., 2013) • Increased number of spines in cortical pyramidal neurons (Dölen et al., 2007)	Decreased number of dendritic spines and synapses via electron microscopy in hippocampal CA1 (Sun, Hongpaisan, & Alkon, 2016)
Immature dendritic spines	• More filopodial and less stubby or mushroom type spines in hippocampal CA1 (Bhattacharya et al., 2012, 2016; Busquets-Garcia et al., 2013; Sun et al., 2016; Sidhu, Dansie, Hickmott, Ethell, & Ethell, 2014; Westmark et al., 2011; Oddi et al., 2015)	—
Synaptic plasticity phenotypes		
Exaggerated hippocampal LTD	• Exaggerated DHPG-induced LTD in hippocampal slices (Michalon et al., 2012; Guo et al., 2016; Osterweil et al., 2013; Bhattacharya et al., 2012; Sidhu et al., 2014; Westmark et al., 2011; Costa, Sardone, Lacivita, Leopoldo, & Ciranna, 2015; Park et al., 2008)	—
Impaired amygdala LTP	• Impaired HFS-induced LTP in slices from the LA (Suvrathan et al., 2010; Zhao et al., 2005) • Decreased mEPSC amplitude and frequency (Suvrathan et al., 2010)	—
Impaired cortical LTP	• Impaired LTP in slices from visual cortex following tetanic stimulation (Wilson & Cox, 2007) • Increased trains of action potentials in visual cortex L5 after white matter stimulation (Osterweil et al., 2013) • Impaired tetanic-induced LTP in slices in the cortical A1 region (Yang, Park, Kirkwood, & Bao, 2014)	—
Prolonged UP states	• Increased duration of UP states in neocortical slices (Guo et al., 2016; Gross et al., 2015b; Guo, Ceolin, Collins, Perroy, & Huber, 2015; Gibson, Bartley, Hays, & Huber, 2008; Hays, Huber, & Gibson, 2011) • Delayed development of UP states in organotypic cortical slices (Motanis & Buonomano, 2015)	—

TABLE 18.1 Main Phenotypes in *Fmr1* Knockout (KO) Mice (*cont.*)

Phenotype	Experimental evidence	Comments
Altered ocular dominance plasticity	• Impaired visual evoked potentials in V1 following monocular deprivation (Dölen et al., 2007)	—
Altered frequency mapping of auditory stimuli	• Decreased representation of 16 kHz tone in A1 (Kim, Gibboni, Kirkhart, & Bao, 2013) • Impaired steady-state habituation to sounds measured with ERP (Lovelace et al., 2016) • Broader tuning, stronger response, and more variability in response to auditory stimulus in KO neurons in the A1 (Rotschafer & Razak, 2013) • Higher hearing threshold in KO mice (Rotschafer, Marshak, & Cramer, 2015)	—
Behavioral phenotypes		
Increased susceptibility to audiogenic seizures	• Increased incidence of audiogenic seizures (Osterweil et al., 2010, 2013; Dansie et al., 2013; Curia et al., 2013; Gross et al., 2015b; Busquets-Garcia et al., 2013; Westmark et al., 2011; Ding, Sethna, & Wang, 2014; Zhao, Wang, Song, Li, & Yuan, 2015; Goebel-Goody et al., 2012; Heulens, D'Hulst, Van Dam, De Deyn, & Kooy, 2012; Chen & Toth, 2001; Thomas et al., 2011)	—
Enhanced PPI	• Increased percent PPI in the range of 68–120 dB (Ding et al., 2014; Veeraragavan et al., 2012; Chen & Toth, 2001; Thomas et al., 2011; Frankland et al., 2004; Olmos-Serrano, Corbin, & Burns, 2011)	PPI seems to be influenced by the genetic background of the mice and is dB dependent (Nielsen, Derber, McClellan, & Crnic, 2002)
Reduced nonsocial anxiety	• Increased time spent in open arms in the EPM (Guo et al., 2016; Dansie et al., 2013; Heulens et al., 2012) • Less time spent in closed arms in the EPM (Goebel-Goody et al., 2012; Heulens et al., 2012; Liu, Chuang, & Smith, 2011) • Increased time spent in the center of the OF (Dansie et al., 2013; Westmark et al., 2011) • Increased locomotion in the center of the OF (Thomas et al., 2011) • Increased time spent in the illuminated compartment in the dark/light box (Goebel-Goody et al., 2012)	Results are quite variable and not always consistent (Veeraragavan et al., 2012; Thomas et al., 2011)
Hyperactivity	• Increased locomotion in the OF (Dansie et al., 2013; Oddi et al., 2015; Ding et al., 2014; Thomas et al., 2011; Liu et al., 2011; Uutela et al., 2012; Pacey et al., 2011) • Increased locomotion in the EPM (Heulens et al., 2012)	—
Deficits in learning and memory	• Deficits in water maze performance (Sun et al., 2016) • Impaired place learning in the MWM (Uutela et al., 2012; Van Dam et al., 2000) • Impaired novel object recognition (Pacey et al., 2011; Franklin et al., 2014; King & Jope, 2013; Pietropaolo et al., 2014) • Deficits in spontaneous alternation in the T-maze or Y-maze (Oddi et al., 2015; Pietropaolo et al., 2014) • Reduced context-dependent freezing in the fear conditioning paradigm (Oddi et al., 2015) • Impaired conditioned place preference (Pacey et al., 2011)	Results are quite variable and several studies found no differences in learning and memory (Veeraragavan et al., 2012; Thomas et al., 2011; Uutela et al., 2012; Van Dam et al., 2000; Leach, Hayes, Pride, Silverman, & Crawley, 2016)

(*Continued*)

TABLE 18.1 Main Phenotypes in Fmr1 Knockout (KO) Mice (*cont.*)

Phenotype	Experimental evidence	Comments
Perservative behavior	• Increased numbers of marbles buried (Dansie et al., 2013; Gross et al., 2015b; Westmark et al., 2011; Veeraragavan et al., 2012)	Results are quite variable and some studies found no difference (Thomas et al., 2011)
Reduced social behaviors	• Reduced social approach in the three-chambers test (Goebel-Goody et al., 2012; Liu et al., 2011) • Reduced interest in social novelty in the three-chambers test (Liu et al., 2011; Pietropaolo et al., 2014) • Reduced wins in the social dominance test (Goebel-Goody et al., 2012; Pacey et al., 2011) • Reduced contact time in the direct social interaction test (Oddi et al., 2015)	Results are quite variable and not always consistent (Thomas et al., 2011; Pietropaolo et al., 2014)
Other		
Increased pain threshold	• Prolonged time to response in hot plate test (Veeraragavan et al., 2012)	Some studies describe no change in pain threshold (Thomas et al., 2011; Uutela et al., 2012)
Macroorchidism	• Increased testicular weight (Dölen et al., 2007; Michalon et al., 2012; Bhattacharya et al., 2012, 2016; Veeraragavan et al., 2012; Thomas et al., 2011; Liu et al., 2011; Pacey et al., 2011)	—
Accelerated body growth	• Increased body weight at different ages (Bhattacharya et al., 2012, 2016; Liu et al., 2011; Pacey et al., 2011)	No change or decrease in body weight or body weight trajectory (Michalon et al., 2012; Oddi et al., 2015; Uutela et al., 2012; Pietropaolo et al., 2014)

This table focuses on phenotypes commonly accepted by the community that were confirmed by multiple groups and with different techniques. It shows selected publications only. BONCAT, Bioorthogonal noncanonical amino acid tagging; EPM, elevated plus maze; ERP, event-related potential; FUNCAT, fluorescent noncanonical amino acid tagging; HFS, high-frequency stimulation; KO, knockout; LA, lateral amygdala; LTD, long-term depression; LTP, long-term potentiation; mEPSC, miniature excitatory postsynaptic current; MWM, Morris water maze; OF, open field; PFC, prefrontal cortex; PPI, prepulse inhibition; rCPS, rates of cerebral protein synthesis; SUnSET, surface sensing of translation.

extinction, have larger amplitudes. The small amplitude of most phenotypes makes it difficult to detect and quantify partial phenotype reversals, and to express the magnitude of the effect for interventions as effect size (effect size = Δ/SD), as it is typically done for clinical studies.

In the last decade, two flavors of conditional *Fmr1* KO mice have been reported. The first type of conditional *Fmr1* KO mice is the well-established approach of a functional *Fmr1* allele, which is rendered inactive when combined with a Cre allele (Mientjes et al., 2006). The second type of conditional *Fmr1* KO mice doesn't express FMRP in all cells of the animal until a trigger (Cre recombinase or tetracycline induction) restores FMRP expression in a time- and/or cell type–specific manner (Guo et al., 2011).

One important difference between *Fmr1* KO mice and FXS patients is the genetics underlying the loss of FMRP. In FXS patients, the loss of FMRP is caused in the majority of the patients by the previously described CGG-trinucleotide expansion, leading to hypermethylation and transcriptional shutdown of the *FMR1* gene. This mechanism leads to certain aspects of FMRP dysregulation, such as residual expression of *FMR1* mRNA and FMRP protein, variability due to different forms of mosaicisms, and potential "off-target" effects of the hypermethylation, which are not present in *Fmr1* KO mice. The KO mice have a complete loss of *Fmr1* mRNA and FMRP protein in all cells and lack the hypermethylation seen in patients. These differences might explain that, contrary to the fairly prominent symptoms in patients, the *Fmr1* KO mice show a generally very mild phenotype, which is profoundly modulated by genetic background or enriched environment housing conditions (Spencer et al., 2011; Restivo et al., 2005).

Fmr1 CGG-Repeat Knockin Mice

In an effort to model the human repeat length expansion more closely, different mouse models with a CGG repeat knockin were generated, such as the knockin of a cloned human premutation allele (98 repeats) and a serial ligation of stable CGG-CCG repeats that reached about 120 repeats (Entezam et al., 2007; Bontekoe et al., 2001). Even though repeat length expansions after germ line transmission up to about 230 repeats has been reported, no DNA methylation of the *Fmr1* gene was detected (Entezam et al., 2007). Of note, the increased CGG repeat number caused a moderate reduction of FMRP expression in spite of the increased abundance of transcripts, indicating that the presence of expanded repeats in the *Fmr1* mRNA, leads to reduced translational efficacy (Brouwer et al., 2008). The repeat length of these animals is mostly in the premutation range, and these mice are being used mainly for research on FXTAS and POI, respectively.

New Disease Models Under Development

Recently, the first studies of *Fmr1* KO rats have been published. While many of the molecular or physiological phenotypes, such as increased spine density, exaggerated long-term depression (LTD), increased protein synthesis rate, and macroorchidism were similar to what is found in mice, important differences have been described in their behavior, for example almost no deficits in learning and memory, but some impairments in social behavior (Till et al., 2015; Hamilton et al., 2014). The *Fmr1* KO rats might offer a marked improvement over *Fmr1* KO mice for investigating aspects of social behavior, as rats show a much more complex range of social behaviors (Blake & McCoy, 2015).

Another strategy is the use of conditional or inducible KO and restoration lines mentioned previously to investigate the role of FMRP in specific sets of cell types, circuits, or specific time windows of development.

A very recent development in the area of transgenic in vivo models is the use of transgenic nonhuman primates. The upcoming research program by the Japanese government to generate a catalog of KO marmorset lines might offer insights especially regarding social behavior, brain circuitry, and pharmacology for neurodevelopmental disorders, which could be much more translational to patients compared to the rodent disease models (Sasaki, 2015). Just recently, the first cynomolgus KO model for another neurodevelopmental disorder (MeCP2 KO

for Rett syndrome) was reported (Liu et al., 2016), showcasing the potential of these models. It is still early days for genetically modified nonhuman primates, and the field will learn how the results obtained with these models translate to humans, and how the ethical considerations around their use will evolve.

TARGETED INTERVENTIONS THAT HAVE BEEN TESTED PRECLINICALLY IN FXS

In the current paragraph we discuss several interventions that have been experimentally tested in FXS. We have chosen to divide the interventions into two sections based on whether conclusive results from double-blind, placebo-controlled human trials have been reported. We are aware that this distinction in some cases is a judgment call: for interventions, such as lithium and lovastatin, significant efforts in patients have been reported, which however were not included into the group of clinically tested interventions in view of factors, such as open-label designs or small sample size. Regardless of this distinction, clinical trial results are being included where available. A summary of the described interventions can be found in Table 18.2.

Interventions Tested in Preclinical Disease Models and in Conclusive Double-Blind Placebo-Controlled Clinical Trials in FXS Patients

mGlu5: Genetic and Pharmacological Intervention

After the discovery of the *FMR1* locus harboring the pathogenic mutation underlying FXS, several observations were made early on, which linked mGlu5 to the disease (Chapter 9). These early observations included the following findings: (1) stimulation of group 1 mGluRs with DHPG leads to an increased protein synthesis rate in hippocampal tissue of proteins, including FMRP (Weiler et al., 1997); (2) FMRP functions as a RNA-binding protein and attenuator of protein biosynthesis (Ashley, Wilkinson, Reines, & Warren, 1993; Li et al., 2001); and (3) the mGlu receptor–dependent form of hippocampal LTD is enhanced in *Fmr1* KO mice, which suggests that FMRP negatively regulates the synthesis of proteins required for LTD (Huber, Gallagher, Warren, & Bear, 2002). These and other data suggested that inhibition of the mGlu5 receptor could have therapeutic benefits in FXS, which was formulated by Bear, Huber, and Warren (2004) in what is now known as the "mGlu receptor hypothesis of FXS."

The reduction of mGlu5 receptor activity in FXS was probed in a vast number of studies in *Fmr1* KO mice using either genetic or pharmacological tools. One landmark study genetically reduced mGlu5 expression levels in *Fmr1* KO mice by 50%, which prevented the onset of a wide range of phenotypes, including increased spine density, elevated protein synthesis rate, and other biochemical alterations, exaggerated hippocampal LTD, as well as several behavioral abnormalities (Dölen et al., 2007). Another landmark study reported that in mice, chronic pharmacological inhibition of the mGlu5 receptor starting in early adulthood, that is, after the onset of FXS phenotypes, corrected a similar range of phenotypes that were previously prevented with the genetic reduction of mGlu5 expression levels (Michalon et al., 2012). This study demonstrated that mGlu5 inhibition can achieve a broad phenotype correction in mammals even when intervention starts after phenotype onset in young adulthood, and that FXS in mammals does not represent an irreversible product of altered brain development. A large number of studies employing a range of different mGlu5 inhibitors showed that most known

TABLE 18.2 Nonexhaustive List of Targeted Interventions Tested in *Fmr1* KO Mice

	mGlu5 inhibition	mGlu5 (genetic)	$GABA_B$ activation	HMG CoA reduct. (lovastatin)	Lithium	STEP (genetic)	MMP9 (minocycline)	S6K inhibition	S6K (genetic)	CB1 (rimonabant)	PAK inhibition	Ampakines
Molecular												
Increased protein synthesis	■	■	■	■	■	■	■	■	■	■	■	■
Increased ERK/mTOR/PI3K activity	■	■	■	■	■	■	■	■	■	■	■	■
Synapse												
Altered synapse architecture	■	■	■	■	■	■	■	■	■	■	■	■
Altered synaptic plasticity	■	■	■	■	■	■	■	■	■	■	■	■
Behavior												
Increased seizure incidence	■	■	■	■	■	■	■	■	■	■	■	■
Impaired sensorimotor gating	■	■	■	■	■	■	■	■	■	■	■	■
Hyperactivity	■	■	■	■	■	■	■	■	■	■	■	■
Impaired memory and cognition	■	■	■	■	■	■	■	■	■	■	■	■
Impaired social interactions	■	■	■	■	■	■	■	■	■	■	■	■

(Continued)

TABLE 18.2 Nonexhaustive List of Targeted Interventions Tested in *Fmr1* KO Mice (*cont.*)

	mGlu5 inhibition	mGlu5 (genetic)	$GABA_B$ activation	HMG CoA reduct. (lovastatin)	Lithium	STEP (genetic)	MMP9 (minocycline)	S6K inhibition	S6K (genetic)	CB1 (rimonabant)	PAK inhibition	Ampakines
Physiology												
Macroorchidism												
Elevated body growth												
Clinical research?[a]	✓[b]	✘	✓[c]	(✓)	(✓)	✘	✓	✘	✘	✘	✘	✓

CB1, Cannabinoid receptor 1; MMP, matrix metalloproteinase-9; PAK, p21-activated kinases; S6K, ribosomal protein tyrosine kinase S6; STEP, striatal enriched protein tyrosine phosphatase.

, Full reversal; , partial reversal; , no reversal; , not tested/no data available; ✓, double-blind placebo-controlled trials sufficiently powered for the defined primary outcome measure, phenotype correction was achieved; (✓), open-label trial *AND/OR* underpowered trial/too small sample size to reach conclusion on primary outcome measure, partial phenotype correction was achieved; ✘, no clinical trial(s) reported (or not applicable in the case of preclinical genetic rescue strategies).

[a] *Information regarding "Clinical research" indicates whether clinical trials have been conducted with a given mechanism irrespective of the trial's outcome.*

[b] *Fenobam, mavoglurant, and basimglurant.*

[c] *Arbaclofen: only tested in social/anxiety context.*

phenotypes in *Fmr1* KO mice can be corrected. Studies with fenobam showed reversal of spine morphology changes, deficits in motor learning, and impaired avoidance discrimination in *Fmr1* KO mice (Wang, Smith, & Mourrain, 2014; de Vrij et al., 2008; Vinueza Veloz et al., 2012). Further studies with MPEP, CTEP, and mavoglurant showed normalization of elevated protein synthesis rates, increased AMPA receptor internalization, enhanced mTOR and ERK activation, increased number of spines and immaturity of spines, increased seizure incidence, exaggerated LTD and machroorchidism (Osterweil et al., 2010; Gross et al., 2010; Yan, Rammal, Tranfaglia, & Bauchwitz, 2005; Gantois et al., 2013; Pop et al., 2014).

mGlu5 inhibitors have been studied extensively in clinical trials in FXS patients, as discussed in more detail further below. The very first study of a mGlu5 inhibitor in FXS patients was a single-dose open-label trial of fenobam in adult male FXS patients (Berry-Kravis et al., 2009). The trial revealed that mGlu5 inhibition intrinsically is not prohibitive in FXS, but the study was neither powered nor designed to demonstrate efficacy. Two other mGlu5 NAMs, mavoglurant (Vranesic et al., 2014) and basimglurant (Lindemann et al., 2015) were studied in a range of well-powered clinical trials in FXS patients across a wide age range. As discussed further, no therapeutic benefits were detected in these trials.

$GABA_B$ Agonism (Baclofen/Arbaclofen)

The GABA neurotransmitter system has been linked to FXS in studies exploring the transmitter synthesis and receptor family (Chapter 10) (Braat & Kooy, 2015). Furthermore, an imbalance between excitatory and inhibitory neurotransmission has been suggested to be an important component of FXS, as well as other autism spectrum disorders (ASD) (Gatto & Broadie, 2010). On this background, the $GABA_B$ agonist baclofen, a marketed drug acting as muscle relaxant, which is used for the treatment of spinal cerebral palsy and other disorders with spasticity, has been explored in FXS (of note, baclofen is a racemate of the inactive S-enantiomer and a prodrug of the active R-enantiomer, dubbed arbaclofen was used as an experimental drug). Treatment of *Fmr1* KO mice with baclofen (Pacey, Heximer, & Hampson, 2009) and arbaclofen (Henderson et al., 2012) corrected the increased protein synthesis rate, increased $AMPA_R$ internalization, increased susceptibility to AGS, and increased synaptic spine density, suggesting a possible therapeutic potential of $GABA_B$ agonists in patients. Arbaclofen has been explored in a clinical trial in FXS patients where it was reported to have missed the primary efficacy endpoint (Berry-Kravis et al., 2012a).

For completeness we would like to mention drugs targeting the $GABA_A$ receptor system, which has been implicated in FXS (Braat et al., 2015a; D'Hulst et al., 2015). Clinically available drugs targeting the $GABA_A$ receptor systems are limited to date to benzodiazepines. These are indeed being used clinically for FXS patients for the treatment of severe anxiety and emotional crisis (Berry-Kravis, Sumis, Hervey, & Mathur, 2012b), but their long-term use is—independent of the indication—limited by their sedative properties and the risk of drug dependence.

Ganoxalone ($GABA_A$ Activation)

During the last decade the glutamatergic system has been the focus of research in FXS and other neurodevelopmental disorders. However, more recently studies tightly link the GABAergic system to FXS (Chapter 10). For example, it was demonstrated that FMRP localizes with several $GABA_A$ receptor mRNAs suggesting a close control of protein synthesis for such selective subunits (Frederikse, Nandanoor, & Kasinathan, 2015). Furthermore, it reduced

mRNA expression of $GABA_A$ receptor subunits and was identified in several brain regions, such as cortex and cerebellum in young *Fmr1* KO mice (Braat et al., 2015b). In addition, reduced expression of specific subunits of the $GABA_A$ receptor system was found to be corrected in "YAC" transgenic rescue mice, containing the full-length human *FMR1* gene in an *Fmr1* KO background (Braat et al., 2015b). Finally, positive allosteric modulation of $GABA_A$ receptors with the neurosteroid ganaxolone (see below) can modulate specific behaviors in *Fmr1* KO mice, emphasizing the therapeutic potential of this approach (Braat et al., 2015b).

Ganaxolone is an investigational drug under development by Marinus Pharmaceuticals. It is a positive modulator of the $GABA_A$ receptor and enhances the GABA transmission (Carter et al., 1997). Ganoxalone has sedative, anxiolytic, and anticonvulsant-like activity in rodent models (Stephen & Brodie, 2011). Currently, ganaxolone is being tested in a Phase 2 proof-of-concept clinical trial in a double-blind, randomized, placebo-controlled, crossover design study in up to 60 subjects with FXS (6–17 years of age). The aim of the study is to assess the safety, tolerability, and efficacy of ganaxolone for treatment of anxiety and attention deficit in subjects with FXS (NCT01725152).

Interventions Tested in Preclinical Disease Models

Statins

One of FXS' molecular hallmarks is the elevated protein synthesis rate. While the exact mechanism by which FMRP exerts its effect on translational control is not fully understood, studies from several labs suggest that mTOR and MAPKinase signaling pathways likely are involved (Gross, Berry-Kravis, & Bassell, 2012). The full activation of MAPKinases is mediated by RAS, which requires tethering to the cell membrane by farnesylation for its full activation. Based on the observation that inhibition of ERK can normalize the elevated protein synthesis rate in *Fmr1* KO mouse hippocampal tissue (Osterweil et al., 2010), it is conceivable that reducing the activity of RAS, acting upstream of ERK, could have a similar effect.

Studies testing this hypothesis took advantage of the fact that statins can attenuate Ras activity (Mendola & Backer, 1990): statins act as inhibitors of the enzyme HMG-CoA reductase, a key part of the early steps in the early cholesterol synthesis. A by-product of dialing down HMG-CoA reductase activity is a reduction in the mevalonate pathway, which serves as a substrate for farnesylation of RAS and other proteins requiring this posttranslational modification for their bioactivity. Treatment of *Fmr1* KO mice with lovastatin was reported to correct multiple core phenotypes, such as exaggerated protein synthesis rate, enhanced hippocampal LTD, increased ERK phosphorylation, mGlu receptor–mediated epileptiform bursting in hippocampal slices, increased incidence of audiogenic seizures, as well as visual cortex hyperexcitability (Osterweil et al., 2013).

A small open-label study in FXS patients with a 3-month treatment period using escalating doses of lovastatin reported good tolerability and signals of efficacy in multiple outcome measures (Caku, Pellerin, Bouvier, Riou, & Corbin, 2014). Two clinical studies investigating lovastatin in combination with either minocycline (open label, NCT02680379) or with parent-implemented language training (NCT02642653) are ongoing. Sufficiently powered double-blind placebo-controlled studies in FXS patients will be needed to determine the therapeutic potential of lovastatin in FXS patients.

Lithium

Lithium is an ion with a complex pharmacology (Malhi, Tanious, Das, Coulston, & Berk, 2013), including the nonspecific inhibition of GSK3β (Chapter 13) (Chiu & Chuang, 2010), which plays an important role in regulating protein biosynthesis (Shin et al., 2014). Lithium treatment of *Fmr1* KO mice was reported to correct elevated protein synthesis rate (Liu et al., 2012) and correct deficits in synaptic transmission, such as enhanced hippocampal LTD (Choi et al., 2011) and impaired hippocampal long-term potentiation (LTP) (Franklin et al., 2014). In addition, lithium treatment ameliorated multiple behavioral deficits in *Fmr1* KO mice, such as increased incidence of audiogenic seizures, augmented locomotor activity, deficits in passive-avoidance behavior, memory deficits, as well as impaired social behaviors (Liu et al., 2011; Yuskaitis et al., 2010a; Min et al., 2009; Chen et al., 2013; Mines, Yuskaitis, King, Beurel, & Jope, 2010). Furthermore, chronic lithium treatment has been reported to partially correct macroorchidism (Yuskaitis, Beurel, & Jope, 2010b), as well as the increased spine density in the medial prefrontal cortex (Liu et al., 2011). Of note, many of the phenotype corrections achieved with lithium have been reproduced with more specific inhibitors of GSK3β (Franklin et al., 2014; Min et al., 2009), suggesting that it might be mainly the inhibition of GSK3β that underlies the efficacy of lithium in the aforementioned studies.

Lithium is being clinically used in FXS patients as a mood stabilizer (Fragile X Clinical and Research Consortium, 2012) which is—as in other clinical target populations—limited by its narrow therapeutic window and the need for recurrent monitoring of drug exposure levels (McKnight et al., 2012). To date one clinical trial has been reported with lithium in FXS (Berry-Kravis et al., 2008): in this open-label trial with a total of 16 participants aged 6–23 years were treated with lithium, and overall good tolerability has been reported. Clinical efficacy measures indicate the possibility of clinical efficacy, which await confirmation in sufficiently powered double-blind, placebo-controlled trials (Liu & Smith, 2014).

STEP

Striatal enriched protein tyrosine phosphatase (STEP) occurs in several alternatively spliced isoforms and is expressed exclusively in neurons in several brain areas, including the cortex, hippocampus, and amygdala (Braithwaite, Paul, Nairn, & Lombroso, 2006). STEP underlies a complex regulation, and substrates of STEP include $AMPA_R$ and $NMDA_R$ receptor subunits, as well as several kinases, including Erk1/2, Fyn, proline-rich kinase 2 (Pyk2), and p38 (Johnson & Lombroso, 2012).

The transcripts of STEP are under translational control of FMRP (Darnell et al., 2011), and elevated STEP expression levels have been found in *Fmr1* KO mice, which were hypothesized to underlie the weakened synaptic strength and some of the behavioral abnormalities in FXS (Goebel-Goody et al., 2012). Expression of STEP in *Fmr1* KO mice was either reduced by 50% or completely abolished by intercross of *Fmr1* KO and STEP KO mice. In the resulting *Fmr1* KO mice with reduced STEP expression normalized susceptibility to audiogenic seizures, as well as ameliorated social deficits and anxiety-related behaviors were reported (Goebel-Goody et al., 2012). To date there have been no studies reported yet with pharmacological inhibition of STEP in FXS.

Minocycline (MMP9)

Minocycline is an approved antibiotic drug belonging to the class of tetracyclines. In addition to its antibiotic action, minocycline has been shown to exert neurorestorative and antineuroinflammatory effects in various animal models. The precise mechanism of action of minocycline in the CNS remains largely unknown, but its pharmacological action through the inhibition of the matrix metalloproteinase-9 (MMP-9) and the inducible nitric oxide synthase (iNOS) was hypothesized for the observed beneficial effect on synapse maturation and on the behavioral symptoms in the *Fmr1* KO mouse model (Chapter 15) (Bilousova et al., 2009). In addition elevated levels of MMP-9 were observed in FXS subject and were shown to be lowered by treatment with minocycline (Dziembowska et al., 2013). An open-label trial with minocycline treatment in FXS patients delivered mixed results with improvements in behavior (Aberrant Behavior Checklist or ABC-C; irritability; inappropriate speech) (Paribello et al., 2010); when minocycline was tested in a double-blind, placebo-controlled trial, no improvement could be confirmed on the aforementioned measures of behavior were found, whereas a significant effect in anxiety and mood-related symptoms were detected (Leigh et al., 2013). Further assessments using electrophysiological investigation using event-related potential (ERPs) showed that minocycline treatment improved the ERP in children with FXS (Schneider et al., 2013). If confirmed with other pharmacological intervention, the assessment of changes in ERPs could be a novel, objective outcome measure for future clinical trials.

S6 Kinase

It was discovered early on that FMRP functions as a repressor of protein translation (Li et al., 2001; Laggerbauer, Ostareck, Keidel, Ostareck-Lederer, & Fischer, 2001), and that the rate of protein synthesis is elevated in the absence of FMRP (Qin et al., 2005). S6K plays a key role in the control of the protein translation machinery, which is closely linked to protein synthesis–dependent forms of hippocampal LTP (Cammalleri et al., 2003). The link between the translational control via the mTOR–S6K pathway and the increased protein synthesis rate in the absence of FMRP was first established by Klann and Dever (2004) and further corroborated with the discovery that S6K is critical for the phosphorylation of FMRP (Narayanan et al., 2008). The identification of S6K as a drug target for a therapeutic intervention in FXS is based on experiments with the genetic ablation of the S6K in the Fmr1 KO mouse (Bhattacharya et al., 2012), which revealed the correction of many important mouse phenotypes, such as the reduction of the exaggerated protein synthesis, enhanced mGlu receptor–dependent LTD, and macroorchidism in FXS model mice. Furthermore, S6K ablation prevented the formation of immature dendritic spine morphology and corrected behavioral phenotypes. Very recently these phenotype corrections achieved with genetic S6K ablation were confirmed pharmacologically with the selective inhibitors PF-4708671 and FS-115, suggesting that clinical evaluation of S6K inhibitors in FXS could be explored (Bhattacharya et al., 2016).

CB1 (Rimonabant)

A crosstalk between mGlu5 and the endocannabinoid system is well established. Activation of mGlu5 promotes the synthesis of endocannabinoids (Varma, Carlson, Ledent, & Alger, 2001), and the release of endocannabinoids induces a cannabinoid receptor 1 (CB1)–mediated LTD of excitatory and inhibitory transmission (Kano, Ohno-Shosaku, Hashimotodani, Uchigashima, & Watanabe, 2009). In *Fmr1* KO mice, an enhanced CB1-mediated

signaling has been observed, which could be normalized with the administration of the CB1 inhibitor, rimonabant (Busquets-Garcia et al., 2013). Furthermore, rimonabant ameliorated the cognitive deficits, reduced the audiogenic-induced seizures, and normalized the spine phenotype in hippocampal dendrites in *Fmr1* KO mice (Busquets-Garcia et al., 2013).

Rimonabant was advanced in clinical development for the treatment of obesity (Xie et al., 2007). The drug was withdrawn from the US market in 2008 (European Medicines Agency, 2009) (rimonabant did not receive market authorization outside the United States) due to its propensity to induce severe neuropsychiatric adverse events, which were deemed related to its mechanism of action (Christensen, Kristensen, Bartels, Bliddal, & Astrup, 2007; Mitchell & Morris, 2007). Due to the lack of a clinically applicable CB1 antagonist, the encouraging results obtained in *Fmr1* KO mice with rimonabant cannot be followed up clinically for the time being.

PAK Inhibitor

The large family of p21-activated kinases (PAKs) includes effector proteins for Rac1 and Cdc42 and is a critical component in pathways driving cell proliferation, survival, and motility. Classically implicated in a variety of cancers, PAKs are presently in the focus for neurodevelopmental disorders (Rudolph, Crawford, Hoeflich, & Wang, 2015). In addition to the findings of PAK3 mutations in X-linked mental retardation (Allen et al., 1998) and altered PAK signaling in FXS mice (Chen et al., 2010), genetic ablation of PAK in *Fmr1* KO mice rescued the spine phenotype, reduced cortical LTP, as well as some behavioral abnormalities (Hayashi et al., 2007). Similar effects were achieved via treatment with the small molecule PAK inhibitor, FRAX486, rescuing elevated spine density, increased seizure incidence, and hyperactivity in Fmr1 KO mice (Dolan et al., 2013).

Ampakines

Ampakines are positive allosteric modulators of the AMPA family of postsynaptic ionotropic glutamate receptors. The potentiation of the glutamate-mediated activity at the AMPA receptor results in a slower decay of the excitatory postsynaptic potential (EPSP) and an increase in hippocampal LTP (Arai et al., 1994; Arai, Guidotti, Costa, & Lynch, 1996). In rats, ampakines have been shown to enhance cognitive performance (learning and memory) (Staubli et al., 1994; Hampson, Rogers, Lynch, & Deadwyler, 1998), an effect that was not confirmed in schizophrenic subjects (Goff et al., 2001) in a Phase 2 clinical trial with the ampakine CX516.

The perturbed synaptic transmission demonstrated in FXS (Huber et al., 2002), such as the reduced GluR1 AMPA subunit (Li, Pelletier, Perez Velazquez, & Carlen, 2002) and impaired LTP (Li et al., 2002; Larson, Jessen, Kim, Fine, & du Hoffmann, 2005), provided evidence of defective AMPA-mediated neurotransmission. The positive effects on AMPA receptor–mediated neurotransmission led to the hypothesis that ampakines could be a useful treatment to restore some of the synaptic deficits in FXS. This hypothesis was tested in a double-blind, placebo-controlled clinical trial with the ampakine drug, CX516. The study did not show significant improvements in cognition (primary outcome) or in language or attention/executive function (secondary outcomes) over placebo (Berry-Kravis et al., 2006).

COMPARING TREATMENT EFFECTS OBSERVED IN *FMR1* KNOCKOUT MICE AND FXS PATIENTS: THE EXAMPLE OF mGlu5 NAMS

Single-Dose Open-Label Fenobam Trial

Fenobam was developed as the first nonbenzodiazepine anxiolytic drug in the 1970s in an effort to improve the side effect profile of benzodiazepines with respect to sedation, dependence, and alcohol interaction. The clinical development of fenobam for general anxiety disorder yielded mixed results, including an erratic pharmacokinetic profile, as well as neuropsychiatric adverse events, which led to the discontinuation of its clinical development (Pecknold, McClure, Appeltauer, Wrzesinski, & Allan, 1982; Friedmann, Davis, Ciccone, & Rubin, 1980; Itil, Seaman, & Huque, 1978). Remarkably, the development of fenobam was solely based on animal data without the knowledge of its molecular target (Pangalos, Schechter, & Hurko, 2007), which was later identified as mGlu5 in the context of a high-throughput screen (Porter et al., 2005).

A single-dose open-label study with fenobam in FXS patients was the first clinical study with a mGlu5 antagonist in this indication (Berry-Kravis et al., 2009). The study conducted in six male and six female FXS patients revealed no significant effect of treatment in measures of prepulse inhibition (PPI) and the Fragile X Continuous Performance Test (FXSCPT). Results from drug exposure monitoring confirmed the variable pharmacokinetic properties of the compound known from earlier studies in human.

Mavoglurant Trials

Mavoglurant/AFQ056 is a selective mGlu5 NAM (Vranesic et al., 2014). Its dosage, formulation, and pharmacokinetics have been extensively characterized in healthy subjects and different patient populations, showing an acceptable safety and tolerability profile in human (Walles et al., 2013). In fragile X patients, three trials have been conducted. The first was a placebo-controlled double-blind trial with a crossover design involving a total of 30 subjects that failed to demonstrate an effect on the full population of the study (NCT00718341). Surprisingly, patient stratification based on the methylation of the *FMR1* promoter region revealed a small subset of seven patients with a complete methylation of the promoter, which showed a positive response during the drug treatment period on multiple elements of the outcome measure scale (ABC-C). The same subjects did not show any improvement during the placebo treatment period. In contrast, the remaining subjects in this trial with an incomplete methylation of the *FMR1* promoter showed no improvement during the drug treatment period as compared to the placebo treatment period. The small number of subjects involved in this study and the absence of response to placebo of the completely methylated subset of patients might have contributed to the positive outcome. These results provided the rationale for conducting two Phase 2b, multinational, double-blind, placebo-controlled, and parallel-design trials (3 months) to assess the effects of multiple doses of mavoglurant in adults (>18 years) and adolescent (12–17 years) FXS patients stratified by their methylation status (NCT01253629 and NCT01357239). No improvement in any of the subgroups was demonstrated with the primary outcome measure (ABC-A), or with the secondary outcome

measures Clinical Global Impression-Improvement (CGI-I) and Repetitive Behavior Scale-Revised (RBS-R) (Berry-Kravis et al., 2016). An additional analysis using the CGI-I measure of the first treatment period (3 months) and a long-term, open-label extension follow up study (32 months) of the adolescent study patients failed to demonstrate an efficacy of the treatment with the highest dose of mavoglurant (100 mg b.i.d.) (Bailey et al., 2016).

Basimglurant Trials

Basimglurant is a potent and selective, orally bioavailable mGlu5 NAM, which is well characterized preclinically (Lindemann et al., 2015), and which was tested in two clinical trials in subjects with fragile X. The FRAGXIS trial (NCT01517698) evaluated the safety and efficacy of basimglurant in 183 adults and adolescents (14–50 years), with a primary endpoint of improvement in the Anxiety Depression and Mood Scale (ADAMS) total score and several secondary scales, as well as biomarker measures. The Foxtail trial (NCT01750957) was designed to evaluate the safety, as well as exploratory efficacy of basimglurant in a total of 47 children aged 5–13 years (Scharf, Jaeschke, Wettstein, & Lindemann, 2015).

The secondary endpoints for the FRAGXIS trial were the CGI-I/CGI-S, ABC total and factor scores, ADAMS total and factor scores, SRS, RBANS-Immediate memory, VABS total and domain scores, and VAS-Most troubling behavioral symptoms (Scharf et al., 2015). For both trials, biomarker assessments included CGG repeat size, methylation status, *FMR1* transcripts measured in peripheral blood, as well as FMRP concentration in blood. In both trials, basimglurant was administered once daily at 1.5 or 0.5 mg in adults and adolescents, and in a body weight adjusted, high or low dose (weight adjusted) in children. Analysis of the FRAGXIS results revealed no therapeutic benefits of basimglurant. For the Foxtail study no conclusions about efficacy could be reached, as the study was designed to evaluate the tolerability and safety of basimglurant, while the patient sample was too small to conclusively evaluate efficacy. In both studies basimglurant was overall well tolerated.

FUTURE DIRECTIONS FOR DRUG DISCOVERY IN FXS

The failure of the two targeted interventions, mGlu5 inhibitors and arbaclofen, to show robust efficacy in carefully designed and sufficiently powered clinical trials is disappointing. In view of the unusually strong preclinical evidence, which was generated and confirmed in multiple labs, this outcome was anything but expected. Here, we will look at both sides of the equation in more detail, discuss possible reasons for the lack of translation and implications for future use of FXS disease models, and suggest future directions for the development of new treatments.

Clinical Trial Outcomes for mGlu5 Inhibitors are Unequivocally Negative and the Trial Designs Were Adequate to Pick Up Efficacy in Multiple Domains

The mGlu5 inhibitors mavoglurant and basimglurant have been clinically tested for efficacy in adult and adolescent FXS patients of both genders across an age range of 14–50 years (basimglurant) and 12–45 years [mavoglurant (Berry-Kravis et al., 2016)] with a treatment

interval of ≥3 months. Earlier studies with shorter-treatment intervals conducted in a small sample of adult subjects with fenobam (Berry-Kravis et al., 2009), mavoglurant (Jacquemont et al., 2011), and basimglurant were mainly focused on establishing the drug's safety and an appropriate dosing regimen on the disease background and can therefore give only limited information about efficacy.

The primary outcome measures—the ABC-C and ADAMS rating scales, respectively—were selected based on the aforementioned smaller studies with mGlu5 inhibitors, as well as on experience with these scales in other drug trials in autistic patients (McCracken et al., 2002; Marcus et al., 2009). During the development of the antipsychotics, Risperdal and Abilify, for treatment of autism these scales turned out to be adequate to capture changes in anxiety, irritability, and aggression, which are some of the major FXS symptoms. A host of additional rating scales were included to assess changes in a range of behavioral domains relevant to the disease. Biomarker measures, such as CGG repeat size, methylation status, *FMR1* transcript, and FMRP abundance in blood were recorded to confirm the diagnosis, to allow stratification of subjects based on their molecular phenotypes, and to record the correlation between these parameters and the clinical phenotype in a significantly sized patient sample. The dose selection for mavoglurant and basimglurant was based on extensive human pharmacokinetic and receptor occupancy data, which, in combination with the drug exposure data recorded in the FXS trials, ensured that an appropriate range of receptor occupancies was covered and that the investigational drugs have indeed "hit their target".

The proof-of-concept trials of both mGlu5 inhibitors, mavoglurant and basimglurant, in adult and adolescent FXS patients were unequivocally negative. The trials missed all primary and secondary endpoints, and there were indications of a slight worsening in the highest dose group of the mavoglurant trial in adult FXS patients. Extensive posthoc analyses and stratification of data with respect to age, gender, and molecular parameters did not reveal indications for efficacy in patient subgroups.

It is important to note that in terms of efficacy, even trends were not observed in any of the primary or secondary outcome measures. Equally, there were no formal design flaws or unexpected events, which could have rendered the trials failed or which could have obscured potential signals of efficacy. The stratification of patients was well balanced with respect to age, gender, and molecular parameters, and the rate of adverse events was overall unremarkable, which means that potential treatment effects would not have gone unnoticed because of side effects.

There have been anecdotal reports from parents of FXS patients participating in the proof-of-concept clinical trials with basimglurant and mavoglurant, suggesting improvements in some of the behavioral issues and somatic symptoms, such as anxiety, social interaction, and gastroesophageal reflux typical for FXS. Some of these reports are most likely linked to the prominent placebo effects observed in the FXS trials, as discussed here.

The gradual reoccurrence of physiological symptoms, such as reflux, suggested by some parents is more difficult to understand. While mGlu5 antagonists have been reported to suppress reflux episodes in healthy subjects and in individuals suffering from gastroesophageal reflux disease (Keywood, Wakefield, & Tack, 2009; Zerbib et al., 2011; Rohof et al., 2012), there have been no studies investigating the maintenance of this effect upon chronic treatment. Moreover, there is only one report of tachyphylaxis in *Fmr1* KO mice, where repeated administration of the short-acting mGlu5 inhibitor tool compound, MPEP (Gasparini et al., 1999),

over multiple days partially lost its efficacy in suppressing audiogenic seizures (Yan et al., 2005). Given the short half-life of MPEP of c. 20 min in mice (Lindemann et al., 2011), it is unclear whether the reoccurence of audiogenic seizures in this study could be due to the pulsatile drug exposure in the study; of note, the genetic reduction of mGlu5 expression in *Fmr1* KO mice that mimics chronic reduction of mGlu5 activity achieved suppression of audiogenic seizures (Dölen et al., 2007), which suggests that chronic inhibition of mGlu5 activity in the context of FXS does not intrinsically underlie tachyphylaxis. Moreover, for most phenotypes of *Fmr1* KO mice, including protein synthesis rate and biochemical measures, such as ERK and MAPK activity, synaptic spine density and architecture, synaptic plasticity, learning and memory, social interaction, and hyperactivity, maintained efficacy over chronic drug treatment has been reported, and indeed chronic drug treatment has been shown to be a prerequisite for efficacy in several of the phenotypes analyzed in *Fmr1* KO mice (Michalon et al., 2012; Gantois et al., 2013; Pop et al., 2014). Taken together, with the possible exception of audiogenic seizures, there is little evidence for mGlu5 inhibitors to show tachyphylaxis in *Fmr1* KO mice, and there is no mechanism by which the molecular target mGlu5 would desensitize to NAMs. On this background, and in the view of therapeutic effects with chronic mGlu5 inhibitor treatment in patients in other indications, it is unlikely that the mentioned reoccurrence of reflux in FXS with chronic treatment could indicate a general tachyphylaxis of the molecular mechanism of action of mGlu5 inhibitors in FXS.

Patient's Age and Treatment Duration: Risk–Benefit Considerations are Key

The duration of treatment, as well as the starting age for treatment are two key parameters for the evaluation of novel pharmacological interventions in FXS and other neurodevelopmental disorders. The underlying assumption in this discussion is that the neuronal plasticity generally decreases with age, and that therefore interventions might have the best chance for unfolding their therapeutic potential when treatment starts as early as possible. In addition, it is expected that the chances for preventing a misrouted brain development are best if an intervention is applied earlier rather than later. Also, the alleviation of disease symptoms can be expected to improve the treatment outcome if applied early enough. In FXS, social anxiety, irritability, and aggression contribute greatly to the patient's impairment to interact with peers and to learn and practice language, social skills, and other competencies; improvements of these symptoms would facilitate social interaction with knockon effects in areas, such as language acquisition. This is particularly relevant in view of the well-known "critical periods" during childhood and adolescence, for example, for language and social skills.

While these points make it seem logical that a treatment should be tested at an age as young as possible for a treatment period as long as possible there are a couple of additional factors for pediatric drug development that deserve close examination.

Animal data don't suggest a very young target age range: The studies with mGlu5 inhibitors in *Fmr1* KO mice have shown that a treatment onset well after phenotype onset in animals aged 5 weeks (Michalon et al., 2012) up to 3 months (Gantois et al., 2013), demonstrate that the full correction of most of the phenotypes studied can be achieved when pharmacological intervention starts in early to fully adult animals. There is of course no doubt that developmental milestones and critical development windows are different in mice compared to humans, and that neuronal plasticity in mammals generally is greater in younger compared to older animals. Nonetheless, these data show that FXS does not cause an irreversible disruption

of brain development in a mammalian organism. Furthermore, there are to date no reports of significant alterations in neuronal numbers, general organization of brain areas, or other structural alterations above a subcellular level in FXS, which would suggest that treatment has to start early to prevent irreversible structural brain damage.

Collectively, the actual *Fmr1* KO mouse studies don't provide evidence that FXS clinical trials would need to be conducted in patients younger than the adolescent age range. Considering critical age windows for human development, it is likely that treatment onset at ages below adulthood ultimately has a better chance for achieving an optimal outcome. Given the available data, it is however essentially impossible to safely predict what the optimal starting age for treatment in FXS patients will be.

On the other hand, drug safety needs to be taken into account when considering the pediatric age range. The pediatric age range is linked to stricter safety requirements. As discussed later, unless a treatment is targeted toward a life-threatening disease that presents itself at a very low age, it is typically required to demonstrate a drug's safety and efficacy in adults and adolescent first, before targeting gradually young patients.

Treatment duration: The treatment duration in the mGlu5 FXS clinical trials was 3 months, with an open-label extension offered to adolescent patients receiving mavoglurant for up to 32 months. The animal data suggest that normalization of protein synthesis rate and synaptic plasticity, as well as blockade of audiogenic seizures was achieved within 48 h of mGlu5 inhibitor exposure. Most other phenotypes, including spine density; biochemical measures, such as ERK and S6K activity; and complex behavioral phenotypes, such as avoidance extinction and social interaction, normalized within 3-4 weeks of mGlu5 inhibitor treatment even though the minimal required treatment period was not systematically tested (Michalon et al., 2012; Gantois et al., 2013).

These data suggest that a potential therapeutic effect in FXS patients could have become apparent within the 3-month duration of the placebo-controlled double-blind portions of both the basimglurant and mavoglurant trials.

It has been proposed that longer-treatment durations could have increased the chances for detecting possible therapeutic effects with mGlu5 inhibitors. While there are no actual data arguing in favor or against this assumption, there are also no data to rule out that a treatment with mGlu5 inhibitors period longer than 3–32 months, even if unlikely, could have shown some efficacy.

Generally it is expected that pharmacological interventions for FXS, such as mGlu5 inhibitors—if proven efficacious—would likely be long term if not lifelong, given that there are currently no modalities being considered that would correct the primary molecular cause of FXS (i.e., reinstating the *FMR1* gene function).

Regulatory framework: It is well beyond the scope of this book chapter to lay out the detailed regulatory framework, the rationale behind the regulations, the typical outline of a drug development path, and what is typically done for a novel investigative drug targeting a neuropsychiatric indication in the autism spectrum. Here we would like to just briefly touch upon a few points that are of particular importance for the discussion in this context.

The current regulatory framework provided by health authorities, such as the FDA and EMEA, foresees that first-in-patient studies for new modalities in nonlife-threatening indications are conducted in adult subjects, with possible extensions to adolescent patients,

provided there is a strong scientific rationale and supportive evidence for the drug's safety. Provided that these studies support a favorable safety profile of the investigative drug in the targeted indication, subsequent studies in gradually younger patients can be granted. As mentioned previously, treatment of young age groups, such as children and infants, is linked to the requirement for dedicated safety studies. Furthermore, the administration of drugs to pediatric patients requires age-specific formulation and dose adjustments, and below an age range of about 6 years, the pharmacokinetic properties can no longer be extrapolated in silico from data obtained in adults and adolescents, necessitating a stepwise lowering of the investigated age range.

The permitted duration of clinical studies with a new investigative drug is based on toxicological and other safety-related studies, which are typically designed such that subsequent preclinical toxicological and safety-related studies run for increasingly longer intervals, which, provided a favorable outcome, enable gradually longer clinical studies. In addition, targeting very young patients requires a set of safety studies that emphasizes the possible impact of a study drug on the development of the brain and other organ systems. It is important to note that there is often little reliable information available around the importance of a drug target in human brain development at young ages in human. For example, inhibiting mGlu5 in an infant brain for prolonged periods of time could risk causing irreversible damage, or alter synaptic wiring or cognitive function in certain domains, an untoward effect that would become apparent only much later after the damage has already occurred.

There is an ongoing debate that future clinical trials with new modalities in FXS should aim for targeting young-aged individuals right away, without prior testing in adult patients (Gross, Hoffmann, Bassell, & Berry-Kravis, 2015). While this push might be understandable, given the disappointing outcome of the mGlu5 inhibitor trials, it is important to balance risk and benefits of clinical trials: The available framework emphasizes patients' safety (first do no harm), which has been successfully overall in preventing harm being inflicted on patients treated with novel investigational drugs in clinical trials and after registration. Clinical investigators would need to closely work with regulators and industry partners to define a revised regulatory framework that ensures patients' safety in new investigational drug studies in FXS, aimed at targeting younger patients more rapidly than currently possible, if indeed desired.

Outcome Measures and Placebo Response

There is currently no approved drug for the treatment of FXS and hence no precedence of a successful clinical development path is available at this time. Based on the efficacy demonstrated by numerous mGlu5 antagonists that corrected—among many other phenotypes—a number of the behavioral symptoms in the mouse *Fmr1* KO model, the assessment of behavioral improvement in human FXS subjects was the first choice for the clinical evaluations; the focus on behavioral measures is further motivated by the fact that most physiological and biochemical measures recorded in mice were simply not applicable to patients. Among the various available scales, the ABC-C (Aman, Singh, Stewart, & Field, 1985), has been applied extensively in trials for intellectual disability and as an endpoint for the approval of the two antipsychotic drugs, Risperdal and Abilify, in ASD (Kent et al., 2013). For FXS the ABC-C

scale was first used in the trial with the Ampakine drug CX516 (Berry-Kravis et al., 2006), and although the drug did not demonstrate any improvement, the trial confirmed that it was technically possible to apply the ABC scale in FXS, which indeed was subsequently used in almost all clinical trials in FXS. This was also the case in the two large studies involving mavoglurant (NCT01253629 and NCT01357239) for which the ABC-C scale was used as a primary outcome measure.

Whereas the lack of efficacy of the drug treatment is not in doubt, the apparent improvement observed during the 4-week placebo run-in period (up to 27 pts) and during the 12-week treatment period in the placebo treatment arms (up to 10 pts) without significant wearing-off is indicative of very strong placebo effects in the mavoglurant trials. The placebo effects in clinical trials in the area of psychiatry have been repetitively observed and several reasons have been hypothesized (Weimer, Colloca, & Enck, 2015); the reasons for the observed very large and sustained effect in the trials with mavoglurant remain unknown. One of the important contributing factors could be related or amplified by the fact that family and/or caregivers were the primary raters of the ABC-C scale, which could have introduced a strong bias, facilitated by a lesser degree of training and practice in parents/caregivers in the use of the scales compared to physicians.

Despite all the intense efforts by pre- and clinical research, no validated objective outcome measures for the assessment of the efficacy of investigational pharmacological treatments in the FXS patient population are available at this time. This applies especially to outcome measures in the area of cognition, even more so when children or infants are to be included into future drug trials. It has furthermore been discussed that future drug trials should be combined with training in specific areas, such as language or with behavioral intervention. While specific training efforts and behavioral therapy indeed have been tested and proven to be effective in ameliorating some of the symptoms in FXS and related ASD (Cohen, Amerine-Dickens, & Smith, 2006; Lovaas, 1987), the integration of these nondrug interventions in drug trials is mostly uncharted territory. Great care needs to be taken when attempting to combine new investigational drugs with training efforts in a clinical trial setting. A potential enhancement of the placebo effect due to the training needs to be taken into consideration and the respective contributions of the drug versus the training effects to the overall treatment outcome need to be dissected. This might require a more complex study design to prove the efficacy of the investigational drug.

Taken together, in parallel to the drug discovery activities, efforts aiming to identify and validate objective outcome measures, for example, in observational trials and to effectively measure the placebo effects are required based on which new targeted treatments for FXS can be developed.

Future Use of Preclinical FXS Disease Models

We are focusing the discussion on the *Fmr1* KO mouse model in view of the limitations of invertebrate models (very large differences to human in terms of genetics and biology in areas, such as cognition and social interaction) and the limited experience with the *Fmr1* KO rat and other vertebrate models in development.

The reasons for the striking discrepancy between the preclinical findings in *Fmr1* KO mice with mGlu5 inhibitors and arbaclofen on the one hand and the lack of efficacy in patients

on the other hand are not yet understood. Moreover, the fact that a large number of diverse molecular mechanisms shows efficacy in the mouse model on many of the same phenotypes might indicate that the *Fmr1* KO mouse is generally overpredictive for the therapeutic potential of novel interventions (Scharf et al., 2015). On this background it is clear that we can't continue to use data obtained in the *Fmr1* KO mouse model as sole predictors for the therapeutic potential of novel interventions in patients.

New preclinical FXS disease model with strong construct and face validity are needed with the primary goal to improve their utility to evaluate the therapeutic potential of novel therapies in patients.

While there is no solution available yet for how to construct new FXS disease models which are better in predicting therapeutic benefits in patients, we want to touch upon several areas that we consider key for improving on the current status of FXS disease models and for evaluating potential new therapies.

- *Understanding the disconnect between preclinical and clinical data for mGlu5 and $GABA_B$*: As discussed elsewhere in this chapter and in other contributions of the book, there is a lot of work needed on the development and validation of clinical outcome measures amenable to FXS patients in the area of cognition and social behavior, and studies in a different age range may have produced a different outcome. However, in view of the results obtained in *Fmr1* KO mice, there was at least some clinically meaningful therapeutic effect to be expected in the trials as they have been conducted. Unrevealing the molecular underpinning of the disconnect between the *Fmr1* KO mouse model and FXS patients in their response to mGlu5 inhibitors and arbaclofen is going to be useful for building a better, more translational model.
- *Establishing* (*a*) *new FXS disease model*(*s*): Building new FXS disease models for the profiling of novel interventions with better translational value is key. It is an open question whether the *Fmr1* KO rat model or stem cell–based models, as well as new models under discussion, for example, in nonhuman primate (Shen, 2013), will perform better in predicting therapeutic potential; mGlu5 inhibitors and arbaclofen should be considered as negative controls for the validation of these models certainly in those areas which were tested clinically.
- *Relying on more than a single disease model in more than one species*: Future profiling of novel interventions should employ more than a single model, as it is typically done in other indication areas. For example, in the areas of depression and schizophrenia, novel mechanisms of action are typically being profiled in a range of different models often in different species, each of which examines different aspects of the disease; only when the readouts across multiple models in more than one species coherently point into the same direction, it is assumed that the intervention might have therapeutic potential.

  One obvious risk factor in the case of mGlu5 inhibitors and arbaclofen was the almost complete reliance on a single disease model, that is, the *Fmr1* KO mouse. As it is known that no single preclinical model can recapitulate the entirety of a human disorder, this overreliance on a single model came with a substantial risk.
- *Including negative controls in model validation*: The validation of new models should include negative controls beyond placebo, which is rarely done and basically not at all

reported. The negative controls for model validations should also include treatments that were clearly not active in patients, such as mGlu5 inhibitors and arbaclofen.

- *Pushing for publication of "negative" data*: As in most research areas there is a strong publication bias toward the success stories, while little, if any attention, is paid to studies with negative outcomes. There is a need for a culture change with the realization that negative data are essential for progress. This means publishing studies with mixed results in their entirety, not reduced to only those parts of a study where a given intervention was active (this is especially important for comparing multiple treatments). In addition, completely negative studies need to be published (i.e., novel interventions completely lacking the desired effects in animals and negative clinical trials), which are important for understanding the specificity of interventions and for improvement, for example, of methodological factors. Publishers will play a key role in this effort, as often even proof-of-concept clinical trials (let alone preclinical studies) are rejected by editorial decision from reputable journals when the study outcomes are negative.
- *Adhering to minimal study design and reporting standards*: There has been a lot of recent attention paid to the topic of reproducibility of data published in life sciences. The two main areas for improvement are the adherence to minimal study design standards, as well as a more complete reporting of data and experimental conditions.
  To touch upon a few design standards, studies should be conducted with the investigator blinded to intervention wherever possible, and the study subjects should be randomized for the study conduct. Appropriate negative and positive controls should be included where available, and experiments should be reproduced at least once to rule out by chance findings (Bailoo, Reichlin, & Wurbel, 2014). For in vivo studies the pharmacokinetic properties of the study drug in the targeted species, as well as appropriate formulation and dosing regimen should be considered; wherever possible more than one drug dose should be tested, and drug exposure should be monitored. Drugs for human use often behave quite differently with respect to species differences around the drug target and pharmacokinetic properties, and experiments, for example, in rodents might require the use of a molecule different from the drug for human use (Arrowsmith et al., 2015).
  The reporting of studies should include all details on, for example, living conditions of animals (group/single housing, food, with/without acoustic background, etc.), age, and genetic background of animals, as well as a meticulous description of experimental protocols. Equally, the reporting of raw data (e.g., individual animal data for behavioral experiments) should become standard, which gives a better sense for variation of data and which allows statistical reanalysis if needed. This reporting should include the indication where parts of datasets have been excluded, for example, because they were considered outliers. Good practice for reporting of in vivo study results is proposed, for example, in the ARRIVE guidelines (https://www.nc3rs.org.uk/arrive-guidelines).
- *Emphasizing preclinical readouts, which are as close as possible to clinical outcome measures*: Most of the preclinical studies using *Fmr1* KO mice and cellular systems focus on measures that cannot be replicated in patients, for example, biochemical readouts on brain tissue, synaptic plasticity measured by slice electrophysiology, synaptic spine morphology, or fear conditioning and extinction. Even though it is well accepted that, for example, measures of synaptic plasticity are correlated to learning and memory function, the extrapolation to any specific learning and memory readout in human is a stretch.

Relatively few studies in *Fmr1* KO mice employ measures, such as functional magnetic resonance imaging (fMRI), measures of sensorimotor gating, or certain learning and memory paradigms, for which there is a close resemblance of a clinical outcome measure available. Focusing on preclinical measures with known direct correlation to clinical measures applicable to FXS patients might help to improve on the translation from preclinical datasets to clinical outcomes.

Taken together, the drug development efforts in FXS with mGlu5 inhibitors and arbaclofen—in spite of their sobering outcome—have significantly advanced the field by proving the feasibility of large international double-blind, placebo-controlled trials with new investigational drugs in this patient population. At the same time these efforts have exposed significant gaps. We learned that the currently available outcome measures that are accepted by regulators in FXS are still fairly crude when investigating, for example, cognitive function and social competencies, especially when looking at very young children. We furthermore were surprised by the striking placebo response in the mGlu5 inhibitor trials, which needs to be understood and better controlled in future trials. For the drug discovery efforts we learned that the currently available FXS disease models failed to predict the lack of therapeutic potential for two modalities that were supported by unusually strong preclinical datasets obtained in these models, and that new appropriately validated models are needed to support drug discovery.

It is hoped that these gaps can be filled in the coming years in close collaboration between patients and their relatives, clinicians, and scientists working in drug discovery, which is expected to ultimately enable the successful development of novel, effective therapies in FXS and related disorders.

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CHAPTER

19

Overview of Targeted Double-Blind, Placebo-Controlled Clinical Trials in Fragile X Syndrome

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INTRODUCTION

The treatment of individuals with fragile X syndrome (FXS) should always involve a multimodality intervention with both behavioral and educational interventions that have been described elsewhere (Braden, 2000, 2002; Scharfenaker, O'Connor, Stackhouse, & Noble, 2002), in addition to the use of psychopharmacological interventions if needed. For many of the medications commonly used in the treatment of children with FXS, either there are no controlled trials (e.g., clonidine or guanfacine) or they have been carried out years ago (e.g., stimulants) (Hagerman, Murphy, & Wittenberger, 1988). The use of these medications have been summarized in clinical surveys (Bailey et al., 2012), documentation from a large fragile X clinic (Berry-Kravis, Sumis, Hervey, & Mathur, 2012) or review papers (Davenport, Schaefer, Friedmann, Fitzpatrick, & Erickson, 2016; Gross, Hoffmann, Bassell, & Berry-Kravis, 2015; Lozano, Hare, & Hagerman, 2014; Schaefer, Davenport, & Erickson, 2015).

The exciting age of targeted treatments for FXS has ushered in new treatments that have the potential to reverse the neurobiological abnormalities that occur in the absence of the fragile X mental retardation protein (FMRP) in FXS. These treatments will be expected to not only improve behavior, but also cognition. Therefore, it is expected that, if considered safe for the young child, initiating these interventions as early as possible in childhood will be most effective in the treatment of FXS. However, the FDA approval process typically requires the demonstration of safety and efficacy in adults first, before attempting treatment in children.

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Therefore, very few treatments have been studied in young children with the exception of minocycline (Leigh et al., 2013) and sertraline (Hess et al., 2016; Winarni et al., 2012) as described further. The lack of FMRP causes dysfunction in several neurotransmitter systems and cell signaling pathways described in this volume. Here we will review the few controlled clinical trials that have been carried out after studies (reviewed in this volume) demonstrated efficacy in fragile X animal models (Table 19.1).

CLINICAL TRIALS IN YOUNG CHILDREN WITH FXS

Sertraline

PET studies in young children with autism under the age of 5 have demonstrated deficits in the production of serotonin in the frontal regions of the brain (Chugani et al., 1999). Additionally, metabolomics studies demonstrated that enzymes that metabolize tryptophan to serotonin are significantly downregulated in all causes of autism compared to conditions that lead to intellectual disabilities without autism (Boccuto et al., 2013). A retrospective study of young children (12–50 months) with FXS who were treated with sertraline, compared to those who were not treated with sertraline, demonstrated a significant improvement in the trajectory of both receptive and expressive language over time on the Mullen Scales of Early Learning (MSEL) (Winarni et al., 2012). Such evidence precipitated a placebo-controlled double-blind trial of low-dose sertraline (2.5–5.0 mg/day) in children of ages 2–6 years with FXS lasting for 6 months. Inclusion criteria led to 57 children who were randomized: 27 to sertraline and 30 to placebo. Two subjects from the sertraline arm and three subjects from the placebo arm discontinued. There were no significant demographic differences between the two treatment arms. The majority of participants were males (78% in sertraline and 90% in placebo) and Caucasian (70% in sertraline and 50% in placebo) with an average age of 3.9 (SD = 1.1) years in the sertraline group and 3.9 (SD = 1.1) years in the placebo group. Fifty-two participants completed 6 months of treatment in this double-blind controlled trial. Although the prespecified outcome measures, including Clinical Global Impressions Scale Improvement (CGI-I) and the receptive and expressive language measure on the MSEL, did not significantly improve, other scales on the MSEL, including the fine motor age equivalent [28.44 (10.91) vs. 25.04 (6.91), $P = 0.005$], the fine motor raw score [27.32 (8.06) vs. 25.19 (4.96), $P = 0.008$], the age equivalents for visual perception [33.68 (15.06) vs. 30.59 (9.6), $P = 0.031$], and the Cognitive *T*-score sum [105.36 (40.27) vs. 93.0 (20.33), $P = 0.047$] were significantly improved. *Posthoc* analysis combining all MSEL age-equivalent scores (expressive, visual, receptive, and fine motor) indicated significant improvements due to sertraline administration as compared to the placebo [30.09 (12.64) vs. 23.60 (10.76), $P = 0.007$]. Observed average scores on other secondary measures typically improved in the sertraline group, although they did not significantly improve as compared to the placebo, with the exception of the Sensory Processing Measure-Parent Version (SPM-P) Social Participation subtest score, which was significantly improved in the sertraline group. Finally, a subgroup of participants with autism spectrum of disorder (ASD) showed significant improvement in the MSEL expressive language raw score: sertraline group compared to the placebo group [23.5 (10.5) vs. 17.6 (6.8), $P = 0.029$] (Hess et al., 2016).

TABLE 19.1 Summary of Randomized Clinical Trials in Fragile X Syndrome (FXS)

Clinical trial registration number	Compound (drug class)	Clinical trial phase	Target population	Principal investigator	Sponsor	Status/results
NCT01474746	Sertraline (SSRI)	Phase II	Children	R. Hagerman, MD	University of California, Davis	Completed/Hess et al., 2016
NCT01053156	Minocycline (tetracycline)	Phase II	Adolescents and children	R. Hagerman, MD	University of California, Davis	Completed/Leigh et al. (2013) and Schneider et al. (2013)
NCT00054730	CX516 (ampakine)	Phase II	Adults	E. Berry-Kravis MD, PhD	FRAXA Research Foundation	Completed/Berry-Kravis et al. (2006)
NCT01357239 NCT01253629	Mavoglurant (AFQ056; mGluR5 antagonist)	Phase IIb Phase IIb	Adolescents Adults	Novartis Pharmaceuticals	Novartis Pharmaceuticals	Completed/Berry-Kravis et al. (2016a)
NCT00718341	Mavoglurant (AFQ056; mGluR5 antagonist)	Phase IIa	Adults	Novartis Pharmaceutical	Novartis Pharmaceuticals	Completed/Jacquemont et al. (2011)
NCT01517698 NCT01750957	Basimglurant (RO4917523; mGluR antagonist)	Phase IIb (adult/adolescents) Phase IIa (children)	Adults and adolescent Children	Hoffmann-La Roche	Hoffmann-La Roche	Completed/Wong et al. (2015) and Youssef et al. (2015)
NCT01282268 NCT00788073	Arbaclofen ($GABA_B$ agonist)	Phase III	Adults and adolescents Children	Seaside Therapeutics	Seaside Therapeutics	Completed/Berry-Kravis et al. (2016b) and De Sonia et al. (2014)
NCT01013480	Arbaclofen ($GABA_B$ agonist)	Phase II	Adults, adolescents, and children	E. Berry-Kravis MD, PhD	Seaside Therapeutics	Completed/Berry-Kravis et al. (2012a)
NCT01911455	Acamprosate (GABA agonist)	Phase II	Adults, adolescents, and children	E. Berry-Kravis, MD, PhD and C. Erickson, MD	Rush University Medical Center and Children's Hospital Medical Center, Cincinnati	Recruiting
NCT01725152	Ganaxolone ($GABA_A$ agonist)	Phase II	Adolescents and children	R. Hagerman, MD	Marinus Pharmaceuticals	Completed/Ligsay et al. (submitted)

(*Continued*)

TABLE 19.1 Summary of Randomized Clinical Trials in Fragile X Syndrome (FXS) (*cont.*)

Clinical trial registration number	Compound (drug class)	Clinical trial phase	Target population	Principal investigator	Sponsor	Status/results
NCT02126995	Metadoxine (ion-pair salt of pyridoxine, GABA activator)	Phase II	Adults and adolescents	E. Berry-Kravis, MD, PhD	Alcobra Ltd.	Completed/Berry-Kravis et al. (2015)
NCT01894958	Trofinetide (NNZ-2566; neurotrophic peptide)	Phase II	Adult and adolescent males	E. Berry-Kravis, MD, PhD	Neuren Pharmaceuticals	Completed/pending
NCT01254045	Oxytocin (neuropeptide)	Phase II	Adults and adolescent	A. Reiss, MD	Stanford University	Completed/Hall, Lightbody, McCarthy, Parker, and Reiss (2012)
NCT01329770	Ascorbic acid and α-tocopherol	Phase II	Adolescents and children	Y. de Diego-Otero, PhD and L. Pérez Costillas, MD, PhD	Yolanda de Diego-Otero, PhD	Completed/de Diego-Otero et al. (2014)
NCT01120626	Donepezil (cholinergic drug)	Phase II	Adults and adolescents	A. Reiss, MD	Stanford University	Completed/Sahu et al. (2013)
NCT02642653	Combined lovastatin (HMG-CoA reductase inhibitor) and PILI	Phase IV	Children	R. Hagerman, MD	University of California, Davis	Open for recruitment
NCT02680379	Combined minocycline (tetracycline) and lovastatin (HMG-CoA reductase inhibitor)	Phase II	Adults and adolescents	F. Corbin, MD, PhD	Université de Sherbrooke	Not yet open for recruitment

GABA, γ-Aminobutyric acid; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; mGluR, metabotropic glutamate receptor; PILI, parent-implemented language intervention; SSRI, selective serotonin reuptake inhibitor.

Adapted from de Diego-Otero, Y., Calvo-Medina, R., Quintero-Navarro, C., Sánchez-Salido, L., García-Guirado, F., del Arco-Herrera, I.,..., & Pérez-Costillas, L. (2014). A combination of ascorbic acid and α-tocopherol to test the effectiveness and safety in the fragile X syndrome: study protocol for a phase II, randomized, placebo-controlled trial. Trials, 15*(1), 345.*

This study demonstrated significant benefit in several areas of development in young children with FXS when treated with a low dose of sertraline, 2.5 mg for 2–3 years and 5.0 mg/day for 4–5 years. This dose was safe and well tolerated in these children and adverse events were no different than in the placebo group (Hess et al., 2016). The rare patient who experienced hyperarousal or an increase in hyperactivity on sertraline did well when the dose was lowered by 50%. All families chose to continue on sertraline after the study. Sertraline not only increases the level of serotonin at the synapse, which improves anxiety, but also increases brain-derived neurotrophic factor (BDNF) levels, which can stimulate connectivity. Sertraline also increases dopamine in the striatum, which helps with attention and auditory processing. All of these effects in combination may generate the therapeutic benefits in young children with FXS (Hanson & Hagerman, 2014; Hess et al., 2016).

Minocycline

There is significant animal data that demonstrates a benefit of minocycline in the fragile X mouse and *Drosophila* models (Rotschafer, Trujillo, Dansie, Ethell, & Razak, 2012; Siller & Broadie, 2011). The lack of FMRP leads to upregulation of many proteins whose translation is normally inhibited by FMRP. Bilousova et al. (2009) demonstrated that lowering of the protein matrix metalloproteinase 9 (MMP9) with the use of minocycline dramatically improved synaptic connections and behavioral and cognitive measures in the *Fmr1* knockout (KO) mouse. Initial open label studies (Paribello et al., 2010; Utari et al., 2010) suggested benefit in adolescents and adults with FXS. These reports led to a randomized, double-blind, placebo-controlled, crossover trial in individuals with FXS, aged 3.5–16 years [mean age 9.2 years (SD = 3.6)]. Participants were randomized into minocycline or placebo groups for 3 months and then switched to the other treatment. Sixty-nine subjects were screened and 66 were randomized. Fifty-five subjects (83.3%) completed the first period and 48 (72.7%) completed the full trial. Intention-to-treat analysis demonstrated significantly greater improvement in one primary outcome, the CGI-I, after comparing the minocycline and placebo groups (2.49 ± 0.13 and 2.97 ± 0.13, respectively, $P = 0.0173$). Greater improvement in a *posthoc* analysis of anxiety and mood-related behaviors on the Visual Analog Scale (VAS) (minocycline: 5.26 ± 0.46 cm, placebo: 4.05 ± 0.46 cm, $P = 0.0488$) was also observed. Side effects did not vary significantly during the minocycline and placebo treatments. No serious adverse events occurred due to minocycline. However, long-term treatment with minocycline can sometimes darken the skin or gums, darken the dentition of the permanent teeth, or lead to autoimmune problems that can resemble a lupus-like syndrome (Leigh et al., 2013; Smith & Leyden, 2005). It is recommended that an antinuclear antibody (ANA) titer be checked every 6 months to 1 year if minocycline is continued long term. In addition, if a rash, swollen joints, or severe headache develops, the minocycline should be discontinued.

A subgroup of patients who participated in the minocycline-controlled trial also underwent an event-related potential (ERP) study utilizing an auditory habituation paradigm (Schneider et al., 2013). Complete baseline and end-of-treatment data for the placebo and minocycline arms were available from 12 subjects [8 male, 4 female; mean age = 10.5 years (SD = 3.7); mean IQ = 64 (SD = 23.7)]. Previous studies show that patients with FXS have exaggerated EEG amplitudes to auditory stimuli, particularly in the N1, N2, and P2 components,

and lack a habituation response after repeated provocations (Castren, Paakkonen, Tarkka, Ryynanen, & Partanen, 2003; Hessl et al., 2009; Van der Molen et al., 2012a,b). In this trial, treatment with minocycline showed statistically significant reductions in temporal N1 and P2 amplitudes to auditory stimuli, as well as significant improvements in habituation, possibly reflecting changes mediated by minocycline at the cellular level. However, there was an increase in ERP response amplitude in the central P2 component, which is contradictory to this hypothesis, although this increase also correlated with improvements on the CGI-I. Given the small sample size, it was not possible to analyze these results in relation to the methylation status. Overall, this study suggests ERP may be an objective and sensitive marker to detect target engagement and can predict treatment response in human subjects with FXS, although further studies are needed.

CLINICAL TRIALS OF AGENTS TARGETING GLUTAMATE RECEPTORS IN FXS

AMPA Receptor Activators (Ampakine)

Based on data from the *Fmr1* KO mouse model showing reduced AMPA receptor activity and a decrease in LTP in cortex (Li, Pelletier, Perez Velazquez, & Carlen, 2002), a Phase II, 4-week randomized, double-blind, placebo-controlled clinical trial was conducted to evaluate the safety and efficacy of the ampakine compound CX516 (RespireRx Pharmaceuticals, New Jersey, USA; *formerly Cortex Pharmaceuticals*) as a potential treatment for the underlying disorder in FXS (Berry-Kravis et al., 2006). After baseline screening, subjects with FXS ($N = 49$) underwent a 1-week placebo lead-in and then were randomized to CX516 ($N = 24$) or placebo ($N = 25$) for a 4-week period. Cognitive and behavioral outcome measures were administered prior to and at the end of treatment and 2-week posttreatment. There were minimal side effects, no significant changes in safety parameters, and no serious adverse events. There was a 12.5% frequency of allergic rash in the CX516 group. There was also no significant improvement in memory, the primary outcome measure, or in secondary measures of language, attention/executive function, behavior, and overall functioning in CX516-treated subjects compared to the placebo group. This study did identify outcome measures that were reproducible in this test–retest setting for the FXS population, yet some were too difficult or variable, providing information to fine-tune subsequent FXS trial design. Problems with potency of CX516 in other studies have suggested that dosing may have been inadequate for therapeutic effect, and thus it remains unclear whether modulation of AMPA-mediated neurotransmission is a viable therapeutic strategy for the treatment of FXS.

mGluR5 Receptor Negative Allosteric Modulators

The "mGluR theory of FXS" proposes that the lack of FMRP leads to increased mGluR-mediated protein translation and AMPA receptor endocytosis, ultimately causing excess mGluR-dependent LTD (Bear, Huber, & Warren, 2004). This correlates with numerous phenotypes in FXS animal models, such as immature neurons with long dendritic spines, impaired memory formation, hyperactivity, and increased susceptibility to seizures

(Berry-Kravis, Knox, & Hervey, 2011; de Vrij et al., 2008; Dockendorff et al., 2002; Lee et al., 2003; Morales et al., 2002; Yan, Rammal, Tranfaglia, & Bauchwitz, 2005; Zhang et al., 2001). Trials of mGluR negative allosteric modulators (NAMs) in these animal models have provided extremely promising results. Treatment with 2-methyl-6-(phenylethynyl)-pyridine (MPEP) rescued prepulse inhibition (PPI) (de Vrij et al., 2008) and treatment with 2-chloro-4-((2,5-dimethyl-1-(4-(trifluoromethoxy)phenyl)-1H-imidazol-4-yl)ethynyl) pyridine (CTEP) corrected audiogenic seizures (Michalon et al., 2012); both also improved learning and cognition (Gandhi, Kogan, & Messier, 2014; McBride et al., 2005; Michalon et al., 2012). Finally, a number of these studies, such as the CTEP study noted earlier, suggested better effectiveness when the drug was started in younger animals and used chronically (Michalon et al., 2012).

On the other hand, studies of mGluR NAMs in humans with FXS have provided mixed results. An open-label, single-dose trial of fenobam in 12 subjects (mean age 23.9 years; range 18–30 years) showed a good safety profile with a significantly positive response on PPI testing compared to data from untreated individuals ($P = 0.01$) (Berry-Kravis et al., 2009). In a double-blind, placebo-controlled, crossover trial of AFQ056 (mavoglurant) in 30 males with FXS, there were no significant differences on the ABC-C (primary outcome measure) or any secondary measures [CGI, Vineland Adaptive Behavior Scale (VABS), Repetitive Behavior Scale-Revised (RBS-R), VAS, and Social Responsiveness Scale (SRS)] after 28 days of treatment with the study drug compared to the placebo (Jacquemont et al., 2011). However, *posthoc* analysis of a subset of patients with fully methylated *FMR1* promoter regions suggested AFQ056 improved stereotypic behavior, hyperactivity, and inappropriate speech on the ABC-C, as well as showed improvements on the RBS-R, SRS, and VAS after AFQ056 treatment versus placebo. There were no statistically significant differences on any test measures in subjects with partial methylation.

These results spurred two multinational, double-blind, placebo-controlled and parallel-group 3-month trials of mavoglurant: one in adults (NCT01253629) and one in adolescents (NCT01357239) (Berry-Kravis et al., 2016a). Subjects were started on a 4-week placebo run-in, followed by a 12-week treatment period during which subjects were randomized to one of three mavoglurant groups (25, 50, or 100 mg BID) or a placebo group. The primary outcome measure for these trials was the Aberrant Behavior Checklist-Community Edition using the FXS-specific algorithm (ABC-C_{FX}) (Sansone et al., 2012), with ABC-C_{FX} subscale scores, the CGI-I, and RBS-R total and subscale scores serving as secondary outcome measures. A total of 175 adults (mean age range = 24.2–26.9 years) and 139 adolescents (mean age range = 14.4–14.6 years) were randomized, with 13 total subjects discontinuing due to adverse effects (AEs) (11 adults and 2 adolescents). Unfortunately, neither adults nor adolescents showed statistically significant improvements on mavoglurant in any outcome measures, regardless of the dose or methylation status. In fact, there was statistically significant deterioration measured on the ABC-C_{FX} total score on mavoglurant 50 mg BID in completely methylated adults (average +1.8 points, $P = 0.018$) and 100 mg BID in completely methylated adolescents (average +8.6 points, $P = 0.004$). *Posthoc* analysis further established no correlation between changes in ABC-C_{FX} total score to age or degree of methylation as continuous variables. A majority of reported AEs were mild in severity with the highest frequency occurring in the mavoglurant 100 mg BID group (adults: 82.2%, adolescents: 87.2%). Within this group, dizziness and insomnia specifically were most frequently reported in adults and

headaches were most frequently reported in adolescents. Four serious AEs occurred in the adult population, three of which occurred in the mavoglurant groups: one patient on mavoglurant 50 mg BID experienced an upper respiratory tract infection, headache, and agitation; one patient on mavoglurant 100 mg BID experienced agitation, visual hallucinations, and insomnia; and another patient on mavoglurant 100 mg BID experienced agitation. Two serious AEs occurred in the adolescent population: one on placebo and another on mavoglurant (appendicitis).

Finally, two trials of the mGlu5-NAM basimglurant were recently completed: one in adolescents and adults (target age 14–50 years) and another in children (target age 5–13 years). The former was a multinational, double-blind, placebo-controlled trial lasting 12 weeks in which subjects were randomized to placebo, basimglurant 0.5 mg QD, or basimglurant 1.5 mg QD (Youssef et al., 2015). One hundred eighty-five subjects were randomized in this study (63 adolescents, 122 adults; mean age range 22.4–24.2 years). Of the three treatment arms, the placebo group had the greatest improvement in the primary end point (ADAMS total score) compared to either doses of basimglurant. Analysis of secondary end points (ABC total and factor scores, CGI-I and CGI-S scales, RBANS, SRS, VABS total and domain scores, and VAS) also did not show statistically significant improvement in favor of the study drug. Basimglurant was safe overall and well-tolerated in adults and adolescents with FXS, although the highest percentage of adverse events occurred within the 1.5 mg QD treatment arm. Additionally, two subjects in the 1.5 mg QD group experienced hallucinations, and one subject in the 0.5 mg QD arm experienced a moderate psychotic disorder (all three subjects discontinued from the study). The second trial mirrored the design of its older counterpart, but used different dosages to match the adult steady state exposure levels (0.5 mg equivalent = 0.2 mg QD in 5–8 years and 0.3 mg QD in 9–13 years; 1.5 mg equivalent = 0.6 mg QD in 5–8 years and 0.9 mg QD in 9–13 years) (Wong et al., 2015). The primary objective of this study was to assess the safety profile of basimglurant in younger children with FXS, with secondary measures exploring changes in the ADAMS, CGI-I and CGI-S, ABC total and factor scores, RBANS, and VAS at end of the treatment period. Forty-seven subjects were randomized to the study (mean age range: 7.9–9.4 years). Results showed basimglurant was overall safe in this younger demographic, with most reported AEs rated mild-to-moderate in severity. No serious AEs were reported. Seven subjects experienced aggression during the active treatment arms, which the authors interpreted as possible target engagement and secondary to activating effects of basimglurant. No statistically significant differences were observed in secondary measure outcomes, although two subgroups (males with low *FMR1* methylation and subjects who were not taking concomitant antipsychotic medication) appeared to have slightly improved performance over placebo on select test measures.

These trials show that despite significant preclinical data for the efficacy of mGluR antagonists in the treatment of FXS, the overall disease process may be more complex in humans with multiple disrupted pathways acting simultaneously with one another (see GABAergic mechanisms further). Ultimately, multiple targeted treatments may be required to treat those affected with FXS. Nonetheless until trials assessing learning in young children with FXS can be performed, it will be impossible to know whether the shortcomings of the trials are due to interspecies differences (mice vs. humans) or trials that are not designed properly to identify changes in plasticity, the predominant finding in the FXS mouse.

CLINICAL TRIALS OF AGENTS TARGETING GABA MECHANISMS IN FXS

Arbaclofen

There has been evidence from numerous studies in the mouse model (Heulens, D'Hulst, Van Dam, De Deyn, & Kooy, 2012) that GABA systems play a role in the pathophysiology of FXS. Thus a number of agents targeting GABA regulation have been studied in clinical trials in FXS. The most extensive work has been done with $GABA_B$ agonist arbaclofen, which lowers presynaptic glutamate release with presumed resultant reduction of group 1 mGluR signaling. Preclinical animal data showed rescue of glutamate-induced lethality in the *dfmr* mutant fly with GABAergic compounds (Chang et al., 2008), reversal of audiogenic seizures in the *Fmr1* KO mouse with racemic baclofen (Pacey, Heximer, & Hampson, 2009), and reversal of excess protein synthesis, excessive AMPA internalization, abnormal spine density, audiogenic seizures, and behavioral phenotypes in the mouse model (Henderson et al., 2012). Based on preclinical work coupled with anecdotal clinical experience suggesting behavioral benefits from racemic baclofen in FXS and autism, and data from TMS studies demonstrating enhancement of cortical inhibition by racemic baclofen (McDonnell, Orekhov, & Ziemann, 2007), a phase II clinical trial of arbaclofen (STX209, Seaside Therapeutics, Massachusetts, USA) in humans with FXS was conducted. Arbaclofen is the enantiomer of racemic baclofen, with more potent $GABA_B$ agonist activity, and contrasts with S-baclofen (the less potent enantiomer) with respect to metabolism, CNS transport, and activity.

The phase II trial was a double-blind, placebo-controlled crossover trial with 4-week treatment periods separated by a washout (E.M. Berry-Kravis et al., 2012) performed at 12 sites in the United States. Sixty-three subjects (55 male) with a full mutation of *FMR1*, and who met severity criteria on the Aberrant Behavior Checklist Irritability (ABC-I) subscale, were enrolled. Up to three concomitant psychoactive medications were allowed. In each treatment period, study drug was flexibly titrated, then continued at the optimal titrated dose for 4 weeks total. Multiple behavioral and cognitive assessments were performed at baseline, 2 weeks, and 4 weeks in each treatment period. The primary endpoint was the ABC-I based on the FDA precedent for use of this scale for prior approval of risperidone and aripiprazole for irritability in ASD.

Arbaclofen showed no safety issues in clinical reports of AEs or in laboratory testing, and improvement over placebo in the entire intent to treat (ITT) ($N = 63$) and per protocol (PP) groups ($N = 54$) on a VAS for the three most severe parent-nominated behaviors ($P = 0.04$) and on the ABC-C_{FX} (Sansone et al., 2012) Social Avoidance (SA) Subscale ($P = 0.008$). Blinded treatment preference as reported by clinicians ($P = 0.05$), parents ($P = 0.09$), CGI-S ($P = 0.09$), and CGI-I ($P = 0.15$) all showed a trend in favor of arbaclofen. The ABC-I (primary outcome) was not different between placebo and arbaclofen. In a *posthoc* analysis, arbaclofen showed improvement over placebo in the more socially impaired subgroup ($N = 27$) for the treatment period preference (both clinician and parent, $P = 0.01$), CGI-I ($P = 0.02$), CGI-S ($P = 0.009$), Vineland Adaptive Behavior Scale Socialization Subscale ($P = 0.03$), ABC-C SW subscale ($P = 0.07$), ABC-FX SA subscale ($P = 0.04$), and a responder analysis (CGI-I of "much" or "very much" improved and at least 25% improvement on ABC-SW was observed, $P = 0.04$). The results were also more robust among subjects who met DSM-IV and ADI-R criteria for autism.

Significantly more subjects were responders on the CGI-I scale when receiving STX209 versus placebo (35% vs. 18% overall; 50% vs. 6% in the autism subgroup), although the ABC-I was not sensitive to these effects. Majority of subjects enrolled in an open-label extension study, and some withdrew from their concomitant medications, including antipsychotics.

Despite this highly promising phase II trial, a large (N = 125, 119 competed) phase III placebo-controlled, flexible dose trial with a treatment period of 8 weeks in adolescents and adults (age 12–50) with FXS did not show benefits for arbaclofen over placebo in the primary outcome of social withdrawal (based on ABC-C_{FX} SA) or in any other outcomes (Berry-Kravis et al., 2016b). An additional phase III placebo-controlled, fixed-dose (three doses and placebo groups) trial with a treatment period of 8 weeks in children (age 5–11) with FXS (N = 172; 159 completed, less than the prespecified study cohort of 200 due to early closure of the study for financial reasons) showed a trend toward improvement in the primary ABC-C_{FX} SA outcome in the highest-dose group (N = 38) as compared to the placebo group (N = 44) and significant improvement in several key secondary outcomes in this group (Berry-Kravis et al., 2016b). Further development of arbaclofen for FXS has not been possible for financial reasons. Strong clinical impression from trial investigators that arbaclofen was highly effective for at least a subgroup of patients with FXS, prompted an analysis of responses, placebo responders, and nonresponders, from the blinded trials who remained in the open-label treatment extension studies for 1–3 years (De Sonia et al., 2014). This analysis suggested that the placebo response did not involve the same symptom pattern as the drug response in prior placebo nonresponders in the controlled primary trial that then had a drug response in the extension. Also the benefit observed clinically in many patients did not align well with the outcome measures in the blinded trials, such that only the reversal of maladaptive behaviors was measured and not the positive adaptive changes (e.g., language and coping skills). Based on experience with the blinded arbaclofen trials and the open-label extensions, it seems numerous other problems also may have contributed to the development failure for arbaclofen. These problems include strong placebo effects, high parent variability on rating scales, and exaggeration of symptoms to include oneself in the study, in a setting where the same measure was being used for the entry criteria and primary outcome. In addition, side effects subtracted from positive responses in the fixed-dose study, as these are in the same domain as key outcome measures, and dose adjustment to eliminate side effects due to interpersonal variability in drug response cannot be done. Only the highest dose in the fixed dose study was effective overall leaving a relatively small cohort to analyze; one of the studies had to be closed before it filled, and the treatment period was likely not long enough. As the arbaclofen trials were the first large FXS trials to be implemented, the problems in these trials have provided important lessons for the field that have been used to improve subsequent trial designs.

Acamprosate

Acamprosate, currently FDA approved for alcohol withdrawal and having agonist properties at both $GABA_A$ and $GABA_B$ receptors, has shown promise in initial naturalistic open-label clinical experience (Erickson, Mullett, & McDougle, 2010) for seven persons (one youth and six adults) with FXS. Six subjects (86%) were defined as treatment responders after a minimum of 10 weeks of treatment as defined by a CGI-I subscale scores “very much improved” (1) or “much improved” (2), and a ≥30% improvement on the Aberrant Behavior Checklist

Social Withdrawal (ABC-SW) subscale score. Three patients experienced mild gastrointestinal AEs, a known side effect of acamprosate. In a 10-week prospective open-label trial (Erickson et al., 2013) of acamprosate in 12 children (mean age 11.9 years) with FXS, 9 subjects (75%) met the criteria for treatment response defined by a CGI-I score of 1 or 2 and a ≥30% improvement on the ABC-SW. The ABC-Hyperactivity subscale ($P = 0.04$), SRS ($P = 0.005$), and the Attention Deficit Hyperactivity Disorder-Rating Scale (ADHD-RS, $P < 0.0001$) also showed significant improvement relative to baseline. Two subjects had gastrointestinal side effects. There were no changes in safety laboratory studies, ECG, or vital signs (including weight) with treatment. These studies must be interpreted with caution given the strong placebo response in FXS; however, the normalization of elevated levels of total soluble amyloid precursor protein (sAPP) and sAPPalpha in the FXS patients treated with acamprosate (Erickson et al., 2014) suggests the drug may target an underlying mechanism related to the absence of FMRP. Acamprosate is currently being tested to determine whether effects on hyperactivity and social functioning observed in the open-label studies can be verified in a small placebo-controlled trial in FXS with a primary outcome of social withdrawal, but with concomitant evaluation of multiple behavioral domains, objective expressive language sampling, and phenotype-directed measures, such as eye-tracking and biomarkers.

Ganaxolone

Ganaxolone is a synthetic analog of the neuroactive steroid allopregnanolone, which acts as a positive allosteric modulator of $GABA_A$ receptors. Recent literature indicate these receptors are reduced in FXS (Braat et al., 2015; D'Hulst et al., 2009; D'Hulst et al., 2015; Gantois et al., 2006) with $GABA_A$ receptor subtypes containing a delta subunit being particularly diminished in the *Fmr1* KO model (D'Hulst et al., 2006). Neuroactive steroids, such as ganaxolone, could be well suited to the treatment of FXS because they potentiate the effects of $GABA_A$ receptors containing the delta subunit. Ganaxolone has also been previously used in the treatment of PTSD and epilepsy, and is generally safe and well-tolerated in children.

Preclinical trials of ganaxolone in the *Fmr1* KO mouse have decreased audiogenic seizures (Heulens et al., 2012), as well as stereotypic and repetitive behavior (Braat et al., 2015). This data encouraged a double-blind, placebo-controlled crossover trial of ganaxolone in children with FXS to monitor its effects on behavioral changes, particularly in areas of anxiety, hyperactivity, and overall clinical improvement. Subjects received 6 weeks of ganaxolone and 6 weeks of placebo with a maximum dosage of 54 mg/kg/day. The phases were separated by a 2-week washout period. Fifty-nine subjects were randomized, with 55 completing the first arm (93.2%) and 51 completing the second arm (89.4%) (Ligsay et al., submitted). The ages of participants ranged from 6–17 years (mean: Placebo-Ganaxolone 11.3 $\pm$ 0.6 years; Ganaxolone-Placebo 10.6 $\pm$ 0.6 years), and there were no significant demographic differences between the two groups. There were no serious adverse events that occurred throughout the study, although there was an increase in fatigue and drowsiness (Ligsay et al., submitted) during the ganaxolone treatment. Previous trials of ganaxolone (Study 1042-0600) have increased reports of fatigue and somnolence at similar frequencies compared to this study (16.3%–14.8% and 13.3%–12.2%, respectively). Efficacy results are currently being analyzed and will be available in this year.

Metadoxine

Metadoxine (pyridoxol L-2-pyrrolidone-5-carboxylate) is an ion-pair salt of pyridoxine (vitamin B6) and 2-pyrrolidone-5-carboxylate (PCA, also known as L-PGA) that has been used for over 30 years in multiple countries outside of the USA as an immediate-release form to treat acute alcohol intoxication, alcohol withdrawal syndrome, and chronic alcoholic liver disease (Manor et al., 2012). Metadoxine extended release (MDX, Alcobra Ltd., Tel Aviv, Israel) is a long-acting formulation of metadoxine, which demonstrated to be a modulator of GABAergic transmission with a monoamine-independent mechanism of action (Berry-Kravis, Rubin, Harary, & Daniely, 2015). In clinical trials of adults with ADHD, MDX has been well tolerated and demonstrated significant improvement in ADHD symptoms, neuropsychological test performance, and quality of life with a rapid onset of action (Manor, Rubin, Daniely, & Adler, 2014; Weisler, Adler, Rubin, Daniely, & Manor, 2014). Preclinical studies in the *Fmr1* KO mouse model suggested significant improvements in working memory, learning, and social interaction, as well as normalization of excessive ERK and Akt activation. These studies, in combination with work showing GABA transmission imbalance in the mouse model (Braat & Kooy, 2015), and the frequent ADHD symptomatology observed in FXS, prompted an exploratory phase II 6-week, randomized, multicenter, double-blind, parallel, flexed- and fixed-dose study of MDX compared with placebo in adolescents and adults with FXS (age 14–50 years) (Berry-Kravis et al., 2015). Sixty-two subjects were randomized in the study (MDX 30; placebo 32), and there were no safety concerns with adverse events in the MDX-treated group as compared to those in the placebo group. Preliminary reports of the efficacy results suggest the MDX group did not show improvement from baseline to week 6 relative to the placebo group on the primary outcome, the inattentive subscale of the ADHD RS-IV. However, the MDX group did show significant improvement over placebo, in the ITT population, on a key secondary endpoint of the VABS Daily Living Skills Domain ($P = 0.023$), as well as on the computerized cognitive Test of Attentional Performance for Children (KiTAP) Go-NoGo subscale ($P = 0.043$). The VABS findings were stronger in the higher-functioning and younger (age 14-18) groups of participants. It is expected that additional larger phase III studies will be performed to confirm these findings, as the VABS Daily Living Skills Domain is a highly clinically relevant measure of adaptive skills and improvement, and this could potentially impact life functioning and quality of life for individuals with FXS and their families.

CLINICAL TRIALS OF AGENTS TARGETING CELLULAR SIGNALING IN FXS

Trofinetide (NNZ-2566)

Trofinetide is a synthetic analog of a naturally occurring neurotrophic peptide, which is the terminal tripeptide of IGF-1, a growth factor produced by brain cells. In animal models, trofinetide has been shown to inhibit neuroinflammation, decrease overactivity of microglia, and correct deficits in synaptic function (Cartagena et al., 2013; Wei et al., 2009). An intravenous form of trofinetide is presently in a phase II clinical trial in patients with moderate to severe traumatic brain injury and an oral form of the drug is being studied in Rett Syndrome and FXS. Preclinical studies done in the *Fmr1* KO mouse showed reversal of cognitive and

behavioral phenotypes, dendritic spine abnormalities, and excessive ERK and Akt activity (Deacon et al., 2015). This prompted a 16-site randomized, double-blind, placebo-controlled, parallel group, fixed-dose trial in adolescent and adult males with FXS (age 12–45). Seventy subjects were randomized to one of three groups: placebo (25 subjects), 35 mg/kg twice per day (24 subjects), and 70 mg/kg twice per day (21 subjects). There were no safety concerns with regard to laboratory abnormalities or clinical reports of adverse events in the treated groups relative to the placebo group.

In this trial, two novel measures directed at measuring FXS characteristics were developed and utilized to define efficacy. The Fragile X Syndrome Rating Scale, which was based on studies that have evaluated the natural history of FXS, was developed in consultation with FXS clinical experts (Berry-Kravis et al., in review). The Fragile X Domain-Specific Concerns VAS is a clinician-completed scale that uses VAS to assess domain-specific individualized symptoms that are identified by the clinician as key areas of impairment. Concerns are identified on an individual, per-subject basis in one of six domains related to the subject's FXS: repetitive behaviors, speech and language, anxiety, phobias and social withdrawal, motor performance, sensory oversensitivity, and cognition (Berry-Kravis et al., in review). In addition, the Caregiver Top Three Concerns was developed as a caregiver-completed VAS that is intended to be syndrome specific. Caregivers identify three priority concerns related to the subject's FXS, which they would like to observe changes in as a result of treatment. This measure is similar to the successful caregiver concerns measure used in the phase II trial of arbaclofen (see earlier).

Five measures (clinician-completed Fragile X Syndrome Rating Scale, Fragile X Domain Specific Concerns, FXS-anchored CGI-I used a standardized scoring rubric that was specific to the clinical features of FXS, caregiver-completed Caregiver Top Three Concerns, and ABC_{FX} total score) were prespecified in the statistical analysis plan as core measures for the efficacy analyses. The analyses compared the mean clinical responses in the three treatment groups for each core measure, as well as compared the collective clinical responses in all core measures for each subject individually. The individual analysis was designed to confirm that the treatment benefit shown by the group mean responses was broadly evident and not simply due to a few large outlier responses. Permutation testing was done to estimate the probability that the observed clinical improvement in both the group-level and subject-level analyses was observed by chance, and this probability in a preliminary report was estimated as 4.5% ($P = 0.045$), providing statistical evidence of improvement in the higher-dose trofinetide-treated group over the placebo group, with respect to FXS-related symptoms (Treagus, 2015). This methodology represented a unique approach in measuring whether the drug might have a global effect on the FXS phenotype rather than modifying a specific behavior, and further development of this type of methodology may be critical to see responses for brain mechanism-targeted therapeutics that treat the whole disease, rather than having an effect on a specific behavior.

Lithium

Lithium is primarily used for the treatment of mood disorders, but may provide additional therapeutic benefit in FXS. It interacts with various pathways, notably through GSK3β, phospholipase C, and ERK, and has been shown to correct fragile X–associated defects in the FXS mouse model, at both the cellular and behavioral level, including mGluR-dependent LTD, memory

deficits, and anxiety (Berry-Kravis et al., 2011; Choi et al., 2011; Liu, Chuang, & Smith, 2011; McBride et al., 2005; Mines, Yuskaitis, King, Beurel, & Jope, 2010; Yuskaitis et al., 2010).

Data from animal models prompted a pilot open-label study at Rush University in 15 patients with FXS (Berry-Kravis et al., 2008). The patient's ages ranged from 6 to 30 years (mean 11 ± 5 years) with a mean IQ of 50.5 ± 4.9. The subjects were enrolled in a 2-month treatment period with 4 weeks uptitration to reach blood levels of 0.8–1.2 mEq/L. This was followed by a 4-week stable dose (mean levels at end of treatment period 0.90 ± 0.26 mEq/L). Study results showed significant improvement in areas of hyperactivity and inappropriate speech on the ABC-C, improvements in personal daily living skills and maladaptive behavior on the VABS, and significant improvements on the CGI and VAS. Laboratory data showed normalization of ERK phosphorylation rates in lymphocytes. There were no serious adverse events, and no discontinuation due to study-drug side effects. Seven of the subjects experienced polydipsia, and four subjects experienced polyuria. These AEs were transient, and resolved with discontinuation of the study medication. While these results showed promise, further studies have not yet been conducted due to concerns regarding lithium toxicity with chronic use.

CONCLUSIONS

Although there have been several controlled trials carried out in FXS, the promise of targeted treatments is just being realized, as we have learned through experience how to design trials with better outcome measures and inclusion strategies. Only subgroups of patients in any trial have a good response ,and a variety of environmental and learning factors may affect the outcome of a study. Although FXS is a single-gene disorder, the level of FMRP that is present varies across individuals and across the genders. In addition, the effect of background genetic changes is important especially when the absence of FMRP will influence many pathways through the regulation of dendritic translation. We have also postulated that combining a targeted treatment with a behavioral intervention, particularly in early childhood, may be a productive way to demonstrate the cognitive promise of targeted treatments. Such a trial design is being carried out in a controlled trial of lovastatin that is combined with a parent implemented language intervention (PILI) in children with FXS ages 10–18 years (NCT02642653). Another trial is planned for young children with FXS ages 3–6years who will be treated with mavoglurant combined with PILI. As new treatments, such as PAK inhibitors or cannabidiol (CBD), are studied and expanded trials of trofinetide and methotrexate are carried out, the design of the trials will improve with increased use of disease-specific quantitative, cognitive, and functional outcome measures and biomarkers, such as ERP, as well as analysis plans that evaluate improvement across the global FXS phenotype, and will be more likely demonstrate the benefits of these agents. The promise of targeted treatments for FXS is very much alive and expanding.

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CHAPTER

20

Reflections on Clinical Trials in Fragile X Syndrome

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INTRODUCTION

Fragile X syndrome (FXS) is a neurodevelopmental disorder with a majority of males presenting with mild-to-severe intellectual disability (Kaufmann, Abrams, Chen, & Reiss, 1999). The average IQ in men with FXS is 40–50, with a mental age of about 5–6 years. The developmental trajectory is slower than neurotypical children and adolescents, resulting in a decline in IQ and adaptive behavior scores throughout childhood (Dykens, Hodapp, & Leckman, 1987; Fisch et al., 1999).

Beyond the intellectual disabilities, there is a pattern of intellectual weaknesses and strengths in individuals with FXS. Relative weaknesses include visuospatial skills, working memory, processing of sequential information, and attention (Dykens et al., 1987), while simultaneous processing and long-term memory are relative strengths.

FXS is an X-linked disorder, and females with FXS are on average less affected than males, with a mean IQ of 80 and a much broader range of involvement from severe impairment to normal cognitive skills. About 25% have cognitive impairment and others are frequently being diagnosed with learning disabilities.

Over 50% of males and 20% of females meet diagnostic criteria for autism spectrum of disorders (ASD) (Lewis et al., 2006; McDuffie et al., 2010). Pragmatic deficits, reduced eye contact, social anxiety, difficulty with regulation of attention and activity level, self-injurious behaviors, and aggression are some of the important symptoms driving autism diagnoses in individuals with FXS. FXS contributes to the group of low-functioning ASDs, and is not identified in ASDs with normal or high IQ. Interestingly, genomic studies in individuals with autism show that genes altered by likely gene disrupting genomic variants are enriched in fragile X mental retardation portein (FMRP)–associated genes. These genes form a large group of approximately 800 postsynaptic proteins thought to play a major role in neuroplasticity.

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This suggests that genes controlled by FMRP (at the translational level) are also individually associated with ASDs.

SYMPTOMATIC TREATMENTS

Current treatments focus on symptomatic management and include stimulants for attention deficit hyperactivity disorder (ADHD) and impulsivity (Hagerman, Murphy, & Wittenberger, 1988); α2-agonists for sensory overstimulation, hyperarousal, hyperactivity, and sleep disturbances (Ingrassia & Turk, 2005); anticonvulsants for seizures and mood instability (Hagerman et al., 2009); and antipsychotics and antidepressants for aggression, anxiety, and sleep disturbance (Erickson, Stigler, Posey, & McDougle, 2010; Hagerman et al., 2009). Only few randomized clinical trials (RCTs) have been conducted for symptomatic management in FXS. Nevertheless, off-label symptomatic treatments are commonly used, but practice varies widely across countries and clinical centers. The available data on RCTs for symptomatic treatments is summarized further and in Table 20.1.

Trials Targeting Attention Deficit and Hyperactivity

ADHD is prevalent in boys with FXS (73%) (Baumgardner, Reiss, Freund, & Abrams, 1995). The first trial targeting ADHD in FXS was a 3-week randomized double-blind placebo-controlled crossover study in 15 children. Participants received methylphenidate, dextroamphetamine, and placebo in a random manner for a 1-week period each. Significantly improved performance in the ADD-H: Comprehensive Teacher Rating Scale (ACTeRS) was reported for methylphenidate, but not for dextroamphetamine; levels of significance reported by the authors were very close: $P = 0.02$ and $P = 0.08$ (Hagerman et al., 1988). L-Acetylcarnitine (LAC) has been studied in two double-blind placebo-controlled trials in FXS. LAC significantly reduced hyperactive behavior ($P = 0.006$) in a first small study (Torrioli et al., 1999), which lead to a larger study replicating the reduction of hyperactivity [Clinical Global Impression Scale-Parent (CGI-P), $P = 0.05$], and further demonstrated improvement in social and adaptive behavior (Vineland ABC domain, $P = 0.04$ and socialization domain, $P = 0.008$) in the treated boys compared to the nontreated (Torrioli et al., 2008). The authors suggested LAC as an alternative treatment to the use of stimulants in children with FXS.

Sleep

Sleep difficulties are reported in 32% of children with FXS who present difficulties falling asleep and multiple awakenings (Kronk et al., 2010). Melatonin was studied in a 4-week placebo-controlled, double-blind, crossover trial in children with autism and FXS. Melatonin significantly decreased sleep latency, and increased sleep duration and sleep-onset time. A trend toward a decrease in the number of awakenings during the night was observed (Wirojanan et al., 2009).

TABLE 20.1 Symptomatic Treatment Trials in Fragile X Syndrome (FXS) With ≥8 Individuals

								Primary outcomes											Biomarkers				
Target	**Drug**	**Design**[a]	**Study period**[b]	***n***[c]	**Age (years)**	**Sex (F/M)**	**Efficacy**[d]	**ABC**	**ACTeRS**	**BGT**	**CGI-I**	**Conners P/T**	**MSEL**	**Impulsivity**[e]	**Sleep**[f]	**VABS**	**WISC-R**	**EGF**	**ET**	**HR**	**LSI**	**RSA**	**SC**
ADHD	Methylphenidate or dextroamphetamine	RCT	0.1 month	15	3–12	2/13	Y	—	√	—	—	√	—	√	—	—	—	—	—	√	—	—	—
	LAC	RCT	12 month	63	6–13	—/63	Y	—	—	—	—	√	—	—	—	√	√	—	—	—	—	—	—
	LAC	RCT	12 month	20	6–13	—/20	Y	—	—	√	—	√	—	—	—	—	√	—	—	—	—	—	—
	Valproic acid	OL	6 month	10	7–16	—/10	Y	—	—	—	—	√	—	—	—	—	—	—	—	—	—	—	—
Sleep	Melatonin	RCT	0.2 month	12	2–15.3	1/11[g]	Y	—	—	—	—	—	—	—	√	—	—	—	—	—	—	—	—
Anxiety	Oxytocin	RCT	1 day	10	13–29	—/10	Y	—	—	—	—	—	—	—	—	—	—	√	—	√	—	√	√
	Sertraline	RCT	6 month	57	2–5.8	9/48	Y	—	—	—	√	—	EL	—	—	—	—	—	√	—	—	—	—
Irritability	Aripiprazole	OL	3 month	12	5–35	1/11	Y	I	—	—	√	—	—	—	—	—	—	—	—	—	—	—	—

(*Continued*)

TABLE 20.1 Symptomatic Treatment Trials in Fragile X Syndrome (FXS) With ≥8 Individuals (*cont.*)

		Secondary outcomes												
Target	**Drug**	**ABC**	**ADOS**	**CGI-I/S**	**Conners-P/T**	**CYBOCS-PDD**	**K-SADS-PL**	**MSEL**	**PLS**	**SNAP-IV**	**SPM/SP**	**SRS**	**VABS**	**VAS C/P**
	Methylphenidate or dextroamphetamine	—	—	—	—	—	—	—	—	—	—	—	—	—
ADHD	LAC	—	—	—	—	—	—	—	—	—	—	—	—	—
	LAC	—	—	—	—	—	—	—	—	—	—	—	—	—
	Valproic acid	—	—	√	√	—	√	—	—	√	—	—	√	—
Sleep	Melatonin	—	—	—	—	—	—	—	—	—	—	—	—	—
Anxiety	Oxytocin	—	—	—	—	—	—	—	—	—	—	—	√	—
	Sertraline	—	√	—	—	—	—	FM, VR, and RL	√	—	√	—	√	√
Irritability	Aripiprazole	C	—	√	—	√	—	—	—	—	—	√	—	—

ABC-C, Aberrant Behavior Checklist-Community Edition with all individual subscales (ABC-H, Hyperactivity Subscale; ABC-I, Irritability Subscale; ABC-IS, Inappropriate Speech Subscale; ABC-SB, Stereotypic Behavior Subscale; ABC-SW, Social Withdrawal/lethargy Subscale); ADOS-2, Autism Diagnostic Observation Scale; BGT, Bender Gestalt test; CGI-I, Clinical Global Impression-Improvement; CGI-S, Clinical Global Impression-Severity; Conner's-P/T, Conner's Parent/Teacher Rating Scale questionnaire; CYBOCS-PDD, Children's Yale–Brown Obsessive Compulsive Scale modified for pervasive developmental disorders; EGF, eye gaze frequency; ET, eye tracking; HR, heart rate; HRV, heart rate variability; K-SADS PL, Kiddie Schedule for affective disorder and schizophrenia for school age children present and lifetime version; LAC, L-acetylcarnitine; LSI, Large Scale Integrated Sensor actometer (locomotion); MSEL, Mullen Scales of Early Learning, Early Learning Composite with four of it's subtests (EL, expressive language; RL, receptive language; FM, fine motor; VR, visual reception skills); PLS, preschool language scale IV; RSA, respiratory sinus arrhythmia; SNAP-IV, Swanson, Nolan, and Pelham-IV Questionnaire for Attention Deficit Hyperactivity Disorder; SP, Sensory Profile; SPM, Sensory Processing Measure (Preschool Home Form); SRS, Social Responsiveness Scale; VABS, Vineland Adaptive Behavior Scales, Second Edition (Vineland-II); VAS, Visual Analog Scale (VAS-C, clinician rated; VAS-P, parent rated); WISC-R, Wechsler Intelligence Scale for Children-Revised.

[a] *Design: OL, open label; RCT, randomized clinical trial.*

[b] *Study period = treatment duration.*

[c] *Number of subject enrolled into the study.*

[d] *Reported Efficacy: N, no; Y, yes.*

[e] *Impulsivity tasks include delay and vigilance tasks.*

[f] *Sleep includes: sleep onset, sleep duration, sleep latency, and number of night awakenings.*

[g] *Six of 12 study subjects were diagnosed with FXS.*

Anxiety

Social anxiety with hyperarousal and eye gaze avoidance is a common behavioral problem especially in males with FXS (Hagerman et al., 2009). In females, anxiety and symptoms interpreted as secondary to anxiety, such as selective mutism, have been treated with selective serotonin reuptake inhibitors (SSRIs). This is based on the large trials performed in separation anxiety disorder, social phobia, or generalized anxiety disorder in children between 7 and 17 years of age (Giles & Martini, 2016), but no RCT in FXS supports this approach (Berry-Kravis & Potanos, 2004; Hagerman et al., 2009). Experiences with autism shows that these benefits may not generalize to similar symptoms in a group of individuals with neurodevelopmental disorders, and the review of the data clearly shows insufficient evidence to demonstrate benefits and evaluate the adverse effects for SSRIs (Hammerness, Vivas, & Geller, 2006; McPheeters et al., 2011). Sertraline was recently tested in a double-blind placebo-controlled trial targeting autistic behavior and language in 57 FXS children aged 2–6 years (NCT01474746) (Greiss Hess et al., 2016). Sertraline was well tolerated during the 6-month treatment, but showed no benefit in primary outcome measures [the Mullen Scales of Early Learning (MSEL) expressive language and CGI-I] over placebo. There were, however, nominal significant improvements of secondary outcome measures, including the age-equivalent combined subtest scores of the MSEL, the cognitive *T*-score sum of the MSEL, and both the age-equivalent and raw scores of fine motor coordination and visual perceptual subtests of the MSEL, as well as raw score of the social participation subscale from the Sensory Processing Measure-Preschool Home Form. Posthoc analysis further demonstrated significant improvement in early expressive language development in a subgroup of participants with ASD (Greiss Hess et al., 2016).

Beyond the interpretation of these results, which may or may not suggest potential benefit of sertraline, this study is of particular interest because it is among the very few RCTs investigating young FXS individuals. In particular, this study demonstrates that very young participants can be assessed and may complete such demanding studies. It also highlights the challenges of working during a period of steep developmental trajectory, where one cannot distinguish between placebo effects and natural development.

Novel symptomatic treatments have been investigated, including oxytocin. In a randomized double-blind placebo-controlled trial, the effect of a single dose of intranasal oxytocin was studied in eight males with FXS. The study measured eye gaze frequency, heart rate, heart rate variability, respiratory sinus arrhythmia, and salivary cortisol concentration during social stressors. Significant improvement in the frequency of eye gaze and salivary cortisol levels were reported (Hall, Lightbody, McCarthy, Parker, & Reiss, 2012).

Irritability

The use of antipsychotics for irritability in FXS stems from the results of large trials showing efficacy of these drugs for disruptive behaviors in autism (McPheeters et al., 2011). Aripiprazole is among the newer-generation antipsychotics that have been approved by the Food and Drug Administration (FDA) for irritability in patients with autism. The only data available in FXS is a small (n = 12) open-label trial of aripiprazole. Improvement of several measures was reported, including primary (Aberrant Behavior

Checklist-Irritability subscale, ABC-I) and secondary outcome measures [Clinical Global Impressions-Severity, Social Responsiveness Scale (SRS), and Aberrant Behavior Checklist-Hyperactivity (ABC-H) subscale] (Erickson et al., 2011). RCTs of antipsychotics have not been conducted in FXS.

Conclusions on Data Supporting Symptomatic Treatments

Symptom-targeted drugs are among the most common approaches for the treatment of patients with FXS. These drugs may be helpful for alleviating problematic behaviors in FXS, but overall studies have not clearly demonstrated the benefit of these approaches in FXS. Well-powered multicentric RCTs studies are therefore warranted, as it is unlikely that any center alone can generate sample sizes required for appropriate power.

Interestingly, authors of many studies report "efficacy," which creates confusion in the field because these claims do not survive critical review. As an example, a significant research effort was required to produce two independent studies of LAC in FXS, including a total of over 80 individuals. Efficacy is reported in the abstract, but a systematic review (Rueda, Guillén, Ballesteros, Tejada, & Solà, 2015) concluded that there were no clear evidence of differences in verbal and nonverbal intellectual functioning, no clear improvement in teacher assessments of hyperactive behavior, and that improvements in parent-assessed hyperactive behavior had no clinical significance. Insufficient information about randomization, allocation, blinding of evaluators, and the presence of employees from the drug company in the research teams, as well as funding by the drug company was noted. A Grading of Recommendations Assessment, Development, and Evaluation (GRADE) rated the evidence as low quality (Rueda et al., 2015). Another systematic review on the trials in FXS concluded that there was no robust evidence to support recommendations on pharmacological treatments in general in patients with FXS or in those with additional diagnosis of ADHD or autism (Rueda, Ballesteros, & Tejada, 2009).

A UNIQUE TARGETED DRUG DEVELOPMENT EFFORT

FXS was among the first molecularly characterized neurodevelopmental disorder. Over a decade of preclinical work has generated robust results demonstrating that metabotropic glutamate receptors (mGluR5) inhibitors rescue multiple phenotypes in animal models caused by the loss of function of the fragile X mental retardation gene (*Fmr1*). This has led to the development of one of the most comprehensive drug development programs undertaken thus far for a neurodevelopmental disorder. It was conducted in parallel through pharmaceutical- and investigator-led trials, assessing the effect of compounds targeting many different steps of FMRP-related signaling pathways. These are also the largest studies conducted in genetically defined subgroups of psychiatric disorders. However, what initially appeared as an optimal translational scenario did not lead to the expected results (Berry-Kravis et al., 2016; Jacquemont et al., 2011).

Overview of Preclinical Studies

Translational research in neurodevelopmental disorders is in its infancy, relative to the other biomedical fields and will likely struggle with similar or even greater issues. In oncology, a field with a much more mature understanding of mechanisms, rates of translation are approximately 8% (Mak, Evaniew, & Ghert, 2014) with many targeted molecular approaches showing extremely different outcomes in mice and humans (M.J. Lee et al., 2012; Zeidler, Hukema, & Willemsen, 2015).

Among the different outcome measures used in preclinical studies, protein synthesis, dendritic spine density, morphology, long-term depression (LTD), and audiogenic seizures are some of the most robust phenotypes observed in FXS mice. They have been consistently rescued by a series of genetic approaches and pharmacological compounds, including mGluR antagonists (MPEP, fenobam, mavoglurant, and CTEP), gamma-aminobutyric acid (GABA) agonist, inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA), mTOR inhibitors, and specific targets of FMRP (such as MMP-9, p110-β, AβPP, PIKE, STEP, BKCA, and Kv4.2) (Bhattacharya et al., 2012; Bilousova et al., 2009; Boda, Mendez, Boury-Jamot, Magara, & Muller, 2014; Busquets-Garcia et al., 2013; Dolan et al., 2013; Gross et al., 2010, 2015; Hayashi et al., 2007; Henderson et al., 2012; Hébert et al., 2014; Liu, Huang, & Smith, 2012; Osterweil, Krueger, Reinhold, & Bear, 2010; Thomas et al., 2011; Tian et al., 2015; Udagawa et al., 2013).

However, many of the classic behavioral phenotypes used as outcome measures in preclinical trials are inconsistently observed across different FXS mice models (open field, rotarod, elevated plus maze, marble burying, self grooming, and most social paradigms). As a result, the rescue of these phenotypes has been difficult to document (Bhattacharya et al., 2012; de Esch et al., 2015; Gantois et al., 2013; Hébert et al., 2014; Westmark et al., 2011).

Overview of Targeted Randomized Placebo-Controlled Clinical Trial

Based on the work with FXS animal models, several therapeutic targets have been tested in clinical trials. We will mainly cover placebo-controlled randomized clinical trials (PC RCT) presented in Table 20.2, and will briefly mention open-label studies that are not designed to evaluate efficacy.

Modulating Translational Control Through Pre- and Postsynaptic Receptors

Two main targets are covered in this section: (1) negative modulators of mGluR5 signaling and (2) GABA agonists.

PRE- AND POSTSYNAPTIC RECEPTORS: METABOTROPIC GLUTAMATE RECEPTORS

Preclinical results indicated that mGluR-dependent LTD signaling is enhanced in the hippocampus of *Fmr1* knockout (KO) mice lacking FMRP (Bear, Huber, & Warren, 2004). This led to the "mGluR theory of FXS," which proposes that the absence of FMRP can cause overactivation of mGluR signaling, leading to enhanced hippocampal LTD and contribute to the features of the FXS phenotype (Chapter 9) (Bear et al., 2004; Dolen & Bear, 2008). Subsequently, multiple genetic and pharmacological preclinical studies have aimed at reducing the mGluR signaling. Selective antagonists of mGluR5, a group 1 mGluR, have been evaluated in a number of preclinical and clinical studies. Protein synthesis, dendritic spine density, and

TABLE 20.2 Randomized Placebo-Controlled Clinical Trials in FXS With ≥8 Individuals

										Primary Outcomes										Biomarkers					
Target	Drug	Phase	Study period (months)[a]	*n*[b]	Age (years)	Sex (F/M)	Stratification[c]	Status[d]	Efficacy[e]	ABC	ADAMS–Total score	ADHDRS	CGI-I	CNT	ELS	IQ (SB)	Memory[f]	VAS	APP	Akt	ERK	ERP	ET	MMP-9	PPI
mGluR5 receptor	AFQ056[g]	2	1	30	18–35	—/30	√	C	Y	C	—	—	—	—	—	—	—	—	—	—	√	—	√	—	√
	AFQ056[g]	2b	3	175	18–45	11/164	√	C	N	C_{FX}	—	—	—	—	—	—	—	—	—	—	—	—	√	—	—
	AFQ056[g]	2b	3	139	12–17	15/124	√	C	N	C_{FX}	—	—	—	—	—	—	—	—	—	—	—	—	√	—	—
	RO4917523	2a	1.5	40	18–50	NA	—	C	NA	—	—	—	—	—	—	—	—	—	—	—	—	—	√	—	√
	RO4917523	2b	3	185	14–50	NA	—	C	N	—	√	—	—	—	—	—	—	—	—	—	—	—	√	—	—
	RO4917523	2a	3	47	5–13	NA	—	C	NA	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Intracellular signaling	NNZ-2256	2	1.5	72	12–45	0/72	—	C	NA	—	—	—	—	—	—	—	—	—	—	√	√	—	√	—	—
	Metadoxine[h]	2	1.5	62	15–55	15/47	—	O	NA	—	—	√	—	—	—	—	—	—	—	√	√	—	√	—	—
	Lovastatin/PILI	4	5	104	10–17	NA	—	O	NA	—	—	—	—	—	√	—	—	—	—	—	—	—	—	—	—
Proteins regulated by FMRP	Minocycline	2	3	66	3.5–16	8/47	—	C	Y	—	—	—	√	—	—	—	—	√	—	—	—	—	—	√	—
AMPA receptor	CX516[i]	2	1	49	18–50	11/38	—	C	N	—	—	—	—	—	—	—	√	—	—	—	—	—	—	—	—
GABA modulators	Arabaclofen	2	1	63	6–40	8/55	√	C	Y	I	—	—	—	—	—	—	—	—	√	—	—	—	—	—	—
	Arabaclofen	3	2	125	12–50	NA	√	C	N	SA	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	Arabaclofen	3	2	172	5–11	NA	√	C	NA	SA	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	Acamprosate	2/3	2.5	48	5–23	NA	—	O	NA	SW	—	—	—	—	—	—	—	—	√	—	√	—	√	—	—
	Ganaxolone	2	1.5	60	6–17	NA	—	O	NA	—	—	—	√	—	—	—	—	—	—	—	—	√	√	—	√
	Donepezil	2	3	42	12–29	15/27	—	C	NA	—	—	—	—	√	—	—	—	—	—	—	—	—	—	—	—
	Donepezil	2	3	20	6–15	—/20	—	C	N	—	—	—	—	—	—	√[j]	—	—	—	—	—	—	—	—	—

		Secondary outcomes																												
			Behavior														Autism					Cognition/language							Others	
Target	Drug	ABC (subscales)	ADAMS	ADHDRS	Conners	CASI	CYBOCS-PDD	GBAS	IVA	PARS	PedsQL	RBANS (LL/SM)	RBS	SNAPI-V	VABS	VAS	ADOS	CARS	GARS	SRS	CELF	ELS	EVT	KiTAP	PLS-4	PPVT	SB5-WM	TEA-Ch	CGI-I/S	CSQ	PSI
mGluR5 receptor	AFQ056	C	—	—	—	—	—	—	—	—	—	√	√	—	√	√	—	—	—	√	—	—	—	√	—	√	—	—	√	—	—
	AFQ056	C	—	—	—	—	—	—	—	—	—	—	√	—	—	—	—	—	—	√	—	—	—	√	—	—	—	—	√	—	—
	AFQ056	C	—	—	—	—	—	—	—	—	—	—	√	—	—	—	—	—	—	√	—	—	—	√	—	—	—	√	√	—	—
	RO4917523	C	√	—	—	—	—	—	—	—	—	√	—	—	—	√	—	—	—	—	—	—	—	√	—	—	—	—	√	—	—
	RO4917523	C	√	—	—	—	—	√	—	—	—	√	—	—	—	√	—	—	—	√	—	—	—	√	—	—	—	—	√	—	—
	RO4917523	C	√	—	—	—	—	√	—	—	—	√	—	—	—	√	—	—	—	—	—	—	—	—	—	—	—	—	√	—	—
Intracellular signaling	NNZ-2256	C_{FX}	—	—	—	√	√	—	—	√	—	—	—	—	√	C, P	—	—	—	√	—	√	—	√	—	—	—	—	√	—	—
	Metadoxine	C_{FX}	—	—	—	—	—	—	—	√	—	√	—	—	√	—	—	—	—	√	—	—	—	√	—	—	—	—	√	—	—
	Lovastatin/PILI	C_{FX}/SA	—	—	—	—	—	—	—	—	—	—	—	—	—	√	—	—	—	—	—	√	—	—	—	—	—	—	√	—	—
Proteins regulated by FMRP	Minocycline	C_{FX}	—	—	—	—	—	—	—	—	—	—	—	—	√	√[k]	—	—	—	—	—	—	√	—	—	—	—	—	—	—	—
AMPA receptor	CX516	C	—	—	—	—	—	—	√	—	—	—	—	√	√	√	√	√	√	—	√	—	—	—	√	√	—	—	√	—	—
GABA modulators	Arabaclofen	C_{FX}	—	√	—	√	—	—	—	—	—	√	√	—	√	√	—	—	—	√	—	—	—	—	—	√	√	—	√	—	—
	Arabaclofen	C_{FX}	—	—	—	—	—	—	—	—	—	—	—	—	√	√	—	—	—	√	—	—	—	—	—	—	—	—	√	—	√
	Arabaclofen	C_{FX}	—	—	—	—	—	—	—	—	—	—	—	—	√	√	—	—	—	√	—	—	—	—	—	—	—	—	√	—	√
	Acamprosate	C_{FX}	√	—	—	—	√	—	—	√	√	—	—	—	√	√	—	—	—	√	—	√	—	—	—	—	—	—	√	√	—
	Ganaxolone	C_{FX}	√	—	—	—	—	—	—	√	—	—	—	√	—	√	—	—	—	—	—	—	—	√	—	—	—	—	—	—	—
	Donepezil	C	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	Donepezil		—	—	√	—	—	—	—	—	—	—	—	—	—	—	—	√	—	—	—	—	—	—	—	—	—	—	—	—	—

(*Continued*)

TABLE 20.2 Randomized Placebo-Controlled Clinical Trials in FXS With ≥8 Individuals *(cont.)*

ABC-C, Aberrant Behavior Checklist-Community Edition with all individual subscales (ABC-H, Hyperactivity Subscale; ABC-I, Irritability Subscale; ABC-IS, Inappropriate Speech Subscale; ABC-SB, Stereotypic Behavior Subscale; ABC-SW, Social Withdrawal/lethargy Subscale); ABC-C_{FX}, Aberrant Behavior Checklist-Community Edition refactored for FXS (all six subscales, ABCFX-SA, Social Avoidance Subscale); ADAMS, Anxiety Depression and Mood Scale; ADHDRS, ADHD Rating Scale IV; ADOS, Autism Diagnostic Observation Scale; Akt, protein kinase B (PKB); APP, amyloid precursor protein; BRIEF, Behavior Rating Inventory of Executive Function; Card Task, Card Task Test of Visual Sequential Memory; CARS, Childhood Autism Rating Scale; CASI, Childhood Anxiety Sensitivity Index; CELF, Clinical Evaluation of Language Skills; CFXCPT, Carolina Project Fragile X Continuous Performance Test; CGI-I/S, Clinical Global Impression-Improvement/Severity; CNT, Contingency Naming Task; CSQ, Caregiver Strain Questionnaire; CYBOCS-PDD, Children's Yale–Brown Obsessive Compulsive Scale modified for pervasive developmental disorders; ELS, expressive language sampling; ERK, extracellular regulated kinase-related kinase activation rate; ERP, evoked response potentials; ET, eye tracking; EVT, expectancy-value theory; GARS, Gillam Autism Rating Scale; GBAS, Global Behavior Assessment Scale; HR, heart rate; HRV, heart rate variability; HVLT, Hopkins Verbal Learning Test; IVA, Integrated Visual–Auditory Continuous Performance Test; KiTAP, Test of Attentional Performance for Children; MMP-9, matrix metalloproteinase-9; NEPSY, developmental NEuroPSYchological assessment (Tower subtest); PARS, Pediatric Anxiety Rating Scale; PedsQL, Pediatric Quality of Life Scale; PLS-4, Preschool Language Scale-version 4; PPI, prepulse inhibition; PPVT, The Peabody Picture Vocabulary Test; PSI, Parenting Stress Index; RBANS (LL/SM), Repeatable Battery for the Assessment of Neuropsychological Status (LL, List Learning; SM, Story Memory); RBS, Repetitive Behavior Scale; RSA, Respiratory Sinus Arrhythmia; SB5-WM, Stanford-Binet Version 5 Working Memory index; SNAP-IV, Swanson, Nolan, and Pelham-IV Questionnaire for Attention Deficit Hyperactivity Disorder; SRS, Social Responsiveness Scale; TEA-Ch, Test of Every Day Attention for Children; TVPS, Test of Visual Perceptual skills; VABS, Vineland Adaptive Behavior Scales; VAS, Visual Analog Scale (VAS-C, clinician rated; VAS-P, parent rated); W-JMem, Woodcock–Johnson Memory for Words.

[a] *Study period = treatment duration.*

[b] *Number of subject enrolled into the study.*

[c] *Stratification strategy: in the AFQ056 studies, participants were divided in strata depending on methylation status in completely methylated (CM) and partial methylated (PM). In the arbaclofen studies, social withdrawal was used for stratifying participants.*

[d] *Status: C, completed; O, ongoing; T, terminated.*

[e] *Reported efficacy: N, no; NA, not available; Y, yes.*

[f] *Memory includes TVPS, W-JMem, and RBANS.*

[g] *AFQ056 studies phase 2b: KiTAP and eye tracking only some sites. In a limited number of study sites (three) in the United States, a cognitive test battery in adults were conducted that included four modules: Visual Memory Test (VIM), Symbol Digit Coding (SDC), Perception of Emotions Test (POET), and three-part Continuous Performance Test (CPT) [Working Memory Test (WMT)] (Berry-Kravis et al., 2016).*

[h] *Metadoxine also targets the GABA modulators group.*

[i] *Z-scores reported for all outcome measures.*

[j] *In this trial the Stanford–Binet Intelligence Scale used is the Hindi adaptation by Kulshrestha.*

[k] *Multiple behaviors.*

morphology, LTD, and audiogenic seizures are some of the most robust phenotypes observed in FXS mice, and were consistently rescued in preclinical studies evaluating mGluR5 antagonists, including MPEP, fenombam, mavoglurant, basmiglurant, and CTEP (Dolen et al., 2007; Gross et al., 2010, 2015; Michalon et al., 2012; Osterweil et al., 2010; Thomas et al., 2011). Among these, mavoglurant and basmiglurant are structurally novel noncompetitive mGluR5 inhibitors, which have successfully rescued molecular, neuronal spine, and behavioral phenotypes in the *Fmr1* KO mice (Gantois et al., 2013; Levenga et al., 2011; Michalon et al., 2012) and were subsequently used in several large RCTs in humans.

Negative modulators of the mGluR5 receptor have been tested in double-blind, PC RCTs. Mavoglurant (AFQ056), a noncompetitive mGluR5 inhibitor, was initially evaluated in a phase 2, randomized, double-blind, placebo-controlled, crossover design trial in 30 adult male FXS patients treated for 28 days (NCT00718341). This study suggested improvement in maladaptive behavior in a posthoc analysis in the subgroup with full methylation of *FMR1* (Jacquemont et al., 2011). However, in a subsequent larger phase 2b, double-blind RCT, no improvement was demonstrated. This trial used the same outcome measures and the same stratification approach based on methylation status. It was a 3-month treatment trial designed with parallel groups evaluating multiple doses of mavoglurant in adult patients with FXS stratified (Berry-Kravis et al., 2016). Simultaneously, an almost identical trial was conducted in adolescents (Berry-Kravis et al., 2016). This was motivated by research in animal models and early intervention studies in other neurodevelopmental disorders, indicating that greater improvement may be achieved when treatment was conducted early in development (Dawson et al., 2010; Silva-Santos et al., 2015). In particular, although selective mGluR5 inhibitors have been effective in adolescent/adult FXS mouse models, improvements were larger in younger animal models (Michalon et al., 2012).

In a second large effort evaluating another mGluR5 negative modulator (RO4917523, basimglurant) the safety and tolerability was studied in two phase 2 trials in children and adolescents (NCT01750957) and in adults (NCT01015430). In a third trial including 185 adolescents and adults (NCT01517698), the primary outcome measure [a change in total score of the Anxiety Depression and Mood Scale (ADAMS)] was not meet and the program was terminated. The results are still unpublished. Of note, none of these studies were designed to addressed cognitive or learning outcomes (Berry-Kravis et al., 2016).

MODULATING GAMMA-AMINOBUTYRIC ACID SIGNALING

Several studies have shown impairment in the inhibitory, GABAergic system in FXS syndrome (Braat & Kooy, 2015). Differential expression of several $GABA_A$ receptor subunits and other components of the GABAergic system, including enzymes involved in GABA synthesis (*Gad1* and *Gad2*), GABA transporters (*Slc6a1* and *Slc6a11*), and an enzyme involved in GABA degradation (*Aldh5a1*). Decreased GABA concentrations have been reported in several brain regions. In addition to an overall down regulation of the GABAergic system that is brain region and age dependent, a delayed excitation–inhibition switch of $GABA_A$ receptors has been reported in the *Fmr1* KO mice from postnatal day 10 to postnatal day 14 (He, Nomura, Xu, & Contractor, 2014). A high-throughput screening of 2000 compounds in a FXS fly model for their ability to rescue the glutamate-induced lethality demonstrated that three of the nine lead compounds were modulators of the GABAergic system (Chang et al., 2008). Subsequent studies with the GABA-mimetic gaboxadol in *Fmr1* KO mice demonstrated a correction of

deficits in the open-field test and modulation of altered sensorimotor gating, measured with prepulse inhibition (PPI) after acute treatment (Olmos-Serrano, Corbin, & Burns, 2011). Acute treatment of a FXS mouse model with ganaxolone corrected audiogenic seizures (Heulens, D'Hulst, Van Dam, De Deyn, & Kooy, 2012) and deficits in the marble-burying assay (Braat & Kooy, 2015). Acute maternal pretreatment with bumetanide was able to restore the excitation–inhibition switch in neonatal FXS mouse model and corrected electrophysiological and behavioral phenotypes (Tyzio et al., 2014).

$GABA_B$ agonist arbaclofen presumably lowers presynaptic glutamate release with resultant reduction of group 1 mGluR signaling. In a phase 2 double-blind placebo-controlled crossover, flexible-dose trial (Berry-Kravis et al., 2012), arbaclofen did not show any improvement in the primary outcome measure (ABC-I) but posthoc analyses showed improvement over placebo in the group with social withdrawal. Improvements in a secondary outcome measures (parent-nominated problem behaviors) were also reported (Berry-Kravis et al., 2012). However, a large phase 3 placebo-controlled trial in adolescents and adults with FXS did not replicate these findings and showed no benefits for arbaclofen over placebo in the primary outcome of social withdrawal (unpublished data). Clinical observations from long-term extension studies with both arbaclofen and AFQ056 have suggested that there may be long-term cognitive and functional benefits of these drugs that were not captured by formal measures employed in the trials.

Acamprosate, currently FDA approved for alcohol withdrawal, with agonist properties at both $GABA_A$ and $GABA_B$ receptors (Boismare et al., 1984) and as mGluR5 antagonist (Harris et al., 2002), has shown promise in an open-label trial for hyperactivity and social functioning in FXS (Erickson et al., 2013). Significant improvement was reported for the ABC-H subscale ($P = 0.009$), ADHD Rating Scale (ADHDRS) ($P < 0.0001$), SRS ($P = 0.005$), and Global Clinical Impression-Severity (CGI-S) ($P < 0.0001$) (Erickson et al., 2013). A phase 2/3 double-blind, placebo-controlled proof-of-concept study of acamprosate in FXS individuals (5–23 year, targeting 48 participants) is currently ongoing (NCT01911455).

Ganaxolone, a $GABA_A$ receptor agonist and neuroactive steroid, was found to prevent audiogenic seizures in the *Fmr1* KO mouse (Braat et al., 2015; Heulens et al., 2012) and to be well tolerated in humans (Monaghan, Navalta, Shum, Ashbrook, & Lee, 1997). It is currently being tested in a small phase 2 placebo-controlled crossover trial in FXS children aged 6–17 years with an estimated enrollment of 60 participants (NCT01725152).

Donepezil is an FDA-approved treatment for Alzheimer's disease that acts as an acetylcholinesterase inhibitor and increases choline levels. *FMR1* is highly expressed in cholinergic neurons during early development (Abitbol et al., 1993). A magnetic resonance spectroscopy study of nine individuals with FXS reported reduced choline levels (Kesler, Lightbody, & Reiss, 2009), and in the *Fmr1* KO mouse model, abnormal cholinergic function in a limbic region involved in learning and memory (the subiculum) was observed (D'Antuono, Merlo, & Avoli, 2003). These results led to an open-label trial of eight FXS subjects reporting significant improvement in working memory and mental flexibility (CNT $P = 0.05$) and behavior (CBCL, $P = 0.009$; ABC-T, $P = 0.002$), including hyperactivity ($P = 0.04$) and irritability ($P = 0.009$) (Kesler et al., 2009). Subsequently, in a study of 20 males with FXS, donepezil was not effective in any of the outcome measures (Sahu et al., 2013). Another study enrolled 42 FXS individuals to evaluate the effect of donepezil on working memory and behavior (NCT01120626). Results are unpublished.

Modulating Translational Control at the Synapse by Targeting Intracellular Signaling

A novel synthetic analog of a naturally occurring neurotrophic peptide derived from insulin-like growth factor-1 (IGF-1), trofinetide (NNZ-2566), was reported to correct memory and learning deficits, normalize dendritic spines, and restore extracellular signal–related kinase (ERK) signaling in a FXS mouse model (Deacon et al., 2015). In a rat model of traumatic brain injury, trofinetide has also been shown to have neuroprotective effects by reducing neuroinflamation, improving recovery and reducing apoptotic cell death (Cartagena et al., 2013; Lu et al., 2009; Wei et al., 2009).

In a small double-blind placebo-controlled trial of adolescent and adult males with FXS, safety and tolerability of trofinetide was recently studied (unpublished results, NCT01894958). Trofinetide has also been the subject of a recent phase 2 clinical trial in adolescent and adults with Rett syndrome (NCT01703533). Safety and tolerability of trofinetide is also being studied in an ongoing trial in children and adolescents with Rett syndrome (unpublished results, NCT02715115). In a small pilot study of nine children with Phelan–McDermid syndrome (another monogenic form of ASD, resulting from haploinsufficiency of SHANK3, that plays a critical role in glutamatergic synaptic functioning) (Boeckers, 2006; Bonaglia et al., 2006, 2011), trofinetide significantly improved social impairment and restricted behavior compared to the placebo (Kolevzon et al., 2014).

Proteins Regulated by FMRP

Minocycline, an antibiotic that inhibits overexpressed synaptic MMP-9 in the *Fmr1* KO mouse model (Bilousova et al., 2009), was initially studied in an open-label add-on trial that showed significant improvement in behavior [ABC-I, $P < 0.001$ and Visual Analog Scale (VAS), $P = 0.003$] and global clinical impression scales ($P < 0.001$) in 20 adolescents and adults with FXS (Paribello et al., 2010). The positive results from this preliminary study led to a double-blind placebo-controlled crossover trial of minocycline in 66 children and adolescents. The study demonstrated mild global clinical improvement ($P = 0.02$) and a reduction of MMP-9 levels in the blood of responders (Leigh et al., 2013).

Lovastatin is a compound approved by the FDA for the long-term treatment of familial hypercholesterolemia (Descamps et al., 2011). Lovastatin has a demonstrated effect on intracellular signaling. In fibroblasts, cultured rat brain neuroblasts (Cerezo-Guisado et al., 2007), and in the FXS mouse lovastatin was shown to inhibit Ras signaling, resulting in reduced ERK1/2 activation. In the FXS mouse protein levels were further lowered to wildtype levels and audiogenic seizures were prevented. Lovastatin is being tested in an ongoing phase 4 randomized placebo-controlled trial in combination with a parent-implemented intervention (PILI) that aims at targeting language and behavior (NCT02642653).

Surface AMPA Receptors

One of the earliest proof-of-concept clinical trials evaluated CX516, an AMPA activator. The study did not show efficacy, but CX516 was most likely used at a subtherapeutic dose, a conclusion that was supported by a suggestion of efficacy in patients cotreated with antipsychotics, known to potentiate effects of CX516 (Berry-Kravis et al., 2006).

Other Targets

Some compounds with minimal preclinical data are being tested and it is unclear whether they represent a targeted approach. As an example, metadoxine, a nonstimulant ion-pair salt of pyridoxine (vitamin B6) and 2-pyrrolidone-5-carboxylate (PCA) is used as a treatment of acute alcohol intoxication and thought to be a modulator of GABAergic transmission with a monoamine-independent mechanism of action (unpublished data). Phase 2 randomized, double-blind, placebo-controlled, single-dose studies in adults (NCT01685281) with ADHD showed no significant adverse events (Manor, Rubin, Daniely, & Adler, 2014). A similar study conducted in adolescents with ADHD (NCT02189772) remains unpublished. Subsequently, a randomized, double-blind, placebo-controlled parallel trial in adults with ADHD (NCT01243242) showed significant improvements in ADHD symptoms (Manor, Newcorn, Faraone, & Adler, 2013). Results of another study are unpublished (NCT02059642) and a phase 3 trial is currently ongoing in adults with ADHD (NCT02477748). Metadoxine has also been studied in FXS and a double-blind placebo-controlled trial of 57 male and females was recently completed (unpublished results).

Open-Label Trials (Table 20.3)

Open-label trials not directly designed to evaluate efficacy were also conducted. The first mGluR5 antagonist tested in humans is the nonbenzodiazepine anxiolytic drug, fenobam. An open-label single-dose trial evaluated safety, pharmacokinetics, sensory gating, attention, impulsivity, and inhibition in 12 adult males and females. The single dose of fenobam was associated with a significant improvement of PPI compared to the untreated control group from a previous study (Berry-Kravis et al., 2009). Development of fenobam was interrupted due to financial challenges of the sponsor Neuropharm Ltd. (Hagerman, Lauterborn, Au, & Berry-Kravis, 2012).

Lithium, which may target mGluR-dependent activation of translation by attenuating GSK3β activity, was tested in a 2-month pilot open-label, proof-of-concept trial in children and young adults with FXS. Behavioral scales, verbal memory, and abnormal ERK phosphorylation rates in lymphocytes were measured (Berry-Kravis et al., 2008). There was no improvement in the primary endpoint measure (ABC-I), but behavioral improvements in a number of secondary outcome measures [total ABC score ($P = 0.005$), CGI ($P = 0.002$), and VAS ($P = 0.003$)] were reported. Despite promising results of lithium on several phenotypes in the FXS mouse (Chapter 13) (Choi et al., 2011; Liu et al., 2012; Liu, Chuang, & Smith, 2011), it is unlikely that lithium will be used in FXS due to its complex safety profile.

In an open-label trial, lovastatin was assessed in 16 children and adolescents with FXS. Improvement in behavioral scores and a modest improvement in CGI-I were reported (Çaku, Pellerin, Bouvier, Riou, & Corbin, 2014). A small open-label parallel pilot study of minocycline and lovastatin is currently underway, in adolescent and adults with FXS, to explore the safety and synergistic effect of the combined treatment on behavior (NCT02680379). The study also aims at validating a new biochemical and neurophysiological markers (LovaMix).

TABLE 20.3 Open-Label Trials in FXS With ≥8 Individuals

Target	Drug	Study period[a]	*n*[b]	Age (years)	Sex (F/M)	Stratifi-cation[c]	Status[d]	Efficacy[e]	Primary outcome	Biomarkers											
									ABC	CGI-I	CNT	AP	BDNF	ERK	ET	HR/HRV	LovaMix	Neuroimaging[f]	RSA	PPI
mGluR5 receptor	AFQ056	>12 months	148	18+	10/138	√	T	N	—	—	—	—	—	—	—	—	—	—	—	—	—
	AFQ056	>12 months	119	12–18	13/106	√	T	N	—	—	—	—	—	—	—	—	—	—	—	—	—
	Fenobam	1 day	12	18–31	6/6	—	C	Y	—	—	—	—	—	—	—	—	—	—	—	—	√
Intracellular signaling	Lithium	2 months	16	6–30	NA	—	C	Y	I	—	—	√	—	√	√	√	—	—	√	—	
Proteins regulated by FMRP	Minocycline[g]	2 months	20	13–35	2/18	—	C	Y	I	—	—	—	—	—	—	—	—	—	—	—	—
	Minocycline/ lovastatin	3 months	26	13–45	NA	—	O	O	C	—	—	—	—	—	—	—	√	√	—	—	
GABA modulators	Acamprosate	2.5 months	12	5–17	2/10	—	C	Y	—	√	—	—	√	—	—	—	—	—	—	—	
	Arbaclofen	12 months	45	6–40	NA	√	T	NA	I	—	—	—	—	—	—	—	—	—	—	—	
	Arbaclofen	>12 months	357	5–50	NA	√	T	NA	—	—	—	—	—	—	—	—	—	—	—	—	
	Donepezil	1.5 months	8	14–44	2/6	—	C	Y	—	—	√	—	—	—	—	—	—	—	—	—	

(Continued)

TABLE 20.3 Open-Label Trials in FXS With ≥8 Individuals (*cont.*)

		Secondary outcomes																					
		Behavior													Cognition/language							Other	
Target	Drug	ABC	ADAMS	ADHDRS	CASI	CBCL	CYBO CS-PDD	RBANS (LL/SM)	RBS	SRS	VABS	VAS	BRIEF	CARD task	CELF	CFXCPT	HVLT	NVALT	NEPSY	PPVT	SB-IV	TEA-Ch	CGI-I/S
mGluR5 receptor	AFQ056	C	—	—	—	—	—	—	√	√	—	—	—	—	—	—	—	—	—	—	—	—	√
	AFQ056	C	—	—	—	—	—	—	√	√	—	—	—	—	—	—	—	—	—	—	—	—	√
	Fenobam	—	—	—	—	—	—	—	—	—	—	—	—	—	—	√	—	—	—	—	—	—	—
Intracellular signaling	Lithium	C	—	—	—	—	—	√	—	—	√	√	—	√	—	√	—	√	Tower	√	—	—	√
Proteins regulated by FMRP	Minocycline	C	—	—	—	—	—	√	—	—	√	√	—	—	—	—	—	√	—	√	√	—	√
	Minocycline/ Lovastatin	—	√	—	—	—	—	√	—	√	√	—	√	—	—	—	—	—	—	—	—	—	√
GABA modulators	Acamprosate	C	—	√	√	—	√	—	—	√	√	—	—	—	√	—	—	—	—	√	√	—	—
	Arbaclofen	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	Arbaclofen	SW	—	—	—	—	—	—	—	—	√	—	—	—	—	—	—	—	—	—	√	—	√
	Donepezil	C	—	—	—	√	—	—	—	—	—	—	—	—	—	—	√	—	—	—	—	—	—

For all open-label studies safety, tolerability, and in some instances pharmacokinetics were also tested.
ABC-C, Aberrant Behavior Checklist-Community Edition with all individual subscales (ABC-H, Hyperactivity Subscale; ABC-I, Irritability Subscale; ABC-IS, Inappropriate Speech Subscale; ABC-SB, Stereotypic Behavior Subscale; ABC-SW, Social Withdrawal/lethargy Subscale); ABC-C_{FX}, Aberrant Behavior Checklist-Community Edition refactored for FXS (all six subscales, ABCFX-SA, Social Avoidance Subscale); ADAMS, Anxiety Depression and Mood Scale; ADHDRS, ADHD Rating Scale IV; AP, auditory processing; BRIEF, Behavior Rating Inventory of Executive Function; BDNF, brain-derived neurotrophic factor; Card Task, Card Task Test of Visual Sequential Memory; CASI, Childhood Anxiety Sensitivity Index; CBCL, Child Behavior Checklist; CELF, Clinical Evaluation of Language Skills; CFXCPT, Carolina Project Fragile X Continuous Performance Test; CGI-I/S, Clinical Global Impression-Improvement/Severity; CNT, Contingency Naming Task; CYBOCS-PDD, Children's Yale–Brown Obsessive Compulsive Scale modified for pervasive developmental disorders; ERK, extracellular regulated kinase–related kinase activation rate; ET, eye tracking; fMRI, functional magnetic resonance imaging; HR, heart rate; HRV, heart rate variability; HVLT, Hopkins Verbal Learning Test; MRS, magnetic resonance spectroscopy; LovaMix, biochemical and neurophysiological markers; NEPSY, developmental NEuroPSYchological assessment (Tower subtest); NVALT, Nonverbal Associative Learning Task; PPVT, The Peabody Picture Vocabulary Test; RBANS (LL/SM), Repeatable Battery for the Assessment of Neuropsychological Status (LL, List Learning; SM, Story Memory); RBS, Repetitive Behavior Scale; RSA, respiratory sinus arrhythmia; SB-IV, Stanford–Binet intelligence scale edition IV; SNAP-IV, Swanson, Nolan, and Pelham-IV Questionnaire for Attention Deficit Hyperactivity Disorder; SRS, Social Responsiveness Scale; TEA-Ch, Test of Every Day Attention for Children; TMS, transcranial magnetic stimulation; VABS, Vineland Adaptive Behavior Scales; VAS, Visual Analog Scale (VAS-C, clinician rated; VAS-P, parent rated).

[a] *Number of subject enrolled into the study.*
[b] *Study period = treatment duration.*
[c] *Stratification strategy: in the AFQ056 studies, participants were divided in strata depending on methylation status in completely methylated (CM) and partial methylated (PM). In the arbaclofen studies, social withdrawal was used for stratifying participants.*
[d] *Status: C, completed; O, ongoing; T, terminated.*
[e] *Reported Efficacy: N, no; NA, not available; O, ongoing; Y, yes.*
[f] *Neuroimaging includes fMRI and TMS.*
[g] *Opportunity for a 1-year extension.*

CONCLUSIONS AND FUTURE PROSPECTS IN CLINICAL TRIALS

The number of pharmacological studies in FXS has greatly expanded due to an increasing understanding of the pathophysiology in FXS. Open-label trials and small controlled trials have served as indicators, but results have so far not been replicated in larger placebo-controlled trials. New drug mechanisms are continuing to be assessed and lessons learned from previously failed trials would help improve future investigations.

Although there are many issues surrounding the design of clinical trials summarized earlier, there are a number of conclusions that can be drawn:

1. Changes in a broad array of behavioral measures are not observed in children > 12 years of age; adolescence and adults treated during approximately 1–3 months.
2. These unequivocal negative findings over a large array of behavioral measures require sample size over 100 participants. None of the smaller trials are able to reach a positive or negative conclusion.
3. These unequivocal findings also question the nature of the relationship between the basic cellular mechanisms (such the regulation of protein synthesis) and the cognitive and behavioral symptoms observed in adolescence and adults with FXS. More work is required to investigate how these mechanisms contribute to neurodevelopmental alterations. It is possible that a combination of mechanisms contribute cumulatively to the neurodevelopmental insult. Thus targeting one of them alone does not result in visible improvement.

However, it is fair to highlight the fact that the FXS field does not unanimously embrace these conclusions. Several prominent groups are focusing on potential methodological issues that may have introduced significant noise in the data captured during the trials, thus masking efficacy in the entire group or subgroups of participants, in particular, the loss of power due to a large placebo effect and issues related to behavioral scales relying on parental report. In addition, almost all trials have used the same measure for the primary outcome, as well as the inclusion criteria to select moderate-to-severely affected participants. This amplifies a well-known phenomenon of regression to the mean especially in the context of a highly variable parental-report measure.

Among the key issues to be addressed for future preclinical and clinical trials, we highlight the following matters.

The Challenge of Assessing Behavioral and Cognitive Changes

Behavior

It is often the primary motive for referral and will remain a major objective for treatment. However, if a disease-modifying drug restores underlying neural mechanisms, the subsequent behavioral changes may be pleiotropic and may occur later in the course of the treatment. Behavioral measures used in the FXS trials were sensitive to placebo effects, as well as worsened behavior at a higher dose. Improvement was also recorded using the ABC in previous ASD trials evaluating risperidone (Dove et al., 2012; McCracken et al., 2002). It is therefore unlikely that behavioral improvement may have escaped the very broad array of measures used in these trials. Nevertheless, further research is warranted to improve quantification of behavior, with an emphasis on direct capture to avoid sole reliance on the caregiver report.

Cognitive Improvement

Intellectual disability is the number one issue in patients with FXS. There is a consensus on domains critical to FXS outcomes, but not on the specific measures to be used. Studies are currently establishing validity, reliability, and sensitivity of cognitive measures in FXS for clinical trials (e.g., validating NIH Toolbox for populations with intellectual disabilities). Related areas of research have struggled with the same issues. As an example, MATRICS was a multipronged NIMH-led effort including academia, FDA, and the industry to improve measurement and treatment research for cognition in schizophrenia. Evidence of cognitive improvement has not yet been unequivocally demonstrated in RCTs evaluating pharmacological treatments in neurodevelopmental disorders. In ASD, and ADHD improvement in measures of cognitive processing and IQ have been reported in pharmacological RCTs ranging from 3 to 12 months and behavioral interventions (Aman et al., 2008; Dawson et al., 2010; Estes et al., 2015; Gillberg et al., 1997). Although, these studies were underpowered, they suggest that some readily available tools are sensitive to cognitive improvement. Recording these improvements will likely require long trials with intensive nonpharmacological learning interventions. Disease modification is conceptually not yet well defined for neurodevelopmental disorders, but it is widely agreed that disease-modifying treatments are expected to improve the core deficits, notably learning and cognition, the developmental , and everyday functioning of affected individuals. Cognitive mediation trials are underway and might be a good platform for measuring the effect of drugs on the learning rate. Whether a drug needs to show benefit alone before designing a learning trial will be an important question.

Early Treatments

Although disease-modifying compounds in neurodevelopmental disorder may show no efficacy in adolescent or adult patients, regulators expect the demonstration of safety, and some therapeutic benefit in adults, before allowing a gradual lowering of the targeted age range or a clear rational why efficacy is expected only in younger patients versus adults. New strategies to safely advance RCTs into younger populations need to be explored.

Quality and Scalability of Clinical Trials in Neurodevelopmental Disorders

Among the many challenges of trials in neurodevelopmental disorders, quality, and power are particularly problematic. A recent review identified 169 trials performed in patients evaluating 32 genetic disorders. In 44% of these studies, authors reported potential efficacy, but this lead to only two approved treatments: dietary restriction for phenylketonuria and miglustat for Niemann–Pick disease type C (van der Vaart, Overwater, Oostenbrink, Moll, & Elgersma, 2015). This review identified several issues: (1) the median sample size for RCTs being 25 (range 2–537); (2) only 30 of 107 RCTs had acceptable Jadad scores exceeding 3; and (3) reporting of key CONSORT items was poor and reported outcome measures almost never matched (<5%) preregistered outcome measures in trial registries. These issues also apply to FXS trials, many of them being underpowered and open label.

As preclinical research will continue to identify treatment targets in FXS, and a growing number of "genetically defined" neurodevelopmental disorders, we will be faced with difficult choices. To achieve the power and quality required to draw unequivocal conclusions on

the benefits of a given compound, trials will have to be conducted through large international consortiums and in the absence of an industrial partner, new international funding strategies are required.

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CHAPTER

21

Outcome Measures in Clinical Trials for Fragile X Syndrome: The Search for Sensitive Neurocognitive Assays

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Previous chapters in this volume have overviewed the molecular, cellular, and systems neuroscience findings that have driven symptomatic and mechanistic pharmacological treatment efforts aimed at ameliorating difficulties for children, adolescents, and adults with FXS. Here, we "take a step back" and identify key considerations emerging from a growing understanding of the developmental cognitive neuroscience of FXS and other neurodevelopmental disorders, all of which we believe will ultimately aid measuring change and stability in cognitive and learning milestones for individuals with FXS. We focus on, first, insights that emerge from investigating the developmental nature (i.e., stability and change) of the cognitive strengths and weaknesses that are characteristic of FXS and then, we point to the significant element of differences across individuals with FXS (whether they are correlated to fragile X mental retardation protein (FMRP) expression, genetic background, and/or differences in environmental input). Next, we identify variable degrees to which behavioral symptoms resembling autism and, in a similar way, attention deficits present in FXS and the challenge of understanding the neurocognitive underpinnings of these symptoms when trying to measure their amelioration by treatment.

As a whole, these considerations point to the need to develop measures that are more sensitive than currently used ones, not just to measure overt behavioral symptoms, but also to target the learning and cognitive mechanisms leading to the emergence of developmentally dynamic and variable profiles of strengths and weaknesses. Measures developed with these considerations in mind may be more sensitive than currently available outcome measures and, in turn, aid in establishing whether treatments are successful, be they focused

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on pharmacological, nonpharmacological (e.g., cognitive training, psychodynamic, family, or individually-focused therapeutic approaches), or combined approaches. Finally, as exemplified by some of the ongoing trials, critical for the future of these attempts is the involvement of stakeholders (parents, families, practitioners, but also adults with FXS) in identifying the goals for cognitive and behavioral measurement of outcomes that are most relevant to people with FXS and their families.

RETHINKING FRAGILE X SYNDROME WITH A VIEW TO MEASURING POSITIVE TREATMENT OUTCOMES: AN OVERVIEW

As summarized across this volume, FXS is the most common known single gene cause of genetically inherited learning disability, with prevalence estimates of 1 in 7,143 in males and 1 in 11,111 in females (Hunter et al., 2014). The disorder results from a methylational silencing on the *FMR1* gene that leads to a reduction or absence of the FMRP. We shall return to this key characteristic of FXS in the context of potential target outcome measures, because, surprisingly, few of the existing trials have included indices that relate to learning and learning disability. Several additional features, ranging from the cellular neuroscience to the systems neuroscience and developmental psychology of FXS, are also useful from the point of view of identifying sensitive assays.

At the cellular level, FXS has been consistently associated with anatomical and functional changes related to the synaptic connections between neurons (Santoro, Bray, & Warren, 2012) but mouse models of FXS suggest that these changes occur at varying time points in development (Meredith, 2015), stressing the importance of measuring changes in a time-sensitive way. In addition, the investigation of FXS at the cognitive neuroscience level suggests that there are correlations between FMRP levels, brain activity, and specific cognitive impairments that impact on learning in FXS. For example, Menon, Kwon, Eliez, Taylor, & Reiss (2000) have demonstrated an association between FMRP levels, working memory impairments, and decreased activation in dorsolateral prefrontal and parietal areas in the same group of female participants with FXS. Similarly, others have shown atypical activity in dorso-striatal brain networks during attention and impulse control tasks (e.g., Hoeft, Hernandez, Parthasarathy, Watson, Hall, & Reiss, 2007), as well as reduced amygdala volume and atypical amygdala activation when individuals with FXS participate in emotion processing tasks (Kim et al., 2014). Despite these interesting links between FMRP, brain, and cognition, it is critical to note that, for some behaviors, including autistic behaviors, environmental factors (such as effectiveness of educational and therapeutic services) are associated with severity whereas FMRP did not predict severity (Hessl et al., 2001). These findings pinpoint the high individual variability in baseline symptom levels such as autism symptoms prior to trial entry in groups of children with FXS. In turn, these individual differences, to which we return later, highlight that when stratifying samples for treatment trials, it will be critical to measure not only markers of FMRP expression, but also differential environmental provisions for individuals in different treatment arms.

From a systems neuroscience perspective, in addition, while much of the early research on the FXS brain focused on older children and adults, recent structural imaging studies have taken advantage of the early age of diagnosis (compared to disorders that are behaviorally

defined later) to study very young children with the syndrome. These recent studies have highlighted patterns of stability and change in global and local brain structure that deserve full attention of researchers evaluating treatment effects (e.g., Hoeft et al., 2010). Finally, cognitive profiles that are dynamically changing over developmental time (to which we now turn) suggest that measures that are solely focused on age-standardized norms may not be sensitive enough to the profile of strengths and weaknesses that compound overall levels of ability or symptom levels.

BEYOND BRAIN-BEHAVIOR LINKS THROUGH DEVELOPMENTAL FINDINGS: IMPLICATIONS FOR TREATMENT

Multiple cognitive factors underpin the learning difficulties that characterize FXS, including significant attentional, working memory, and sociocognitive deficits. Subtle behavioral and cognitive delays are evident as early as infancy (Bailey, Raspa, Bishop & Holiday, 2009). In addition to these early concerning signs, from infancy FXS presents with a distinct cognitive profile: poor response inhibition (Scerif, Cornish, Wilding, Driver, & Karmiloff-Smith, 2007), poor saccadic eye-movement control (Scerif, Karmiloff-Smith, Campus, Elsabbagh, Driver, & Cornish, 2005), and prolonged visual attention to objects (Roberts, Hatton, Long, Anello, & Colombo, 2012), suggesting that these early markers of attentional differences are measurable very early and should be modifiable by intervention, even if in this age range the treatment of choice is likely to be nonpharmacological. Later in development, school children and adolescents also display attentional control difficulties, with poor response inhibition (Sullivan et al., 2007) and atypical patterns of visual attention, including distinctive impairments in selective, sustained, and divided attention (Munir, Cornish, & Wilding, 2000). These findings again suggest that indices of change should include sensitive attentional measures, although early treatment trials that included indices of sustained attention (e.g., Berry-Kravis et al., 2009) in the form of a continuous performance test, were plagued by ceiling effects (i.e., performance that was initially already high) because the target population was a group of adults. Finally, executive function (e.g., inhibitory control, working memory, cognitive flexibility) is clearly affected in FXS in childhood (e.g., Lanfranchi, Cornoldi, Drigo, & Vianello, 2009), despite relative strengths in long-term memory and daily living skills (Hatton et al., 2003), making indices of executive function and working memory likely to be a good target assay not only because their amelioration would be desirable, but also because these indices have previously been demonstrated to be malleable to intervention even in young children (e.g., Diamond & Lee, 2011; but see Melby-Lervåg, Redick, & Hulme, 2016, for a critical review of previous working memory training successes).

Additional implications for the selection of sensitive neurocognitive assays come from studying changes in patterns of strengths and weaknesses in FXS longitudinally. Here we focus on attentional control as an example because attention modifies interactions of developing systems with their environment (e.g., Amso & Scerif, 2015), but we also refer to other cognitive domains for a broader perspective. Although the findings reviewed earlier tell us that boys with FXS demonstrate significant delays in attentional control, this turns out not to be a case of developmental arrest. Instead, longitudinal research has identified trajectories of delayed development: a prospective longitudinal study examined early profiles of

attention and working memory impairment in boys with FXS, discovering improvements over time despite significant delays (Cornish, Cole, Longhi, Karmiloff-Smith, & Scerif, 2013). Of note, these longitudinal developmental improvements in inhibitory control have since been measured in children as young as 3-years old (Tonnsen, Grefer, Hatton, & Roberts, 2014) and dynamic trajectories of more specific cognitive abilities have been revealed using longitudinal designs, including gaps in cognitive functioning that both narrow and widen over adolescence, demonstrating profiles of strengths and weaknesses over developmental time. While verbal comprehension, perceptual organization, and processing speed demonstrate widening gaps compared to typically developing individuals over development, freedom from distractibility shows a narrowing gap (Quintin et al., 2016). These changes are important from the point of view of identifying appropriate outcome measures, because the measures selected to index in this case attentional control and memory (or any other target domain) need to be able to capture change that might occur even in an untreated control sample.

The second insight that developmental studies offer is in highlighting that even physiological markers (such as indices of brain structure and function) that could be identified as a sensitive future assay for treatment effects are also highly sensitive to developmental changes. For example, Hoeft and coworkers (2010) followed 1–3-year-old boys with FXS longitudinally over a 2-year-period to investigate brain maturation. These researchers identified areas of the brain that were either enlarged or reduced, and these differences held across both time points, thereby highlighting from a very early point in development quite stable regional effects. Interestingly, other brain regions revealed initial volume comparable to that of typically developing controls, but these areas subsequently increased in size in FXS, again stressing the need for a longitudinal/developmental perspective when considering the atypical brain. These brain regions include those related to inattention and socialization and have been implicated previously in autism and attention deficit hyperactivity disorder (ADHD). In general, then, these results underscore how abnormalities within different brain regions develop differently over time in FXS, reflecting again the time-dependent effects of *FMR1* silencing. Indeed, differing trajectories of brain growth can distinguish children with idiopathic autism and children with FXS (Hazlett et al., 2012), a point to which we return later. These divergences and atypical trajectories in brain development would not have been detected in cross-sectional designs alone, but their measurement as baseline changes becomes critical in the context of identifying long-term treatment effects on brain structure or functions.

In addition to the group-level differences in trajectories, a final developmental insight expands upon what clinicians working with individuals with FXS can readily verify—there are striking individual differences in attention outcomes present in the disorder, with some individuals much more seriously affected by inattention than others despite equivalent IQ levels. In the broadest sense, there are marked sex differences in the presentation of the FXS phenotype: *FMR1* is located on the X chromosome and females, for whom one of the two X chromosomes is randomly inactivated, have more variable and overall less severe impairment than males (Grigsby, Kemper, Hagerman, & Myers, 1990). However, even in boys with the full mutation, large longitudinal studies have dissected within-syndrome variability in FXS, to ask how within-syndrome variability predicts subsequent outcomes. For example, visual, auditory, and multimodal attention were measured in young boys with FXS, aged between 4 and 10 years at time 1 and again 12 months later (Scerif, Longhi, Cole, Karmiloff-Smith, & Cornish, 2012). At an individual level, while visual attention was a significant longitudinal predictor of

ADHD symptoms in the boys with FXS (Scerif et al., 2012), auditory attention predicted later symptoms related to autism (Cornish, Cole, Longhi, Karmiloff-Smith, & Scerif, 2012). Different indices of attention (visual, auditory) may therefore be sensitive to baseline individual differences across a group of individuals with FXS, and predict differential improvement in response to treatment for those dimensions. In turn, charting these individual differences highlights two further important caveats in identifying cognitive assays that are sensitive to treatment in FXS: first, there is considerable variability in symptom severity for dimensions, such as inattention/hyperactivity, or autism; second, and perhaps most importantly, the cognitive underpinnings of these symptoms and their variability may differ in individuals with FXS compared to, for example, idiopathic ADHD or autism (i.e., ADHD or autism without an identified genetic aetiology). In turn, outcome measures that are selected based on (idiopathic) symptoms alone may entirely miss why these symptoms are present in FXS, and therefore not be able to capture any change by FXS specific pharmacological agents or nonpharmacological treatments. We turn to the challenge posed for measuring selection by individual differences and by potentially differing neurocognitive underpinnings of target symptoms in the next section.

UNDERSTANDING COGNITIVE UNDERPINNINGS OF TARGET SYMPTOMS: INSIGHTS FROM AUTISM

Individuals with FXS are at high risk for behaviorally-defined disorders, including autism and ADHD. In fact, a significant percentage of individuals with FXS meet criteria for an autism diagnosis, with recent estimates suggesting upwards of 50% (Harris et al., 2008; Klusek, Martin, & Losh, 2014). This is unsurprising, given that both conditions share similar behavioral difficulties, including impairments in social interactions and communication, as well as delays in adaptive and cognitive development (Budimirovic & Kaufmann, 2011; Clifford et al., 2007). There is still some debate, however, whether FXS with autism represents a distinct phenotype or simply a continuum of impairments in individuals that may be more severely affected (Hall, Lightbody, Hirt, Rezvani, & Reiss, 2010). Moreover, it is unclear whether the autism symptoms underlying this putative phenotype are distinct from those found in idiopathic autism (discussed in Abbeduto, McDuffie, & Thurman, 2013). On the one hand, some research suggests that the behavioral profile of autism symptoms in FXS are different than those found in idiopathic autism (McDuffie, Thurman, Hagerman, & Abbeduto, 2015; Thurman, McDuffie, Kover, Hagerman, & Abbeduto, 2015; Wolff et al., 2012), while on the other hand, some research does not (Rogers, Wehner, & Hagerman, 2001).

Why do many children with FXS present with social and cognitive control problems, such as those experienced by children diagnosed with autism and ADHD, while others do not? A second central point for debate is whether the autism symptoms in FXS are the same as those existing in individuals with idiopathic autism. Alternatively, do these behaviors represent the severe end of a continuum of cognitive and behavioral difficulties present in more affected individuals (Hall et al., 2010)? Even if the symptoms appear the same, could autism symptoms in FXS be manifestations of different underlying cognitive or emotional mechanisms to those that drive similar behaviors in idiopathic autism? Separating these interpretations is not an easy endeavor, yet is critical for the development of treatments that address the core impairments in autism and FXS.

A strategy used to address these questions focuses on whether there are differences in autism symptoms in autism cooccuring with FXS and idiopathic autism. For example, one prominent account suggests that autism symptoms in FXS are a "category mistake," meaning that autism and FXS are placed in the same category despite stemming from different levels of explanation (behaviorally vs. biologically defined). These researchers argue that autistic behaviors in FXS are in fact explained by the low IQ associated with these individuals (Hall et al., 2010). These researchers found dissimilar autism symptoms in those diagnosed with FXS and autism and those with idiopathic autism, and showed a negative association between autistic behaviors and IQ. However, other studies have reported little to no differences in autism symptoms across the IQ range (e.g., Hernandez et al., 2009). This debate is relevant to measurement issues: if autism symptoms are underpinned by individual differences in the severity of learning disability in FXS (unlike in idiopathic autism), then in the context of FXS treatment trials, measures that are sensitive to intellectual functioning differences and improvements will be a better outcome measure than an index of symptom severity alone. Again, longitudinal data provide some further insights into this debate. Recent data have shown that the greatest predictor of autism symptoms in FXS is not overall cognitive delay or IQ, but rather adaptive socialization (Hernandez et al., 2009). In turn, this would suggest that measures focused on sensitive indices of adaptive socialization are a better assay in this case than measures of broad intellectual functioning.

A further way of understanding autism symptoms in FXS may be to avoid comparing individuals in terms of their symptoms alone, but rather investigate specific pathways/mechanisms that might drive them. For example, Hall et al. (2015) showed that although social gaze avoidance in neurotypical individuals was related to the degree of autism symptoms, in FXS communication ability and not autism symptoms predicted social gaze. These results again suggest that researchers should be cautious when interpreting change or no change in autism symptoms for individuals with cooccurring FXS and autism—although they may have similar levels of symptoms prior to entry into a trial, such as gaze avoidance, these behaviors may be caused by different underlying mechanisms and therefore measured best by different indices. Novel work in this area suggests that selecting indices that relate to predictors of later emerging autism symptoms in children with FXS well before autism can be diagnosed, may be an additionally important route. Tonnsen, Malone, Hatton, and Roberts (2013) found that early negative affect predicted later anxiety symptoms in these young children, but not autism symptoms, in contrast to previous studies that found negative affect to be related to autism symptoms in infants at familial risk for autism without FXS. This in turn suggests that the developmental pathways leading to autism symptoms targeted by intervention trials may be rather different in FXS, and therefore measures that optimally capture these mechanisms, either at the cognitive or neural level, are preferable to symptom-only measures.

UNDERSTANDING COGNITIVE AND NEURAL UNDERPINNINGS OF SYMPTOMS: IMPLICATIONS FOR MEASURE SELECTION

The open debate on understanding the underpinnings of target symptom domains highlights one of many challenges in selecting sensitive outcome measures for clinical trials. If we are unclear about whether the autism symptoms present in FXS are like those in idiopathic

autism, how are we to assess these effectively, and in turn measure treatment effects? Moreover, will measures validated for autism effectively detect the presence and severity of autism symptoms in FXS? The current focus has centered on parent or caregiver rating scales to assess the presence or frequency of autism or autism-like symptoms in individuals with FXS. Such measures, however, may not be sensitive enough to capture improvements because their reliability, sensitivity, and validity in FXS are unknown. For example, the Social Responsiveness Scale (SRS; Constantino & Gruber, 2005) has been used to evaluate the severity of autism symptoms at the end of several clinical trials (Berry-Kravis et al., 2012; Erickson et al., 2011a,b; Jacquemont et al., 2011). Though these measures are validated to detect autism symptoms and cover the social domain broadly, they may fail to tap into the social difficulties that distinguish individuals with FXS and autism. Specifically, the items listed on the SRS may capture aspects of social indifference rather than social anxiety. Some research suggests that individuals with FXS are interested in interacting socially, but are hindered by high levels of arousal and anxiety (reviewed in Klusek, Roberts, & Losh, 2015; Tonnsen & Roberts, 2016). The focus of the SRS may therefore fail to detect the subtle differences in social difficulties that characterize individuals with FXS (e.g., Berry-Kravis et al., 2012; Erickson et al., 2011a; Jacquemont et al., 2011).

A similar problem has occurred from the investigation of motor behaviors in FXS. Previous clinical trials have recognized that atypical motor behaviors form a part of the broader restricted and repetitive impairments in FXS, and accordingly, have included rating scales to document improvements in this domain. As an example, the Repetitive Behavior Scale-Revised (RBS-R; Bodfish, Symons, Parker, & Lewis, 2000), has been incorporated in several pharmacological intervention trials in individuals with FXS (Berry-Kravis et al., 2012; Jacquemont et al., 2011). Unfortunately, this scale has not been sufficiently sensitive in detecting change or improvement across these trials. Again, it is likely that the validation of this measure for autistic populations is insufficient to detect nuances in the symptom profile that characterizes FXS (Lam & Aman, 2007). However, the need to identify or develop better assessments of motor behaviors in FXS is pressing. Growing evidence suggests that motor abilities may be a predictor of autism in several high-risk groups, including individuals with FXS (Bhat, Galloway, & Landa, 2012; Roberts, Tonnsen, McCary, Caravella, & Shinkareva, 2016). Similarly, other studies have shown that atypical motor abilities, such as fine motor skills, are associated with the severity of autism symptoms in FXS (Roberts et al., 2009; Zingerevich et al., 2009). These findings are also consistent with others that have noted that atypical motor behaviors may be an endophenotype of autism (Esposito & Pasca, 2013, Roberts, Tonnsen, McCary, Caravella, & Shinkareva, 2016). Together, this evidence suggests that the assessment of motor behaviors may be promising in stratifying and differentiating the efficacy of treatments in individuals with FXS and FXS with comorbid autism, however new and more sensitive measures are required.

As noted above, the insensitivity of informant reports and rating scales suggests that the emphasis on examining symptoms and behavioral profiles alone may be insufficient for tracking improvements in FXS. This approach is falsely based on the assumption that common phenotypic end-states reflect the same cognitive mechanisms or etiology (Cornish, Scerif, & Karmiloff-Smith, 2007). Some have suggested that more objective and direct methods focused on underlying mechanisms may be more ideal in identifying change or improvements in FXS, and better inform the debate concerning the similarity of idiopathic autism and FXS and autism (Berry-Kravis, Knox, & Hervey, 2011). Understanding whether different mechanisms account for a similar

behavioral expressions will be critical in assessing the core autism symptomology in FXS. Hall et al., 2015 have provided some preliminary evidence to suggest the gaze avoidance noted in both autismand FXS stems from different underlying mechanisms. Using a naturalistic social gaze paradigm, these researchers found that a higher level of autism symptoms predicted lower levels of social gaze in control participants matched on autism symptomology. This was not the case in participants with FXS. Instead, stronger communication abilities predicted higher levels of social gaze in participants with FXS, suggesting that abnormal eye gaze is not driven by levels of autism symptomology. The findings illustrate the pitfall of assuming an equivalence of symptoms or behaviors in FXS and idiopathic autism. Further, they suggest that an objective measure, such as eye tracking, may be more sensitive in tapping the core FXS phenotype and hold greater promise as clinical trial endpoints than subjective, categorical measures.

An alternative to characterizing autism symptoms in individuals with FXS may be to focus on sensory issues, such as visual information processing. Just like measures of brain structure and function, perceptual profiles offer an attractive endpoint measure because they may be closely linked to the fundamental mechanisms or etiology (Belmonte et al., 2004). Indeed, Bertone, Hanck, Kogan, Chaudhuri, & Cornish, 2010a,b identified condition-specific signatures that underlie low-level visual processes in autism and FXS. Based on their findings and those of others (Bertone, Mottron, Jelenic, & Faubert, 2003, 2005; Farzin, Whitney, Hagerman, & Rivera, 2008; Kogan et al., 2004a,b), these researchers noted that while individuals with autism and FXS both showed a decreased ability to process complex, static and dynamic information, important differences emerged for simple information: individuals with autism showed an enhanced sensitivity to simple, static information, whereas individuals with FXS showed a decreased sensitivity to dynamic information. Further, they argued that these profiles relate to alterations in the local connectivity of neural networks mediating low-level information processing in early visual cortices, which may be linked to an imbalance of excitation and inhibition mediated by glutamate and GABA receptors. Future research is most certainly warranted to establish whether such signatures are associated with specific behaviors and autism symptoms. Once associations between perceptual signatures and autism symptomology are established, these could provide a useful framework for understanding how autism manifests in FXS. For example, it may be reasonable to expect that individuals with FXS who display a similar perceptual profile to those with idiopathic autism would perhaps express greater levels of autism symptoms.

A complementary focus may be on using direct (i.e., noninformat dependent) cognitive measures that have been designed with the aim of measuring individual differences along cognitive dimensions outside the FXS research agenda. Recently, Hessl et al. (2016) assessed feasibility, test-retest reliability, construct validity and ecological validity of the National Institutes of Health Cognitive ToolBox in a sample of individuals with FXS and Down's syndrome, as well as idiopathic intellectual disability (ID). These measures were designed to go beyond the gross nature of symptom checklists in understanding dimensions of difficulties across diagnostic categories in mental health (Insel & wang, 2010). Hessl et al. (2016) are to our knowledge the first to have assessed the feasibility and reliability of using these measures to capture individual differences in FXS. Most importantly, these measures correlated with additional indices of adaptive behavior and intellectual disability, suggesting good ecological validity of these measures. The next step involves gathering more evidence of sensitivity to change, longitudinally and in response to treatment.

Neural correlates have also largely been neglected as potential outcome measures in clinical trials, because of the invasive nature of neuroimaging environments for people with particular hypersensory sensitivity to noise or touch. However, the increased availability of noninvasive, portable imaging techniques has made their application to FXS more likely, as, for example, by using electroencephalography (EEG), near-infrared-spectroscopy (NIRS; Cui, Briant & Reiss, 2012), and resting state connectivity (e.g., van der Molen, Stam, & van der Molen, 2014). Functional and structural imaging studies have made pioneering steps in capturing long-term developmental changes in children and even young infants with FXS, opening a window onto neural phenotypes that may be modified by intervention. The future of this approach may include measures that allow mapping the temporal dynamics of brain functions that are severely affected in FXS: for example, attentional difficulties have been reported for infants, children, adolescents, and adults with FXS. As techniques, such as EEG acquire greater portability, in addition to their limited noise, these markers of neural activity may become even more easily accessible to the full range of abilities characteristic of people with FXS. In addition, the development of assays that require more limited verbal or manual responses (e.g., eye-movements instead of button presses, or neural responses to passively presented stimuli) may also provide interim markers that are closer to those identified in preclinical trials in the laboratory.

CONCLUDING REMARKS ON CURRENT AND FUTURE MEASURE SELECTION CHOICES

To summarize, in the previous sections we have argued that measure selection needs to be informed by: (1) changes in cognitive and neural profiles over development for people with FXS, which entail measures that are sensitive to change even in no treatment groups; (2) the high degree of individual differences in any symptom dimension within a group with FXS, which at a minimum requires careful stratification across treatment arms; (3) the potentially different underpinnings of symptoms in FXS compared to other neurodevelopmental disorders that also display these symptoms, which point to the need for FXS-specific and sensitive assays.

With these considerations in mind, it is useful to briefly survey what the current trends in measure selection have been. As very clearly reviewed in Chapter 20 (Ligsay & Hargerman, 2016), although intellectual disability and difficulties with social anxiety and social cognition are part of those key targets, there is a paucity of measures that can capture improvements in cognitive functions underpinning these. As reviewed earlier, there have been increasing attempts to select and/or modify measures so that they are more specifically tailored to individuals with FXS: for example, they employed the Aberrant behavior checklist (FX modification, Social Withdrawal subscale) and the Vineland Adaptive Behavior Scales (e.g., daily living skills) (see Ligsay & Hargerman, 2016). These outcome measures have shown some evidence of treatment effects, in specific age groups (i.e., treatment effects only for some age brackets) and individuals (e.g., fully methylated cases, very severely affected cases). For example, in an improvement on tools originally generated for many aetiologically distinct groups with behavioral difficulties, FXS specific measurement tools have also been attempted (e.g., Gross, Hoffmann, Bassell & Berry-Kravis, 2015), such as the FXS Rating scales and the Fragile X Domain-Specific Concerns Visual Analog Scale, with moderate success.

However, even these more specific measures fall prey to two very important caveats: they are informant-dependent report measures (parent report, or clinician report), and they focus on quantifying behavioral symptoms, rather than what underpins them. This is problematic, because behaviors can be multiplied and determined, both at each time point in development, and over developmental time. As detailed earlier, for example, behavioral symptoms, such as averting contact with unfamiliar individuals may arise out of distinct cognitive pathways (e.g., a lack of motivational engagement in social interactions on the one hand, or instead a high degree of social anxiety), but current measures do not easily discriminate between these routes. In contrast, new sensitive cognitive assays that are objective do not depend on informant report and yet are noninvasive, such as eye tracking, may be a much better choice (Hall et al., 2015).

From a developmental point of view, what in childhood might initially have presented as resistance to change and overreactivity to uncertainty can develop into anxiety in adolescence, especially given the interactions with peers in an increasingly complex social world. In part, this lack of precision in measuring the cognitive and developmental underpinnings of target outcomes in clinical trials depends on the measures used: parental or clinical report may not be as sensitive to these different cognitive and learning routes to a behavior. A very important limitation is that questionnaire data depend on observer report, and metaanalyses of these measures, for example, in the context of treatment of ADHD, have demonstrated that they are too liable to placebo effects (Sonuga-Barke et al., 2013).

In summary, the current state of the art suggests that FXS specific measures are a better choice for to assess treatment effects than measures designed for other neurodevevelopmental disorders (Lygsay et al., this volume); and that individual level analyses are necessary for analytical purposes. Here we have argued that reliable and sensitive assays that target cognition and learning have only recently been attempted. Finally, it is critical to involve stakeholders in identifying priorities and explain that behavioral markers/symptoms of maladaptive behaviors can generate through rather different cognitive and developmental routes, and therefore these latter pathways need to be studied more directly.

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CHAPTER

22

Fragile X Research From a Parental Perspective

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INTRODUCTION

Research can be driven by many motivations, curiosity in gaining knowledge, exploring the unknown, the quest for breakthroughs in understanding, and the joy of collaboration and problem solving. For those working in the area of health and intellectual disability, there is also the motivation of the potential to conduct research, which has a profound and meaningful impact on people's quality of life. This is particularly the case for those researching fragile X syndrome, the most common inherited cause of intellectual disability and autism. Since the first description of fragile X syndrome in the 1940s and the discovery of the gene in 1991, there have been vast and exciting developments in our understanding of fragile X (the more generic fragile X is used here and throughout this chapter to include all conditions associated with the full range of mutations of the *FMR1* gene and not only fragile X syndrome) thanks to the work of researchers and clinicians across the world. Research has made strides forward in terms of understanding the phenotype (physical and behavioral) and how to best provide support to those living with fragile X syndrome. In addition, there have been significant developments in our understanding of the neurobiology of the condition, including several trials of targeted pharmacological agents, though a successful treatment still remains elusive. In more recent years, research has also highlighted issues, which may directly face carriers of the fragile X premutation. This includes the identification of two other fragile X-related conditions. First, fragile X–associated tremor/ataxia syndrome (FXTAS) is a condition which may affect premutation carriers in later life, typically after the age of 50, which is characterized by tremor, as well as movement and memory problems. In addition, female fragile X carriers may experience primary ovarian insufficiency (FXPOI), which may be associated with premature menopausal symptoms and fertility problems or infertility.

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It is clear that the information gained from high quality research is the basis for developing effective interventions, environmental, and pharmacological, which may have a profound impact on people's lives. However, such research cannot be conducted without the engagement and participation of people living with fragile X and their families. Yet, so often the views and opinions of these groups are overlooked. In addition, families and affected individuals have a unique perspective and important role to play in terms of highlighting the lived experiences, joys, and challenges of having a condition, which too may inform and guide research. Moreover, it's the living conditions and requirements of people living with fragile X that must play the most important role in determining focuses and directions of future fragile X research.

For research to have the greatest impact, findings need to be disseminated, not only among the research and clinical community, but also to families living with fragile X "on the ground." To do this requires researchers to appreciate and understand how families may be accessing this information.

Therefore, this chapter shall help to shed some light on how affected families feel about research in fragile X, what their opinions and preferences are. It offers an important opportunity for the voices of families living with fragile X to be heard. We describe unique data on the opinions of parents on research into fragile X, based on a survey of over 1300 families, from 10 countries.

The first two authors of this chapter have children living with fragile X syndrome. Jeffrey Cohen has been associated with the United States' National Fragile X Foundation (NFXF, www.fragilex.org) for more than 20 years in myriad leadership roles and now serves as its Director of Governmental Affairs organizing a national outreach to the US government to increase funding for fragile X research. Jörg Richstein is the chair of the German Fragile X Association (Interessengemeinschaft Fragiles-X), coordinator of the European Fragile X Network (EFXN, www.fragilex.eu) and is chairman of the German Rare Disease Alliance. Becky Hardiman is the CEO of the UK Fragile X Society and conducting research into behavior in individuals with fragile X syndrome.

The research survey is a cooperation between the NFXF and member associations of EFXN.

In this chapter, the first section addresses the survey methodology, the second section presents tabulated results, the third section discusses conclusions reached by the authors, and in the last section the key theme of communication is considered in more detail, including implications for the future.

SURVEY METHODOLOGY

The aim of the survey was to gain a concise but broad overview of parent perspectives on key topics, such as: expectations and desires for the outcomes of research, willingness and concerns about participation in, and the desire to understand research. Families living with a child with special needs have limited time, therefore a short number of questions were developed (by the chapter authors), in order to facilitate participation. An English language "master copy" was created and translated as needed. During translation, care was taken to preserve the original meanings of both the questions and answers, across languages.

The online survey was disseminated via the NFXF (USA) and the EFXN (participating organizations: Association X Fragile Belgique (Belgium), Mosaïques and Le Goëland (both

France), Interessengemeinschaft Fragiles-X (Germany), Irish Fragile X Society (Ireland), Associazione Italiana Sindrome X Fragile (Italy), Fragiele X Vereniging Nederland (Netherlands), Associação Portuguesa da Síndrome do X-Frágil (Portugal), Federación Española del Síndrome X Frágil (Spain), Fragile X Society (United Kingdom). Participation invitations to families were sent out by the individual associations to their members (for whom email addresses were available). Those invited to take the survey were predominantly family members or caregivers of someone living with fragile X syndrome, many of whom were themselves fragile X carriers experiencing or at future risk of developing symptoms too. The connection of each respondent to fragile X was not recorded, however based on the known characteristics of the distributing organization's databases, the respondents were likely and predominantly parents of an individual living with fragile X syndrome.

The survey remained open online for 3 weeks, yielding a total of 1346 responses. The authors believe this is the largest such survey carried out soliciting the opinions of Fragile X families and caregivers specifically regarding fragile X research. The magnitude of this response highlights the power of such international networks and the broad reach of the participating organizations.

At present, the overall number of family members/contacts in all EFXN member organizations plus the NFXF well exceeds 10,000 families, an impressive number for a rare condition. Furthermore, a global International Fragile X Alliance (IFXA, www.ifxa.net) has recently been established, which is expected to expand this number significantly. Fig. 22.1 shows the number of responses from the individual countries (France including both families from Mosaïques and Le Goëland associations).

Descriptive statistics were calculated for the categorical responses and, in addition, the free-write responses from the open questions were translated and categorized. The responses

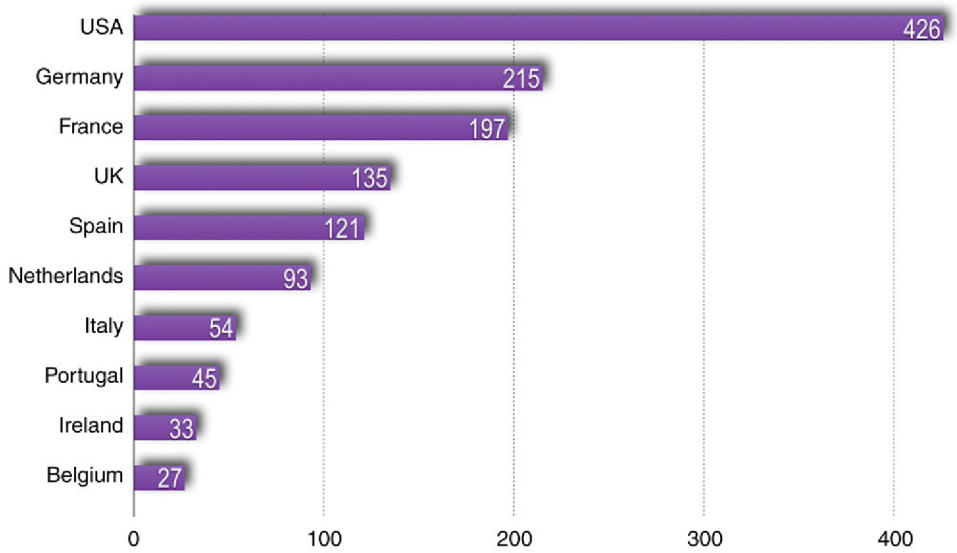

FIGURE 22.1 **Participation in the EFXN/NFXF research survey.**

from all countries were collated and analyzed together. Collapsing the data in this way does not allow systematic investigation of cultural differences. Anecdotally, there were broad similarities in the proportion of many of the responses across countries, though areas where there appeared to be cultural differences are highlighted. Individual results from countries can be obtained from the authors on request.

The fragile X research survey was carried out solely by the participating Fragile X organizations without any third party financial subsidies or other support.

RESULTS/DATA DISCUSSION

Importance of Fragile X Research

> We entrust you with our lives. (Respondent answer to "Is there anything you wish to communicate to the scientific/research community?")

The first section of the survey addressed respondents' views on the importance of research.

Overwhelmingly, the survey highlighted families' positive attitudes to research, with 93% of the responders rating Fragile X research as important or very important (with some national variances ranging from 97% in Germany and Ireland to 72% in Italy) (It is unclear whether this difference represents a genuine cross-cultural difference, as the number of respondents from Italy was quite small and may not be representative of the wider fragile X community in the country). Corresponding to this, a large majority (93%) found it important or very important that their national Fragile X organization support research, with only 3% considering this unimportant. Again, however, there were cross-cultural variations in these findings, with a higher proportion of respondents in Italy (19%) who rated it as unimportant that their association support research.

The survey also allowed families to express their expectations of the outcomes of research. There was a large variation in expectations, with 67% of the responders expecting Fragile X research to eventually lead to a cure or a life-improving treatment while 37% did not. The wide variation in these responses may be at least in part associated with families' differing expectations or opinions about what a "cure" for a developmental condition may look like (Section "Looking to the Future: Communication is Key").

Fig. 22.2 shows the subtopics of fragile X research that families selected as most important (multiple answers were possible).

It is understandable that the areas of greatest interest are those addressing the practical challenges faced by those living with fragile X syndrome, such as: behavior, social life, work, and school. A similar focus was put on clinical trials, which hold the possibility of leading to new drug therapies. However, the findings are a clear signal that families value research with clear practical applicability. Interestingly, carrier-related research (including into other Fragile X-related conditions: FXTAS and FXPOI) were rated at relatively low priority. This may relate to the still low awareness and understanding of these conditions. Alternatively, as fragile X is typically identified in families through a diagnosis of fragile X syndrome, making this an issue of high focus, and there will not be clear premutation carrier issues present in members of all of these families. In this context, it was surprising that basic research was not considered

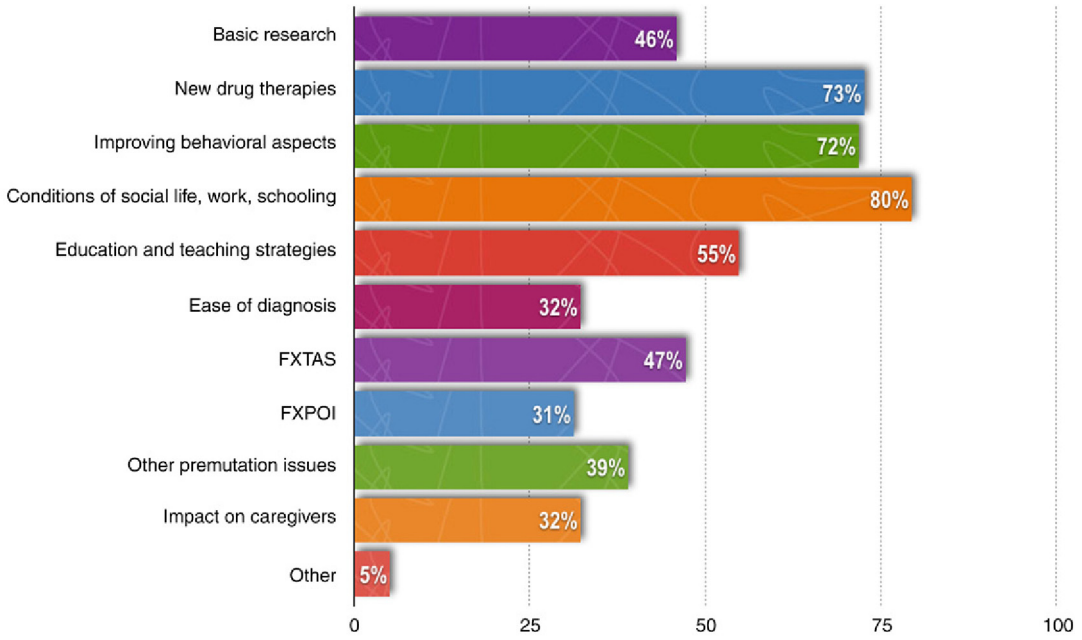

FIGURE 22.2 **Most important research areas.**

an important research area by the families, given its importance for the aforementioned drug therapies. Improved communication about this more basic research, and its potential application to later research addressing practical challenges faced, may help to address this gap.

> Keep doing the incredible work you do and thank you for all working together. That's what makes Fragile X research different. (Respondent answer to "Is there anything you wish to communicate to the scientific/research community?")

Information About Fragile X Research

Given the clear interest in and regard for research, it is essential that information about the findings of research, as well as current opportunities are effectively communicated with families and individuals living with fragile X. This is both important for the understanding and application of findings but also to encourage engagement with current work. First, we asked about how well people have been informed about research two projects in the past. Respondents rated their awareness on a scale of 1 (not informed at all) to 5 (very well informed). The overall response was neutral (a mean of 2.77 on the scale), highlighting a clear lack of information. There were, however, variations between different countries. For instance, 40% of respondents in Spain felt uninformed or not well informed, the highest rate among the countries. In contrast, a good information flow seems to be given in the United Kingdom and the Netherlands, where 64 and 58%, respectively, feel very well informed or well informed

about past projects. Though, clearly, even in the best-informed countries there is still work to be done.

As for current research projects, it is promising that around three out of four responders (77%) had heard about at least one ongoing or current project. Again, however, there was significant variation across different countries in the awareness of current research. The minimum awareness was rated in Germany, where only about half of the people have heard of recent fragile X research. In contrast, about 9 out of 10 Italians know of a recent project. However, the level of information about ongoing research is still lacking, as only 11% of respondents felt very well informed (and a further 24% well informed) about ongoing research.

It is telling that almost all respondents (97%) answered that they are interested in receiving more information about research projects. In order to effectively disseminate this information, it is important that efforts for research teams and support groups to disseminate information about research utilize the preferred methods of communication. When queried about the preferred methods of accessing such information, there was a clear and strong preference for communication about research via email (82%), followed by newsletters (33%), and websites (30%). The convenience of digital access clearly needs to be embraced to ensure that information is being communicated effectively.

In order to better inform families, efforts from and collaborations between both research organization and support organizations will be required.

Personal Involvement in Fragile X Research Projects

Research cannot take place without the participation of individuals living with fragile X and their families. Therefore, it is promising that respondents rated a strong willingness to participate in research, with just 13% answering that they would not consider doing so. There was a significant difference in answers received from different countries; in particular, about half of the responders from Italy oppose research project participation. Furthermore, there was variation (within the collated results) in the willingness to participate in different types of projects. About half of the survey attendees (48%) answered that they would both participate in behavioral/educational, as well as basic research/clinical trial related projects. About one quarter (27%) would participate in behavioral/educational projects, but not be willing to join a clinical trial or a basic research project.

Despite the expressed willingness to participate, actual engagement with research was much lower. Close to a third (38%) of the respondents answered that they have been involved in a previous or current research project, while 62% never actively took part in fragile X research. Again, there were significant international differences with about only one in five having been involved in fragile X research in Belgium, Germany, and Portugal and over half (55%) in the United States.

Among those who had participated, most responders rated their participation experience as positive. However, there were some international differences as shown in Fig. 22.3.

In order to find out how family participation in fragile X research projects can be improved, we asked about problems in case of a negative response in the rating of personal participation in a project. Relatively few responses were received, though the categorized responses are provided in Fig. 22.4. A significant number of responses criticized a lack of information, particularly regarding the feedback after the research project was finished.

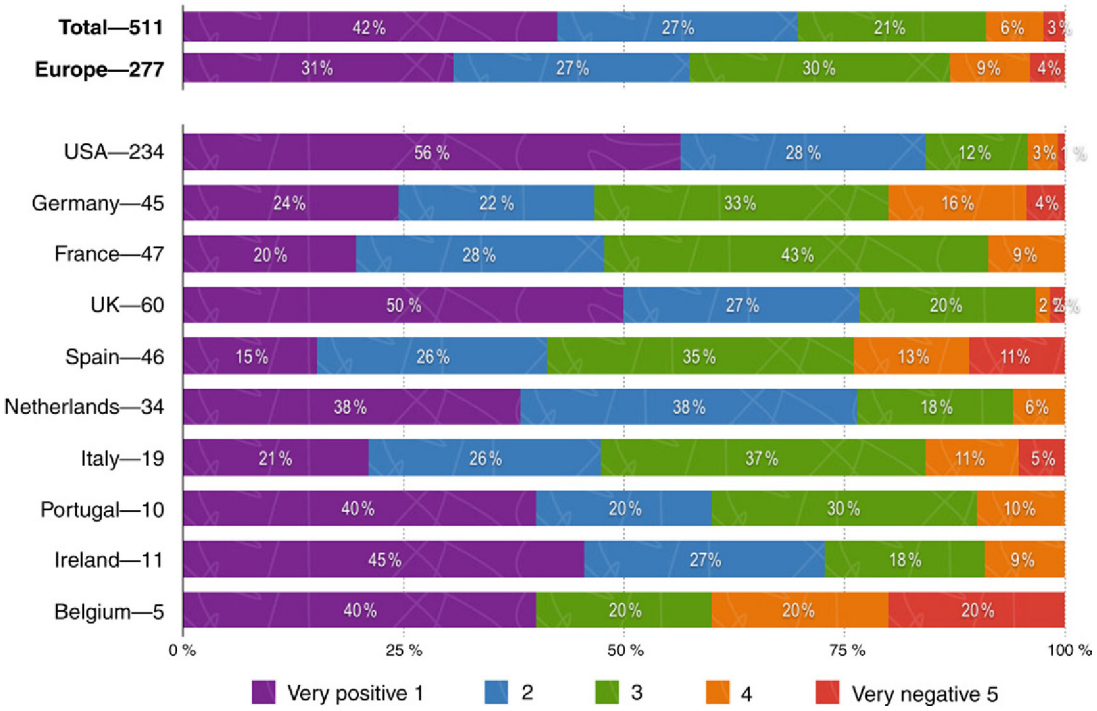

FIGURE 22.3 **Rating of personal experiences in fragile X research projects.**

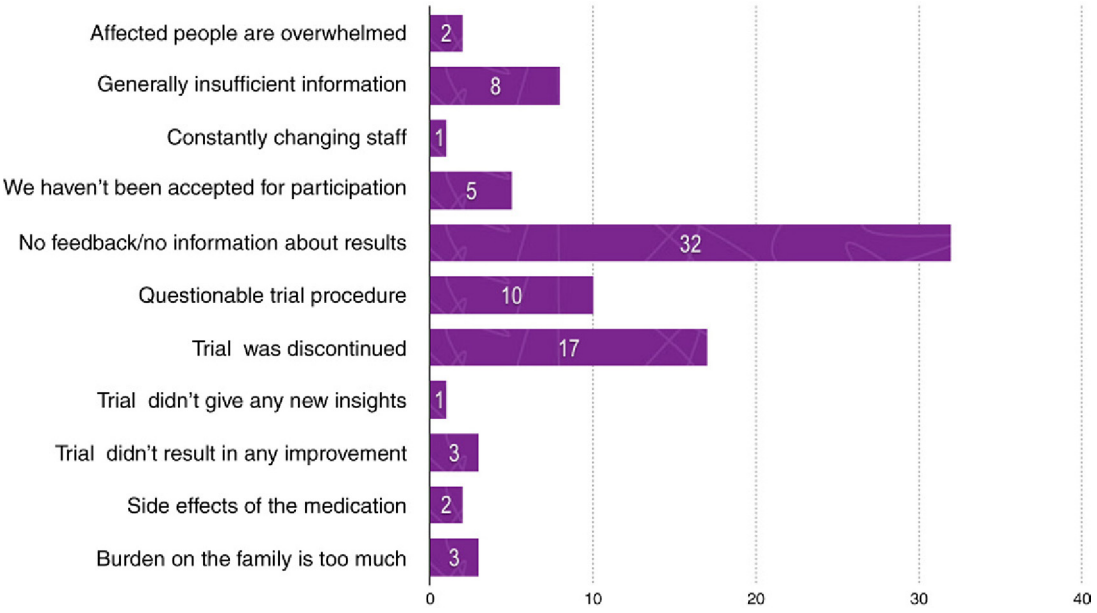

FIGURE 22.4 **Negative experiences during or after fragile X research project participation.**

In order to further investigate the possible barriers to participation, respondents were asked about their concerns about research. Although one third (31%) of the responders have no concerns about a participation in a fragile X research project, a significant number of important issues arose.

The primary concern expressed (as rated by 41% of respondents) was that participation would be too challenging for people with fragile X, as well as 22% who expressed that participation may be harmful for the person with fragile X. In the free comments, there were some specific practical challenges listed by multiple families, which included travel to the clinic and challenges with aspects of the research, such as blood draws. For instance, one family responded:

> We know that research has a very stringent methodology but the challenges and behaviors of individuals with Fragile X must be considered when developing a drug trial or other research protocol. Distance to travel, number of visits, blood draws, other tests, etc. all will be more difficult for our children. But we are willing to try! (Respondent answer to "Is there anything you wish to communicate to the scientific/research community?")

Furthermore, every fifth responder feels that research could be done for the sake of research itself only. More broadly, scientific research may be conducted with an exploratory element, with the aim of accruing knowledge, rather than for a defined outcome. However, these results clearly highlight families desire for research with a clear impact. This highlights the need for researchers to clearly communicate the potential impact and findings of their work, as well as consulting and engaging with the Fragile X community to ensure that research aligns with the concerns and needs of those living with the condition. Of course, not every project can have immediate impact and basic science and descriptive research is a vital part of the process. However, further transparency about the need for this work may help to address such concerns.

> It would be much more motivating if the research resulted in some positive benefit for the Fragile X subject and/or the family. Lots of research proves minute scientific points, many of which are very apparent to families and not really helpful as "new findings", other than in a scientific realm. Many families are struggling and the research does not address that fact. (Respondent answer to "Is there anything you wish to communicate to thescientic/research community".)

CONCLUSIONS

The large and diverse group of respondents allowed us to conclude that nearly all consider fragile X research important. The rich dataset affords the opportunity to draw further conclusions and the authors have brought their unique perspectives to this task. Two of the authors have dependents living with fragile X and all three have served in leadership roles in national Fragile X organizations in the US and across Europe. This has afforded all three authors direct and substantive regular contact with such families for a combined 30+ years and we likewise utilized this background of experience to draw our conclusions. We however, also encourage the reader to bring their own unique perspective to the task on analyzing the dataset and to guide your future patient interactions.

The Importance of Research

Conclusion 1. Notwithstanding that families' ability to cope with the myriad challenges associated with caring for or living with individuals diagnosed with fragile X varies widely, nearly all consider it important that a broad range of research be carried out aimed at improving the lives of those impacted by fragile X.

The area of research rated most important by the greatest number of respondents was "Conditions of Social Life, Work and Schooling." While this suggests a desired, focus on research interventions capable of allowing impacted individuals to live a better quality of life without altering the underlying biological mechanism of fragile X (Social), this was followed, closely by "New Drug Therapies" and "Improving Behavioral Aspects" (Scientific and Social), and "Education and Teaching Strategies", another Social focus. The "most important" ranking of these varied research topics demonstrates the importance of research across the broad, spectrum of impact associated, with fragile X.

Conclusion 2. Fragile X research, generally, is important to the vast majority of respondents surveyed. While social or quality of life focused research is rated most important by a greater percentage of respondents, scientific focused research is likewise classified as most important by a majority of respondents.

Conclusion 3. Nearly all respondents indicated that it was a priority that their respective national Fragile X organizations also support research. Irrespective of whether this support is expressed via direct or indirect financial support, oversight and advice, marshalling research participants, or facilitating collaborations, the message is clear, that from the parents' perspective, national Fragile X organizations play an important and necessary role in advancing research.

Implications. When considering the parent perspective, researchers would he well advised to apply their specialized skills across the full spectrum of research topics and encompass *both*; research leading to biological disease improving interventions of all conditions related to FMR1 mutations, and; research leading to improvements in daily living, such as social, emotional, or educational interventions. The national organizations representing and serving the needs of these same families would be equally well advised to support these efforts. Indeed, from the parent perspective, the hope and promise of biologically altering fragile X is important, but so too is improving the quality of life of impacted individuals until these scientific breakthroughs reach market.

Information

Conclusion 1. Irrespective of who is providing information about fragile X research, only a very small minority of parents consider themselves very well informed about research projects. Most respondents have heard about research projects in the last couple of years, but far less classify themselves very well informed about research that has been conducted in the past. Even when including the next group of respondents who considered themselves well informed, (and with the exception of the United Kingdom and the Netherlands), less than half of respondents were satisfied with the amount of information received. When the questions turned to ongoing research, (again with the exception of the United Kingdom and the Netherlands) the results were no better.

Conclusion 2. Given that nearly all respondents expressed the *desire to know more* about fragile X research, there exists both a challenge and an opportunity. A challenge because so few consider themselves well informed, and an opportunity because nearly all want to know more. The disparity between those who consider themselves well informed and those who want to know more is striking. The desire for more information is near universal and the number who consider themselves well informed is quite small.

Implications. The task before us is clear. Wide majorities of respondents consider research important, yet consider themselves not very well informed. Similar majorities want the national organizations, which represent them, involved in this process and, have made clear their preferred, method, to receive this information. Those conducting the research and the national organizations supporting these efforts must take heed. If we want families participating in future research we must do a better job of keeping them informed.

Participation

Conclusion 1. Deficiencies in information dissemination notwithstanding, across all countries surveyed, there remains strong support and willingness to participate in a broad spectrum of research. With the exception of the Netherlands and Italy, where there are sizeable segments of respondents who would not consider participating in any research, there is a robust willingness to participate in all fragile X research.

Conclusion 2. Despite the high degree of willingness to participate in research, in the countries other than the USA and the United Kingdom, the percentage of respondents that have actually participated in research averages less than one-third. In the USA and, the United Kingdom this rate approaches one-half.

Conclusion 3. The countries with the lowest number of respondents who have participated in research have the highest number of respondents reporting negative experiences. When those who were ambivalent about the experience are added, the average percentage of those NOT reporting a positive experience approaches one-half.

Conclusion 4. Of all respondents reporting a negative experience, two of the three most cited reasons for their negativity related to a failure to provide information.

Conclusion 5. Although nearly half of all respondents reported that their biggest concern about participating in research was the challenge which participation would pose, nearly all expressed willingness to participate.

Implications. From the perspective of parents surveyed, the news is generally good. Fully aware of the challenges they'll face, the vast majority reported a willingness to participate. Despite such willingness, across the European Union, less than one third had availed themselves of the opportunity representing a sizeable bank of individuals who are available for future participation. While we did not ask why they had not participated, the generally low rate of participation across the EU seemingly correlates with a high rate of ambivalent-to-negative experiences. While other cultural factors may need to be explored, we postulate that these negative experiences, once shared with other members of their communities, may suppress the willingness to participate. Most often the reason for such negativity centered on communication and we are obligated to pay attention.

As science continues to identify pathways in the brain and body that are disrupted because of *FMR1* mutations, thus identifying possible targets for treatment, the need for human-trials

participants will only grow. A vast majority of participants in this survey reported a willingness to participate in a wide range of studies.

From the parents' perspective the message to the fragile X research community is clear. The intended beneficiaries of that research:

- Remain keenly interested in exploring all options to improve the life experience of the individuals who are living with the consequences of FMR1 mutations.
- Understand that participation in such research could be very challenging and yet, are willing to participate in a broad range of studies from basic science to schooling and everything in between; and
- Are willing to work together with the research community and in return ask to be always promptly and fully informed at all phases of our participation and thereafter.

LOOKING TO THE FUTURE: COMMUNICATION IS KEY

As highlighted in the discussions earlier, a key theme throughout the findings is communication, with issues identified at all stages of the research process. Therefore, we wish to close this chapter with some broader considerations and messages around this important topic, and how best to move forward.

Involvement and Communication From the Word Go

One of the challenges highlighted by parents with research participation is practical issues and challenges relating to the communication. In addition, recent clinical trials have led to discussion as to the extent to which some standardized measures are able to assess the real issues experienced by individuals with fragile X, and possible changes occurring as a result of interventions. Though some of these issues may be inherent in the research process, it is possible that the input and perspective of families, with their practical experience, may for instance help to identify meaningful behaviors, which could be assessed as outcome measures. Therefore, researchers must not forget the fantastic resource that is available to them: this international community of families. By establishing methods of interacting with those with lived experience of the condition, and drawing on their perspectives, some of these issues may be able to be addressed at the earliest stages of the research design process.

Then, an open and transparent line of communication needs to be maintained through each project to encourage participation and understanding of the results.

This also applies where challenges arise with the research, such as in the recent clinical trials (2013–15), where poor communication left many families feeling blindsided, lied to, or abandoned.

Dissemination Does Not End With Academic Publication

Academics are increasingly evaluated based upon the number and impact factor of their publications. This culture may underlie some of the expressed challenges around the lack of feedback following research participation and the broader lack of information

about research findings. It is clear that, broader avenues for communication with individuals and families living with fragile X need to become ingrained in the dissemination process, as those on the ground' are unlikely to be accessing these academic resources. Furthermore, effective dissemination allows for the application of the important findings from the research in people's lives (where applicable), which is surely where the true impact factor lies.

Watch Your Language!

Medicalized language (such as "disease") and technical jargon are used as standard among the academic community. However, there needs to be greater sensitivity as to the way that these terms may be interpreted or received, particularly when communicating with the wider community.

"Cure." Most pertinent is the use of the word "cure." Recent years have seen substantial efforts and challenges in the search for a targeted pharmacological intervention for fragile X. It has been widely publicized that these prospective interventions will be the "cure" for fragile X syndrome. There are several considerations around this point. First, as one parent eloquently commented in the survey "Be clear what a 'cure' might mean," as this evocative term is variably interpreted. For many, a "cure" means an approach which would result in an instant reversal of symptoms, across all individuals with a certain condition. However, such an outcome is unlikely to be the outcome of even the most successful of trials. The expectation, and subsequent heartache, created via the use of this language was clear to see with the null results of the recent trials. Of course, future communications relating to clinical trials do not need to suppress optimism, but should be mindful of language in order to manage expectations. The road ahead of us may still be long as, despite being a "single gene" condition, the efforts to identify targeted treatments pose significant challenges. In addition, though pharmacological interventions may well form a significant part of supports available to individuals with fragile X, environmental and educational interventions remain vital and must remain to be promoted and researched, as a priority.

A further point relating to cure is the consideration around whether people should be "cured." This point is highly evocative and divides opinion among families. There are many who feel that their loved ones do not need curing of their differences, and that they should not be seen as being "sick" or having a "disease." We urge that through all of the considerations about interventions, an individuals' quality of life is kept central in the discussion and that these differing perspectives are taken into account.

Avoid jargon. The accessibility of communications is paramount and research-related jargon can be a real barrier to people interacting with information about the findings of research.

Think Digital

Online interactions are increasingly becoming a central part of the support network for the Fragile X community. Equally, families in the survey expressed a strong preference for improving digital research communications, such as by developing email bulletins. This fast

and inexpensive mode of communication highlights an exciting opportunity, either by research organizations themselves, or in partnership with support organizations.

Power of the Support Organizations

The results from this survey clearly highlight strong support from families for the work that researchers are doing, which is echoed strongly at the level of the national organizations. As organizations we are keen and passionate about supporting science, which could lead to real improvements in the quality of life of those living with all of the varying features of fragile X. The vast response to this survey highlights the power that we have to support this work.

Addressing the Missing Voice in Research

The views expressed in this survey are those of parents of individuals with fragile X syndrome. However, a voice which remains to be heard is that of self-advocates living with fragile X syndrome, or carrier-related conditions, such as FXTAS. The involvement of self-advocates in support organizations is an exciting area currently under development. These individuals with direct, lived experience have great value to add to both the design and content of research, particularly to projects which are more applied or social-focused. We believe that there is scope for support organizations and research teams to work in partnership on this issue, in order to ensure productive and meaningful involvement which is beneficial both for research and the self-advocates.

SOME CLOSING THOUGHTS

At the conclusion of our survey, respondents were asked whether there was anything else they wanted to communicate to the research community and were provided the opportunity to write in their own personal message. Nearly half of all respondents offered comments.

The most common sentiments expressed were:

1. Optimism.

   We continue to have hope that life improving interventions will come to market.

2. Persistence.

   Please, don't give up. Continue your research.

3. Appreciation.

   Thank you. Your hard work is appreciated.

Please contact the authors of this chapter for further details about the survey results. The National Fragile X Foundation and the European Fragile X Network are a valuable resource that should be fully engaged as we look to the future.

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