Chapter 23
Molecular and Cellular Aspects of Mental Retardation in the Fragile X Syndrome: From Gene Mutation/s to Spine Dysmorphogenesis

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Abstract The Fragile X syndrome (FXS) is the most frequent form of inherited mental retardation and also considered a monogenic cause of Autism Spectrum Disorder. FXS symptoms include neurodevelopmental delay, anxiety, hyperactivity, and autistic-like behavior. The disease is due to mutations or loss of the Fragile X Mental Retardation Protein (FMRP), an RNA-binding protein abundant in the brain and gonads, the two organs mainly affected in FXS patients. FMRP has multiple functions in RNA metabolism, including mRNA decay, dendritic targeting of mRNAs, and protein synthesis. In neurons lacking FMRP, a wide array of mRNAs encoding proteins involved in synaptic structure and function are altered. As a result of this complex dysregulation, in the absence of FMRP, spine morphology and functioning is impaired. Consistently, model organisms for the study of the syndrome recapitulate the phenotype observed in FXS patients, such as dendritic spine anomalies and defects in learning.

Here, we review the fundamentals of genetic and clinical aspects of FXS, devoting a specific attention to ASD comorbidity and FXS-related diseases. We also review the current knowledge on FMRP functions through structural, molecular, and cellular findings. Finally, we discuss the neuroanatomical, electrophysiological, and

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behavioral defects caused by FMRP loss, as well as the current treatments able to partially revert some of the FXS abnormalities.

23.1 Genetics of Fragile X (FXS) and Fragile X Tremor Ataxia (FXTAS) Syndromes

23.1.1 Fragile Mental Retardation 1: A Gene Associated to Two Neurological Diseases

The Fragile X syndrome (FXS) is the most frequent form of inherited intellectual disability (Jacquemont et al. 2007). Patients with FXS show physical features, such as large ears, elongated face, and high-arched palate, which have been reported in 60% of prepubertal FXS boys. Other symptoms include connective tissue anomalies, which can lead to mitral valve prolapse, scoliosis, flat feet, and joint laxity. Recurrent otitis media and strabismus are also common. Macroorchidism due to a hypothalamic dysfunction affects about 90% of boys with FXS by the age of 14 (Jacquemont et al. 2007).

The neurological involvement displays a broad spectrum of cognitive and behavioral deficits. The developmental delay is the most consistent feature, with a mean IQ of 42 in boys and severe mental retardation in about 25% of cases. Since the disorder is X-linked and the penetrance is variable, females are usually in a low–normal range, with an IQ ranging from 70 to 90 (Jacquemont et al. 2007). Moreover, epilepsy has been described in 13–18% of boys and 4% in girls, but normally, the seizures and EEG alterations tend to resolve during childhood or early adulthood (Berry-Kravis 2002; Musumeci et al. 1999). Despite the severe neurobehavioral symptoms, the anatomical studies revealed minor abnormalities in postmortem brains from FXS patients (Hallahan et al. 2010; Reiss et al. 1995). The most prominent neuroanatomical feature is the dysgenesis of the dendritic spines, which appear longer and thinner than normal, likely due to a developmental delay in spine dynamics and transition from immature to mature spines (Cruz-Martin et al. 2010; Irwin et al. 2001). FXS is also the most common monogenic cause of Autism Spectrum Disorder, ASD (Hatton et al. 2006), a heterogenous group of neurodevelopmental pathologies affecting approximately 37 individuals in 10,000 (Fombonne 2005) and present in more than 40% of patients with intellectual disability (Moss and Howlin 2009). About 25% of FXS boys and 6% of girls meet criteria for ASD, while 1–2% of patients affected by ASD have FXS (Abrahams and Geschwind 2008; Hatton et al. 2006). In particular, recent reports estimated that about 30% of FXS subjects meet criteria for Autistic Disorder and 30% for Pervasive Developmental Disorder Not Otherwise Specified (Harris et al. 2008). However, up to 90% of children with Fragile X display behavioral alterations which resemble ASD, such as social anxiety, gaze avoidance, delayed speech development,

echolalia, sensory hypersensitivity, tactile defensiveness, stereotypic movements, and poor motor coordination (Belmonte and Bourgeron 2006; Hernandez et al. 2009). The cognitive delay is more severe in FXS children with ASD, and additional neurological disorders, genetic problems, or seizures may increase the risk of autism (Garcia-Nonell et al. 2008).

FXS is due to triplet repeat expansion or point mutations in the Fragile X mental retardation 1 (FMR1) gene, located on chromosome Xq27-3 (Fig. 23.1). A severe FXS form has also been documented in a patient with a mutation in the coding region of the gene, leading to the substitution of isoleucine 304 for asparagine (Ile304Asn, see below) (De Boulle et al. 1993). Few cases with deletions in the coding regions have also been identified (Gedeon et al. 1992; Meijer et al. 1994; Mila et al. 2000; Wohrle et al. 1992). In over 90% of patients, a CGG triplet in the 5′ UTR of the gene is expanded over 200 copies, leading to hypermethylation of the CGG, transcriptional silencing, and abolished production of the Fragile X Mental Retardation Protein (FMRP) (Jacquemont et al. 2007). The CGG triplet region is highly polymorphic in the population. Normal alleles (5–44 CGG copies) are stably transmitted to the offspring; "gray-zone" alleles (45–54 copies) and "premutation" alleles (55–200 copies) are rather unstable and can evolve into a "full mutation" (>200 repeats) during the maternal transmission (Fig. 23.1). While the gray-zone alleles require at least two generations before expanding to a full mutation (Fernandez-Carvajal et al. 2009), the premutation is highly unstable, and the risk of transmitting an allele with the full mutation is function of the repeat length (Hagerman and Hagerman 2002). The carriers of premutation alleles were considered clinically unaffected since the discovery of a dominant late-onset neurodegenerative disorder: the Fragile X Tremor Ataxia Syndrome (FXTAS). In 2001, the Hagerman laboratory described for the first time action tremor associated with executive function impairments and brain atrophy in five elderly men with the premutation (Hagerman et al. 2001). The frequency of premutation carriers has
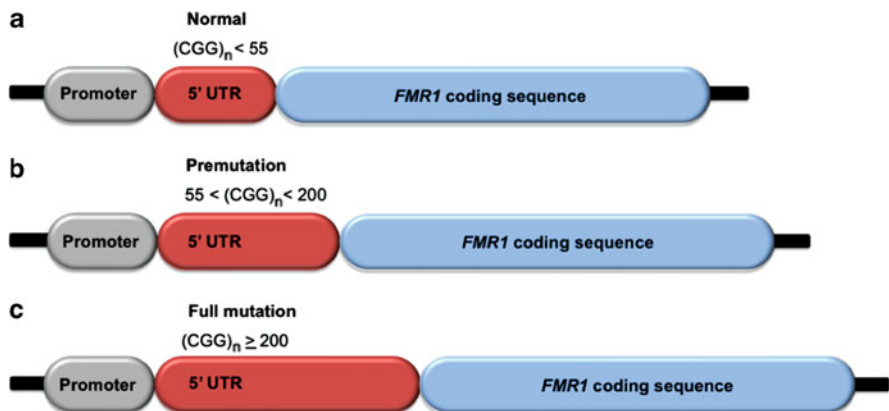

Fig. 23.1 Scheme of the *FMR1* gene which includes the promoter, the 5′ UTR, and the *FMR1*-coding sequence in a normal allele (5–44 CGG copies) (**a**), a premutated allele (55–200 copies) (**b**), and a full mutated allele (>200 repeats) (**c**)

been estimated in 1:800 in men and 1:300 in women, although FXTAS displays reduced penetrance (~33% in men, not yet determined in women). Major behavioral diagnostic criteria for FXTAS are gait ataxia and intention tremor, eventually associated with parkinsonism and cognitive decline, often progressing to dementia (Hagerman and Hagerman 2007). Psychiatric disturbances often observed in FXTAS patients include anxiety, depression, and hostility (Bacalman et al. 2006). In some premutation patients, a psychiatric phenotype with features resembling attention deficit hyperactivity disorder (ADHD) and ASD can also appear in childhood or adolescence (Farzin et al. 2006). Further studies on postmortem brains from premutation carriers revealed a generalized brain atrophy, white matter disease, and middle cerebellar peduncle lesions (Hagerman and Hagerman 2007). One of the key cytological hallmarks of FXTAS is the presence of intranuclear ubiquitin-positive inclusions in neurons and astrocytes throughout the brain (Greco et al. 2006). The intranuclear foci were consistently observed in model organisms for FXTAS, both mouse (Berman and Willemsen 2009) and *Drosophila* (Jin et al. 2007; Sofola et al. 2007). Remarkably, the number of inclusions correlates with the size of the CGG expansion (Greco et al. 2002, 2006).

FXS and FXTAS are both due to triplet expansions. While FXS is a loss-of-function disease, FXTAS is thought to be a consequence of RNA toxic gain-of-function mechanism. First, a consistent molecular feature in both FXTAS patients and mouse models is the elevation of aberrant CGG expanded *FMR1* mRNA levels (Allen et al. 2004; Kenneson et al. 2001; Tassone et al. 2000), due to increased transcription (Tassone et al. 2007). Nevertheless, carriers of premutation alleles show decreased levels of FMRP (Brouwer et al. 2008; Entezam et al. 2007) caused by the reduced translational efficiency of the *FMR1* mRNA carrying the CGG expansion (Primerano et al. 2002), as well as by a differential use of the *FMR1* mRNA 3′ UTR (Tassone et al. 2011). It has been proposed that the CGG expansion in the 5′ UTR would form a secondary structure inhibiting the ribosome scanning and thus leading to a scarce translational efficiency (Feng et al. 1995). The intranuclear foci contain in addition to the aberrant *FMR1* mRNA (Greco et al. 2002; Tassone et al. 2004) a variety of RNA-binding proteins (RBPs) interacting with the rCGG tract, such as PURalpha, hnRNP A2/B1, and CUG-BP1 (Iwahashi et al. 2006).

The *FMR1* gene encodes for FMRP (Bassell and Warren 2008; De Rubeis and Bagni 2010), an RNA-binding protein that contributes to the posttranscriptional control of gene expression (see below). In neurons, FMRP is part of messenger ribonucleoparticles (mRNPs) and regulates dendritic transport of associated mRNAs, their stability and local translation (Bassell and Warren 2008; De Rubeis and Bagni 2010). The roles of FMRP have been mainly addressed by using animal models that mimic FXS.

23.1.2   Model Organisms for the Study of the Fragile X Syndrome

The *FMR1* gene is conserved along evolution, and this allowed researchers to develop murine (Bakker 1994; Mientjes et al. 2006), *Drosophila* (Zhang et al. 2001), and

zebrafish (den Broeder et al. 2009; Tucker et al. 2006) animal models to study the molecular, cellular, and behavioral phenotypes of the syndrome.

Mouse models. The first model available, the *Fmr1* knockout (KO) mouse, was generated by interrupting exon 5 of the *Fmr1* gene with a neomycin cassette (Bakker 1994). Although the insertional mutation does not mimic FXS in humans, it leads to the functional ablation of *Fmr1* gene since the interrupted *Fmr1* mRNA prevents the translation of a functional FMRP (Bakker 1994). This mouse model presents an array of anatomical, behavioral, and neurological similarities to FXS patients (see below). Recently, a conditional KO (*Fmr1* CKO) and a second generation *Fmr1* KO null for *Fmr1* mRNA (*Fmr1* KO 2) have been generated by flanking the murine promoter and the first exon with loxP sites (Mientjes et al. 2006). In *Fmr1* CKO, *Fmr1* expression can be suppressed at specific developmental stages or in specific cell types, as showed by crossing these mice with a line carrying the Cre recombinase driven by a Purkinje cell–specific promoter (Mientjes et al. 2006). Moreover, a mouse model mimicking the mutation Ile304Asn that leads to a severe FXS manifestation (De Boulle et al. 1993) has been recently generated; of interest, this model phenocopies the behavioral and electrophysiological defects observed in *Fmr1* KO mice (Zang et al. 2009) (see below).

Fruit fly Models. In *Drosophila melanogaster*, several loss-of-function mutations, ranging from hypomorphs to nulls, have been generated (Dockendorff et al. 2002; Inoue et al. 2002; Morales et al. 2002; Zhang et al. 2001). Such models display a variety of behavioral and developmental defects (Zarnescu et al. 2005).

Zebrafish Models. The first attempt to produce a model for FXS in *Danio rerio* was performed in 2006 using a knockdown approach by microinjecting morpholinos in early embryos (Tucker et al. 2006). Although the authors described defects in craniofacial development and neuronal branching in embryos, further studies failed in reproducing this phenotype in two *Fmr1* KO lines (den Broeder et al. 2009).

23.2 An Insight into the Structure of the Fragile X Mental Retardation Protein

The *FMR1* gene is composed of 17 exons and subjected to alternative splicing, occurring preferentially at the level of exons 12, 14, 15, and 17. This generates up to 12 different protein isoforms, with a molecular weight ranging between 70 and 80 kDa, with the longest isoform containing 632 amino acidic residues (Bassell and Warren 2008). The role of each isoform still needs to be clarified (Bassell and Warren 2008). Whereas FMRP isoforms are similarly expressed in many tissues and organs, the relative abundance of each isoform seems to be tissue specific (Kaufmann et al. 2002; Xie et al. 2009).

FMRP is a multidomain RNA-binding protein able to recognize several coding and noncoding RNAs, including the brain cytoplasmic RNA BC1/BC200 (Ashley et al. 1993; Johnson et al. 2006; Napoli et al. 2008; O'Donnell and Warren 2002;

Siomi et al. 1993; Zalfa et al. 2005) and microRNAs (Edbauer et al. 2010). Moreover, FMRP homodimerizes and interacts with several cytoplasmic and nuclear proteins, including the two paralogs Fragile-X-related proteins 1 and 2 (FXRP1 and FXRP2) (O'Donnell and Warren 2002; Tamanini et al. 1999; Zhang et al. 1995).

The protein can be structurally divided into three main regions: N-terminal region, central region, and C-terminal region (Fig. 23.2a). The N-terminal region is characterized by the presence of two Tudor domains (TD), a putative Helix-Loop-Helix domain (HLH), and a Nuclear Localization Signal (NLS) (Sjekloca et al. 2009) (Fig. 23.2a). The central region contains two K Homology domains (KH) that share a high degree of homology with the hnRNP K domain and a Nuclear Export Signal (NES) (Valverde et al. 2008) (Fig. 23.2a). The C-terminal region, which is the less conserved region among the different species, is characterized by the presence of an RGG box containing a conserved Arg-Gly-Gly triplet (Darnell et al. 2001; Menon et al. 2004; Sjekloca et al. 2009) (Fig. 23.2a).

A region modulated by phosphorylation is localized between the FMRP central portion and the RGG box, specifically located between the amino acids 483 and 521 and conserved along different species. Ceman and colleagues showed that the phosphorylation of serine 499 triggers hierarchical phosphorylation events of nearby serines (Ceman et al. 2003). This phosphorylation modulates the association of FMRP with Dicer involving FMRP in the miRNA pathway (Cheever and Ceman 2009).
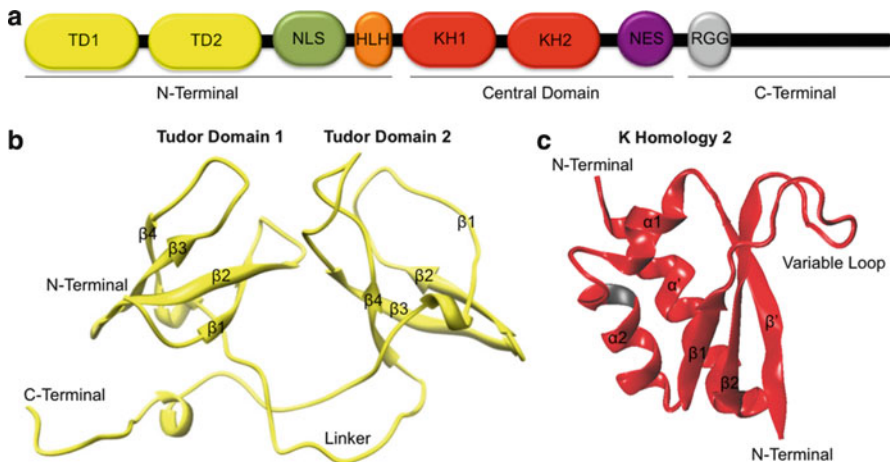

Fig. 23.2 Structural modules of FMRP. (**a**) The different domains that compose the protein are represented following the color code: *yellow* = Tudor domains, *blue* = nuclear localization signal, *green* = helix-loop-helix motif, *red* = K homology domains, *purple* = nuclear export signal, *gray* = RGG box. The division in N-terminal, central, and C-terminal regions is also represented. (**b**) Ribbon representation of the structure of the two Tudor domains represented in *yellow* and (**c**) of the KH2 domain represented in *red*. In both panels, the succession of the secondary structure elements is *underlined*. The position of residue Ile304 is highlighted in *gray*

The N-terminal region of FMRP contains two Tudor domains able to bind single-strand (ss) nucleic acids. The same activity is shared by the two KH domains in the central region (Darnell et al. 2001; Musco et al. 1996; Ramos et al. 2006; Valverde et al. 2007) and the RGG box in the C-terminal region. The structure of the entire protein has not been resolved so far, but NMR and X-ray structures of single domains are available (Ramos et al. 2006; Valverde et al. 2007). The structure of the first 134 residues of the N-terminal domain, resolved by NMR, reveals the three-dimensional organization of the two Tudor domains, each one formed by a barrel-like fold made of four-stranded antiparallel β sheet (Fig. 23.2b). The two Tudor domains are linked by an unstructured fragment (linker) (Ramos et al. 2006) (Fig. 23.2b). The structure of the first 134 residues reveals three structural motifs, the two Tudor domains repeats and one α helix (Ramos et al. 2006) (Fig. 23.2b). Extensive interactions are observed between these elements, strongly suggesting that all the elements are necessary for the stability of the overall N-terminal domain (Ramos et al. 2006). The structure also reveals hydrophobic pockets on the surface of the two Tudor domains, in analogy with other Tudor domains, such as the Survival of Motor Neuron (SMN) Tudor domain and the heterochromatin-associated protein 1 (HP1) chromo domains (Nielsen et al. 2002b; Sprangers et al. 2003). These hydrophobic pockets can bind methylated amino acids (Ramos et al. 2006).

The three-dimensional structure of the two KH domains located in the central region of FMRP has been solved by X-ray (Valverde et al. 2007). The KH domains, usually present in multiple copies in a protein, contain the consensus (ILV)-I-G-X_2-G-X_2-I sequence and are responsible for the interaction with ssDNA, mRNA, and rRNA. The KH domains consist of a $\beta^1\alpha^1\alpha^2\beta^2\beta'\alpha'$ fold, three-dimensionally oriented as a three-stranded β-sheet domain opposed to a three α-helices domain (Valverde et al. 2008), as evidenced by the X-ray structure (Valverde et al. 2007) (Fig. 23.2c). The KH domains are classified as Type I or Type II folds. Both contain the minimal KH motif but with different C- or N-terminal extensions giving $\beta^1\alpha^1\alpha^2\beta^1\beta'\alpha'$ and $\alpha'\beta'\beta^1\alpha^1\alpha^2\beta^2$ for Type I and Type II, respectively (Valverde et al. 2007) (Fig. 23.2c). KH domains in eukaryotic proteins are exclusively Type I, whereas in prokaryotic proteins are exclusively Type II (Grishin 2001; Siomi et al. 1993). β-Sheets β^2 and β' are connected by a variable loop, while α-helices α^2 and α' are connected by the so-called GXXG loop (Fig. 23.2c). This HLH (Helix-Loop-Helix) region of the domain forms the nucleic acid binding site (Valverde et al. 2008).

The best characterized missense mutation for FMRP is Ile304Asn, located on the KH2 domain (De Boulle et al. 1993). As previously mentioned, this mutation has been reported in an individual with a severe manifestation of FXS, including very low IQ, macroorchidism, and severe social and behavioral impairment (De Boulle et al. 1993). This underlines the importance of the KH2 domain for the neuronal functions of FMRP. The structure of the two KH domains revealed that the Ile304 residue is located on helix α2 of the KH2 domain (Fig. 23.2c). This residue is part of the hydrophobic core that stabilizes the three-dimensional folding of the domain. Indeed, the hydrophobic residues present at the interface of the α-helices and β-sheets domain establish a hydrophobic network of interactions that maintains the domain structure. This network of hydrophobic packing and van der Waals interactions is hypothesized to be conserved among all the Fragile-X-related proteins, such as FXRP1 and FXRP2,

since all the hydrophobic residues abovementioned are conserved (Valverde et al. 2007). The substitution of residue Ile304 by an Asn, located in this hydrophobic core may disrupt this network, affecting the structure of the domain (Fig. 23.2c). Furthermore, being Ile304 buried in the domain core and not solvent accessible, it seems that the residue is not directly involved in the binding with nucleic acids, but the structural rearrangements caused by the mutation could affect the nucleic acid binding (Valverde et al. 2007).

FMRP belongs to a multiprotein complex and not only interacts with nucleic acids but also with a series of other proteins. Among all, the best characterized are the Cytoplasmic Fragile X Mental-Retardation-Interacting Protein 1 and 2 (CYFIP1 and 2), the Eukaryotic Translation Initiation Factor 4E (eIF4E), the Insulin-like Growth Factor 2 mRNA-Binding Protein (IGF2BP1), the Survival of Motor Neuron (SMN), the Tudor-Domain-Containing Protein 3 (TDRD3), the FXR1 and 2, the 58-kDa Microspherule Protein (MSP58), and the Nuclear Fragile X Mental-Retardation-Interacting Protein 1 and 2 (NUFIP1 and 2). FMRP also interacts with a series of other proteins involved in several diseases (Table 23.1). The extensive network of interactions explains the presence of FMRP in several multiprotein complexes.

Table 23.1 FMRP interactors found in mammalian cells and tissues. (*ND*) Indicates no diagnosed disease associated to the protein

Protein name	Molecular function/properties
FXR1P	RNA-binding protein
FXR2P	RNA-binding protein
CYFIP1	Rac1-binding protein translational repressor tumor suppressor
CYFIP2	Regulator of actin cytoskeleton
NUFIP1	DNA and RNA binding
82-FIP/NUFP2	ND
NUCLEOLIN	DNA and RNA binding
YB1/p50	DNA and RNA binding
STAUFEN1	Double-stranded RNA binding
PURα	DNA and RNA binding
PURβ	DNA and RNA binding
MYOSIN VA	mRNP and organelle transporter
RanBPM	Scaffolding protein: protein interaction and cytoskeletal-binding domain
elF2C2/AGO1	mRNA processing, translational control
DICER	RNase III endonuclease, RNA interference pathway
PABP1	RNA binding
Kinesin heavy chain 5A/C	Motor protein
Dynein intermediate chain	Microtubule and protein binding, motor activity
elF4E	RNA and protein binding
TDRD3	Nucleic acid binding
UBE21(UBC9)	Ubiquitin-ligase activity, SUMO-ligase activity, transcription factor binding
NXR2	Nuclear mRNA export
	Posttranscriptional mRNA metabolism
APC	Protein and microtubule binding

Most of the protein–protein and protein–RNA interactions occur at the N-terminal and central regions of FMRP. These two regions display a high degree of conservation among different family members (FMRP-FXR1P–FXR2P), while the C-terminal region, where the RGG box is located, is found to be the less conserved (Menon et al. 2004).

Among the best characterized interactions, the region encoded by exon 7 (residues 173–218) of FMRP is responsible for the interaction with CYFIP1 and 2. This interaction leads to the formation of the eIF4E-CYFIP1-FMRP complex that blocks mRNAs translation process (Napoli et al. 2008) (see below). Interaction of FMRP with NUFIP (Nuclear FMRP Interacting Protein) occurs through the N-terminal region (residues 1–217) (Bardoni et al. 2003). Residues 470–485 of the N terminus are also essential for the interaction with SMN, while residues 430–486 as well as the second KH domain are crucial for the binding with TDRD3 (Linder et al. 2008). Interestingly, the Ile304Asn mutation affects the FMRP–TDRD3 interaction (Linder et al. 2008). The C terminus is involved in the interaction with RanBPM, and this interaction also modulates the FMRP RNA binding activity (Menon et al. 2004).

23.3   Cellular and Molecular Functions of FMRP

FMRP is mainly expressed in the brain and gonads (Khandjian et al. 1995; Verheij et al. 1993) where it is mostly confined to the cytoplasm. FMRP has also been localized in nucleus (Willemsen et al. 1996). Despite existing a clear shuttling process of the protein from both compartments (Eberhart et al. 1996; Sittler et al. 1996), the neuronal cytoplasmic function of FMRP has been mainly addressed. FMRP forms large cytoplasmic ribonucleoparticles (RNPs) containing several other proteins and RNAs (Johnson et al. 2006; Zalfa et al. 2005; Zalfa and Bagni 2005; Zalfa et al. 2003). FMRP-RNPs have also been found to cosediment with both polyribosomes and mRNPs (Zalfa et al. 2006) consequently being involved not only in the traffic and stability of the transported mRNAs but also in their translation. FMRP has also been detected in P bodies (PB) and stress granules (SG) containing translationally silent preinitiation complexes (Anderson and Kedersha 2006). Several studies have shown that FMRP plays a critical role in regulating mRNA translation, transport, and stability (Bagni and Greenough 2005; Bassell and Warren 2008; De Rubeis and Bagni 2010) (see Table 23.2 for a list of validated mRNA targets). In addition, the expression of FMRP in dendrites increases after synaptic stimulation suggesting a direct link between FMRP function and synaptic plasticity activation (Antar et al. 2004, 2006; Ferrari et al. 2007).

23.3.1   Regulation of Protein Synthesis

The translational dysregulation of a subset of FMRP target mRNAs is probably the major contribution to FXS (Table 23.2) (Bassell and Warren 2008). In neurons, protein synthesis occurs not only in the soma but also along axons (Holt and

Table 23.2 Shortlist of FMRP mRNA targets whose association has been validated by applying in vivo or in vitro methods

mRNA	Dendritic localization	References
App		Westmark and Malter (2007)
Arc	+	Zalfa et al. (2003), Park et al. (2008)
CamKlla	+	Zalfa et al. (2003), Hou et al. (2006), Muddashetty et al. (2007)
eEF1A	+	Sung et al. (2003)
Fmr1	+	Weiler et al. (1997), Schaeffer et al. (2001)
GluR1/2	+	Muddashetty et al. (2007)
Map1b	+	Brown et al. (2001), Darnell et al. (2001), Zalfa et al. (2003)
NR1/NR2B		Schütt et al. (2009)
NR2A	+	Edbauer et al. 2010
PSD-95	+	Zalfa et al. (2007), Muddashetty et al. (2007)
SAPAP 1/2/3/4	+	Brown et al. (2001), Kindler et al. (2004), Narayanan et al. (2007), Dictenberg et al. (2008), Schütt et al. (2009)
Shank1/2	+	Schütt et al. (2009)
Rgs5	+	Miyashiro et al. (2003), Dictenberg et al. (2008)
GABA-Ad	+	Miyashiro et al. (2003), Dictenberg et al. (2008)

(+) Indicates the evidence for dendritic mRNA localization or synaptic synthesis, while no symbol reflects lack of any experimental conclusive result. This table has been updated from Table 1 of Bassell and Warren (2008)

Bullock 2009), dendrites, and postsynaptic sites (Steward and Schuman 2003). Local protein synthesis is required for long-lasting forms of synaptic plasticity that underlie consolidation of long-term memories (Flavell and Greenberg 2008).

In the brain, protein synthesis is a mechanism that follows different states of synaptic plasticity activation, and it is orchestrated by the action of glutamate receptors. The ionotropic receptors N-methyl-D-aspartate (NMDAR), alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPAR), and the metabotrobic glutamate receptor (mGluR) play a key role in basic synaptic plasticity as well as in the activation of different synaptic plasticity states (Massey and Bashir 2007; Shi et al. 1999). In vitro models to study synaptic plasticity have been developed by depolarization of the postsynaptic membrane. Specifically, activation of mGluR by applying dihydroxyphenylglycine (DHPG) induces a synaptic plasticity state called long-term depression (LTD) (DHPG-induced LTD) (Massey and Bashir 2007) which involves several synaptic events including mRNA targeting and local protein synthesis and degradation (Gladding et al. 2009). However, in the *Fmr1* KO mice, DHPG-induced LTD plasticity is strongly increased (Huber et al. 2002), and it is also protein synthesis independent. This effect on LTD is likely due to deregulated local protein synthesis (Lu et al. 2004; Muddashetty et al. 2007; Zalfa et al. 2007; Huber et al. 2002; Nosyreva and Huber 2006; Ronesi and Huber 2008) and has settled the bases to describe the "mGluR theory" (Bear et al. 2004) (see below).

FMRP is involved in both basal and activity-dependent local protein synthesis by repressing in vivo and in vitro translation (Laggerbauer et al. 2001; Li et al. 2001; Lu et al. 2004; Muddashetty et al. 2007; Napoli et al. 2008; Zalfa et al. 2003). This has

been proved in lymphoblastoid cells from individuals affected by FXS, in which 251 FMRP mRNA targets showed an abnormal polysomal distribution, explaining an increased translation (Brown et al. 2001). Moreover, protein synthesis of FMRP target mRNAs is increased in *Fmr1* KO mice, especially in purified synaptosomes, extending to synapses the function of FMRP as a translation repressor (Muddashetty et al. 2007; Schütt et al. 2009; Zalfa et al. 2003). Regarding proteins localized near the postsynaptic membrane, a single study performed by Schütt and colleagues detected an increase in the expression levels of the postsynaptic proteins SAPAP1, SAPAP2, SAPAP3, Shank1, Shank3, IRSp53 as well as the NMDA receptor subunits NR1 and NR2B and GluR1 (Schütt et al. 2009). These differences were either cortical or hippocampus specific. However, while FMRP was shown to bind the mRNAs encoding SAPAP1, SAPAP2, SAPAP3, Shank1, and the NMDA receptor subunits NR1 and NR2B, the loss of FMRP did not affect their total and synaptic mRNA levels indicating the role of FMRP in their translation control and not in mRNA stability (Schütt et al. 2009). In addition to this, Bassell and collaborators have recently found that FMRP binds and represses the translation of the mRNA encoding phosphatidy-linositol-4,5-bisphosphate 3-kinase 110 kDa catalytic subunit beta (p110β), the catalytic subunit of PI3K, a signaling molecule downstream activation of mGluRs (Gross et al. 2010). Further studies extending the proteomic analysis to the entire synaptosome are required to extend the role of FMRP and its mRNA targets at synapses.

FMRP expression and consequently its function can be regulated by posttranslational modifications such as ubiquitination (Hou et al. 2006) and/or phosphorylation. FMRP is rapidly translated at synapses in response to chemically induced LTD (Antar et al. 2004; Ferrari et al. 2007; Kao et al. 2010) and followed by a quick degradation (5 min) through the ubiquitin–proteasome system after LTD induction (Hou et al. 2006). In parallel, FMRP has also been found to be highly phosphorylated when it cosediments with polyribosomes whereas its dephosphorylation releases FMRP from polysomes allowing protein synthesis (Ceman et al. 2003). The activation of mTOR pathway, through protein phosphatase 2A (PP2A) and ribosomal protein S6 kinase (S6K) activation, seems to be involved in the dephosphorylation and phosphorylation of FMRP at different time points during LTD stimulation (Narayanan et al. 2007). These investigations highlight the fine-tuned mechanism that regulates translation and ultimately gene expression. However, whether FMRP represses translation during initiation and/or elongation step is still a controversy. While some laboratories have found FMRP mainly cosedimenting with polyribosomes (Ceman et al. 2003; Khandjian et al. 2004; Stefani et al. 2004), others have found FMRP cosedimenting with mRNPs (Ishizuka et al. 2002; Monzo et al. 2006; Napoli et al. 2008; Papoulas et al. 2010; Siomi et al. 1996; Zalfa et al. 2003), and one laboratory found FMRP equally distributed among the two fractions (Brown et al. 2001). The different distribution could be explained through the association of FMRP to a variety of mRNPs that may aggregate and form different neuronal granules such as P bodies, stress, and transport granules (Anderson and Kedersha 2006; Kanai et al. 2004; Zalfa et al. 2006). The shuttle of FMRP from polysomes to mRNPs might be influenced also by post-translational modifications (Ceman et al. 2003). However, recent findings suggested that FMRP is involved in the repression of translation initiation through CYFIP1, early

identified as a partner of FMRP in neurons (Napoli et al. 2008; Schenck et al. 2003). In fact, CYFIP1 can act as a binding protein for the eukaryotic initiation factor 4E (eIF-4E), sequestering and repressing the assembly of the translation machinery. Specific mRNAs are tethered on CYFIP1-eIF4E by FMRP, and thus, only a subclass of mRNAs is repressed in a CYFIP1-dependent manner. Upon stimuli, CYFIP1-FMRP is released from eIF4E, and translation is activated (Napoli et al. 2008).

23.3.2 Regulation of mRNA Transport

It is worthwhile to mention that the levels of all FMRP-bound mRNAs are not necessarily translationally dysregulated in the absence of FMRP. Indeed, some experimental evidence indicate that FMRP is also involved in mRNA transport by delivering mRNAs which are thought to be in a dormant state from cell body, through dendrites, to spines where protein synthesis occurs (Fig. 23.3)
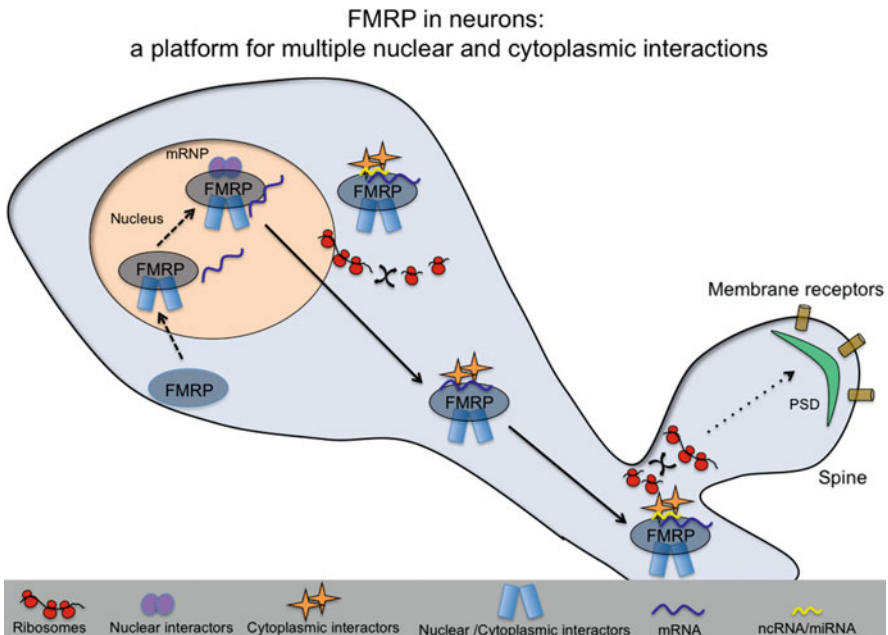

Fig. 23.3 FMRP forms part of a protein complex together with translationally arrested mRNAs. FMRP travels within an RNA–protein complex from the cell body to the synapses transporting dendritically localized mRNAs. After synaptic stimulation, FMRP liberates its mRNA targets allowing their local translation. The reversible translational repression and activation of the mRNA targets are regulated by a signaling pathway described in the text (see Sect. 23.3). Transported mRNAs are then locally translated in dendrites contributing to local protein synthesis and synaptic rearrangement that occurs after synaptic stimulation. FMRP can bind its mRNA targets through direct interaction or through noncoding RNAs such as BC1 RNA and microRNAs

(Bramham and Wells 2007). Upon synaptic LTD stimulation with DHPG, *Fmr1* mRNA is transported to dendrites and newly synthesized in proximity to metabotrobic receptor mGluR5 (Antar et al. 2004; Ferrari et al. 2007; Kao et al. 2010) where is further recruited along microtubules. Following DHPG stimulation, FMRP also interacts with motor proteins on microtubules, promoting the activity-dependent localization of bound mRNAs into synaptic spines (Antar et al. 2004, 2005; Davidovic et al. 2007; Dictenberg et al. 2008; Ferrari et al. 2007; Kanai et al. 2004). Therefore, certain FMRP targets such as *Map1b* and *Sapap4* have been found to be mislocalized in neurons of *Fmr1* KO mice (Table 23.2) (Dictenberg et al. 2008; Kao et al. 2010).

23.3.3   *Regulation of mRNA Stability*

For some FMRP-bound mRNAs, FMRP is a direct modulator of mRNA stability either by sustaining or preventing mRNA decay (De Rubeis and Bagni 2010). This has been revealed by the difference on the abundance of several mRNAs in *Fmr1* KO mice in comparison with wild type (WT) in three independent upscale screenings (Brown et al. 2001; Gantois et al. 2006; Miyashiro et al. 2003). In the first analysis, Brown et al. identified 144 mRNAs dysregulated in lymphoblastoid cells from FXS patients (Brown et al. 2001). Some of these mRNAs were also found to be dysregulated in a second analysis carried out in hippocampus of *Fmr1* KO mice (Miyashiro et al. 2003). Further analysis on the shared mRNAs showed that the dendritic localization of the mRNAs coding for the ribosomal component p40/LRP and the G-protein-coupled receptor kinase 4 (GRK4) was unaffected, while for the *dystroglycan-associated glycoprotein 1* (*DAG1*) mRNA, both localization and abundance were reduced (Miyashiro et al. 2003). The latter is an example of how FMRP may act on two different regulation mechanisms on the same mRNA.

Another example which shows how FMRP can regulate an mRNA at different levels is described by its action on *PSD-95* mRNA. Specifically, FMRP protects *PSD-95* mRNA from decay (Zalfa et al. 2007), and its stabilization is activity dependent. Downregulation of *PSD-95* mRNA occurs only in hippocampus and not in cortex, leading to decreased protein levels in hippocampus of *Fmr1* KO mice (Zalfa et al. 2007). However, PSD-95 protein levels are also downregulated in cortex of *Fmr1* KO mice indicating a defect on the synaptic translation of this *mRNA* in the cortex (Muddashetty et al. 2007). A different effect of FMRP on stability has been reported for the *Nxf1* mRNA. In this context, FMRP together with the nuclear export factor NXF2 facilitates the decay of *Nxf1* mRNA in a neuroblastoma cell line (Zhang et al. 2007). Following NXF2 overexpression, *Nxf1* is rapidly degraded. However, the degradation is impaired in absence of FMRP, suggesting that FMRP mediates *Nxf1* mRNA decay induced by NXF2 (Zhang et al. 2007).

In a genome-wide expression profiling study performed in hippocampus of *Fmr1* KO mice (Gantois et al. 2006), 224 mRNAs showed differences in the expression

levels between *fmr1* KO and WT, being 143 mRNA underexpressed and 81 overexpressed. However, further analyses using microarrays and real-time PCR confirmed that only eight mRNAs were underexpressed in *Fmr1* KO mice. Among those eight mRNAs, it is worth to highlight that the GABA$_A$ receptor subunit δ shows almost two times of underexpression (Gantois et al. 2006), decrease that was already reported by El Idrissi et al. (2005). These results together with the finding that GABA$_A$ receptor subunit δ mRNA was found to be a FMRP target (Dictenberg et al. 2008; Miyashiro et al. 2003) further suggest the role of FMRP as a modulator of this mRNA stability. Noteworthy, eight GABA receptor subunits(α_1, α_3, α_4, β_1, β_2, γ_1, γ_2, and δ) were significantly reduced in cortex but not in cerebellum of *Fmr1* KO mice (D'Hulst et al. 2006).

All these results lead to the hypothesis that different FMRP protein complexes might play different roles in cortex and hippocampus and that FMRP regulates mRNAs through different mechanisms accordingly to cell type and subcellular localization.

23.3.4   RNA Sequence–Structure Recognition

Up to now, several different mechanisms through which FMRP binds to its mRNA targets have been described. One mechanism is mediated through the direct binding of FMRP to the mRNAs, as described for *PSD-95* mRNA (Zalfa et al. 2007). In this case, FMRP binds to G-rich sequences that can, in some cases (i.e., *Map1B* mRNA), also be folded as G-quartets (Darnell et al. 2001) Another mechanism is through the binding of FMRP to noncoding RNAs. The first example is via *BC1*, a small noncoding RNA that acts as a bridge between FMRP and its mRNA targets (Zalfa et al. 2003). A third mechanism of action involves the interaction of FMRP with its targets through microRNAs. Edbauer and colleagues have recently reported that FMRP is associated with at least 12 different miRNAs, and few of them have indeed a relevant effect on spine morphology (Edbauer et al. 2010). NMDA receptor subunit 2A (NR2A) mRNA is an FMRP target that indeed partially depends on miR-125b binding and whose expression has a direct impact on synaptic plasticity (Edbauer et al. 2010). Further studies are required to address which are the other molecular mechanisms mediated by FMRP which affect NR2A translation in neurons.

Considering the involvement of FMRP in the regulation of several genes, both in the cell body and at synapses, as a consequence, its absence causes several cellular phenotypic abnormalities including dendritic spine dysmorphogenesis and behavioral deficits that summarize the impaired molecular synaptic plasticity events. Figure 23.3 summarizes the current model on the multiple FMRP functions in neurons.

23.4 Learning, Memory, and Behavioral Phenotypes: Learning from the Mouse Model

Behavior impairment is one of the most compelling evidence in FXS. Since the animal model for FXS (*Fmr1* KO) has been developed (Bakker 1994), several behavioral analysis have been performed on this mouse model. *Fmr1* KO mice showed behavioral similarities to the syndrome affecting human individuals (see Table 23.3). This includes hyperactivity, abnormal anxiety-related responses, hyperreactivity to auditory stimuli, abnormal sensorimotor gating, and impaired motor coordination (Bakker 1994; Peier et al. 2000). Learning and memory tests performed with *Fmr1* KO mice have shown minor differences compared to their control WT. Morris water maze and radial arm maze tests showed only mild cognitive impairment in *Fmr1* KO mice (Bakker 1994; D'Hooge et al. 1997; Dobkin et al. 2000; Kooy et al. 1996; Mineur et al. 2002; Paradee et al. 1999; Peier et al. 2000; Van Dam et al. 2000; Yan et al. 2004), indicating that *Fmr1* KO mice have only a slight impairment in spatial learning. Only recently, *Fmr1* KO mice generated in the C57 albino genetic background showed a consistent impairment in spatial learning (Baker et al. 2010). Moreover, no impairment in associative aversive learning or memory has been reported for the *Fmr1* KO, since they successfully expressed conditioned taste aversion (Nielsen et al. 2009). Transfer of learning, or reversal learning, based on measures of learning rate is not impaired either (Moon et al. 2008). The major impairment revealing a learning and memory deficiency is reported for tests such as object recognition, eyeblink conditioning, and lever press avoidance (Brennan et al. 2006; Koekkoek et al. 2005; Ventura et al. 2004; Yan et al. 2004). In the learning paradigm of conditioned and contextual fear tests, which involve hippocampal and amygdaloid tasks, in two separate studies, *Fmr1* KO mice show no significant differences compared to their WT (Peier et al. 2000; Van Dam et al. 2000).

Genetic background has also a major impact on behavioral phenotypes (Bucan and Abel 2002; Wolfer and Lipp 2000). For example, the recently generated *Fmr1* KO C57-albino mice showed impaired contextual fear but unaltered conditioned fear (Baker et al. 2010). However, these characteristics were not fully detected in other *Fmr1* KO mice generated in other genetic background (FVB-129 mice) (Zhao et al. 2005) (Table 23.3). In prepulse inhibition (PPI) test, measuring sensory gating, *Fmr1* KO mice perform even better, and these findings contrast the human phenotype in which there is a decrease in PPI (Frankland et al. 2004; Van Dam et al. 2000). Nonetheless, in the PPI test, *Fmr1* KO mice are more reactive than their controls (Chen and Toth 2001; Nielsen et al. 2002a) but only at a near-threshold level of the startle stimulus (Chen and Toth 2001; Nielsen et al. 2002a), indicating a general impairment in sensorimotor gating. This is somehow in agreement with the overall decrease in functioning of the neuronal network of Fragile X patients and also with the decreased ratio of excitatory to inhibitory amino acids observed in the brain of *Fmr1* KO mice (Gruss and Braun 2001; Kooy 2003). Importantly, impaired attention and inhibitory control, two features clearly impaired in humans with FXS (Cornish et al. 2008; Garber et al. 2008), have not been studied in the mouse model for FXS, although a heightened emotional reactivity has been described in *Fmr1* KO by Moon and colleagues (Moon et al. 2008).

Table 23.3 Comparison between human and mouse *FMR1* deletion phenotypes

	Human	Mouse	References
Physical phenotype	Facial features (large ears, elongated face, and high-arched palate in ~60% of prepubertal FXS boys)	No major body phenotype	Bakker et al. (1994)
	Macroorchidism (~40% of prepubertal FXS boys; ~90% adult males)	Macroorchidism	Bakker et al. (1994), Kooy et al. (1996), Peier et al. (2000), Nielsen et al. (2002a)
Neuroanatomy	Increased spine density, excessive immature spines	Increased spine density, excessive immature spines	Rudelli et al. (1985), Comery et al. (1997), Irwin et al. (2001), Nimchinsky et al. (2001), Irwin et al. (2002), Galvez and Greenough (2005), Restivo et al. (2005), Antar et al. (2006), Dolen et al. (2007), Bilousova et al. (2009), Cruz-Martin et al. (2010), Grossman et al. (2010), Pan et al. (2010)
Cognition	Developmental delay (mean IQ ~42 in boys)	Learning and memory impairment	Bakker (1994), Kooy et al. (1996), D'Hooge et al. (1997), Paradee et al. (1999), Dobkin et al. (2000), Peier et al. (2000), Van Dam et al. (2000), Mineur et al. (2002), Frankland et al. (2004), Ventura et al. (2004), Yan et al. (2004), Koekkoek et al. (2005), Brennan et al. (2006), Moon et al. (2008), Nielsen et al. (2009), Baker et al. (2010)
Behavior	ADHD(~80%)	Hyperactivity	Bakker (1994), Peier et al. (2000), Liu and Smith (2009)
	ASD (25% boys, 6% girls)	Social behavior alterations	Spencer et al. (2005), Mineur et al. (2006), McNaughton et al. (2008), Mines et al. (2010)
	Epilepsy (13–18% boys, 4% girls)	Higher susceptibility to audiogenic seizures	Musumeci et al. (2000), Todd and Mack (2000), Chen and Toth (2001), Kooy (2003)

23.4.1 Social Behavior Phenotypes

Fmr1 KO mice are impaired in social dominance with unfamiliar mice and, even though they show interest in social interaction, they spend a longer period of time than WT mice before approaching an unfamiliar mouse (Spencer et al. 2005). Anxiety is a psychological and physiological state characterized by somatic, emotional, cognitive, and behavioral components observed in patients with FXS (Cornish et al. 2008; Garber et al. 2008). When the *Fmr1* KO were studied for this specific state, Mines and colleagues reported that they displayed more anxiety-related behaviors during social interaction (grooming, rearing, and digging) than WT mice (Mines et al. 2010) and reduced social approach and response to social novelty (Liu and Smith 2009). *Fmr1* KO males have also a reduced interest in social interaction with novel females (Mineur et al. 2006). Moreover, KO mice seem to prefer social involvement as their WT controls, even though they show social anxiety in approaching the novel conspecific (McNaughton et al. 2008). *Fmr1* KO mice also show impairment in social discrimination between positive and negative social interactions (McNaughton et al. 2008). In the acoustic startle reflex test, a response of mind and body to a sudden unexpected stimulus, and a measure of anxiety, *Fmr1* KO mice react less that their WT controls (Nielsen et al. 2002a) in contrast with the human behavior, in which FXS patients show an excessive reaction to external stimuli (Jacquemont et al. 2007). Finally, *Fmr1* KO mice show hyperactivity, decreased spatial and environmental anxiety-related responses, and altered motor coordination (Bakker 1994; Liu and Smith 2009; Peier et al. 2000). Since sleep problems are common in children with FXS, it was interesting that Zhang and colleagues reported an altered expression of clock genes in the FXS mouse model (Zhang et al. 2008).

Finally, the recently generated mouse model carrying the Ile304Asn point mutation in the C57BL/6J background seems to recapitulate some of the above reported behavioral characteristics (Zang et al. 2009).

Recently, Spencer and colleague also demonstrate that almost all the abovementioned behavioral characteristics depend on the genetic background of the FXS mouse model; therefore, modifier genes may play a role in phenotype expression (Spencer et al. 2011). This implicates a strong correlation with the variable phenotype in patients and also the difficulty to have a reliable animal model for the study of this pathology.

23.4.2 Rescue of the FXS Behavioral Phenotypes

Genetic, behavioral, and pharmacological rescues have been developed over the last few years. Overexpression of FMRP in the *Fmr1* KO mouse model using a YAC vector reduces to some extent the described behavioral abnormalities (Paylor et al. 2008; Spencer et al. 2005, 2008). Improvement in social behavior, such as in chamber test of social interaction, direct social interaction test, and resident–intruder

task, has been demonstrated after enhanced neuroligin1 expression in *Fmr1* KO, even though no rescue is seen in learning and memory tasks (novel object recognition and plus shaped water maze tests) (Dahlhaus and El-Husseini 2010). One of the FMRP-dysregulated mRNAs encodes for the RGS4 (regulator of G-protein signaling) (Tervonen et al. 2005). *Fmr1* × RGS4 double KO shows a rescue of some of the behavioral phenotypes observed in the *Fmr1* KO such as tube test for social dominance, conditioned place preference, and reduced susceptibility to audiogenic seizures (Pacey et al. 2009, 2011). Moreover, treatment with group I mGluRs antagonist (MPEP) and lithium, through intervention on glycogen synthase kinase-3 (GSK3), ameliorates several behavioral aspects (Choi et al. 2010; Dolen et al. 2010; Liu et al. 2010; Min et al. 2009; Mines et al. 2010; Yuskaitis et al. 2010). Finally, it has been proposed that the absence of FMRP leads to higher levels of matrix metalloproteinase-9 activity (MMP-9) in the brain. In agreement, minocycline inhibits MMP-9 activity and alleviates behavioral measurement of the Aberrant Behavior Checklist – Community Edition (ABC-C) (irritability subscale, clinical global improvement scale (CGI), and the visual analog scale for behavior (VAS)) and synapse abnormalities in *Fmr1* knockout mice (Bilousova et al. 2009). Recently, minocycline administration was shown to provide significant functional benefits to FXS patients. These findings are consistent with the *Fmr1* knockout mouse model results, suggesting that minocycline modifies underlying neural defects that account for behavioral abnormalities. As the authors correctly report, a placebo-controlled trial of minocycline in FXS is warranted (Bilousova et al. 2009; Paribello et al. 2010).

As described below, absence of FMRP leads to an impaired GABA pathway due to its control on the mRNAs encoding different $GABA_A$ receptor subunits; therefore, a GABAergic approach to treat FXS treatment has been considered through the use of taurine, a $GABA_A$ agonist (El Idrissi et al. 2009). The authors show that taurine supplementation to Fragile X mice resulted in a significant improvement in acquisition of a passive avoidance task. Since taurine is an agonist for $GABA_A$ receptor, they suggest that chronic activation of $GABA_A$ receptors may have beneficial effects in ameliorating the learning deficits characteristic of the Fragile X syndrome (El Idrissi et al. 2009). Additionally, environmental enrichment has been proved of some efficacy in ameliorating the outcome of the pathology (Restivo et al. 2005). In this study, the authors showed that some behavior phenotypes such as habituation to object and motor activity as well as spine morphology were rescued. In addition, an increase of glutamate receptor subunit 1 (GluR1) levels in both genotypes was observed suggesting that FMRP-independent pathways activating glutamatergic signaling are preserved in *Fmr1* KO mice and that they can be elicited by environmental stimulation (Restivo et al. 2005). These findings indicate that the environment is of extreme importance for the patients. A follow-up study showed that in *Fmr1* KO mice that were raised in enriched environments, LTP was restored to WT levels, indicating that mechanisms for synaptic plasticity are in place in the *Fmr1* KO mouse but require stronger neuronal activity to be triggered (Meredith et al. 2007). Patients with FXS show in about 20% of the cases epileptic seizures (Garber et al. 2008). Even if the mouse model for FXS does

not display spontaneous seizures, it is susceptible to audiogenic-induced seizures (Bakker 1994; Chen and Toth 2001; Kooy 2003; Kooy et al. 1996; Musumeci et al. 2000). Although seizure age dependency is still debated, a trend in the impaired response to acoustic seizures at P17 and P21 (postnatal days 17 and 21) has been reported (Chen and Toth 2001; Kooy 2003; Musumeci et al. 2000). This effect may be related to an increased cortical excitability or due to a deficit in long-term plasticity (Kooy 2003). Chemically induced seizures, by kainic acid, bicuculline, and pentylenetatrazole injection, do not show difference between *Fmr1* KO and their controls, suggesting that *Fmr1* KO mice have specific susceptibility to audiogenic stimuli (Chen and Toth 2001; Todd and Mack 2000) This is in agreement with a possible developmental impairment of the auditory system in Fragile X mice (Brown et al. 2010) proposed by Chen and Toth (Chen and Toth 2001). Only recently, a study on amygdala showed that *Fmr1* KO mice have a more accelerated kindling development and longer electrographic seizure duration. Both NMDA antagonist, MK-801, and mGluR5 antagonist, MPEP, were able to repress accelerated rate of kindling development (Qiu et al. 2009).

23.4.3 Electrophysiological Phenotypes

Due to the learning and memory alterations, characteristics of FXS, synaptic plasticity events monitored through *ex vivo* LTP and LTD paradigms have been extensively investigated in Fmr1 KO mice.

Effects on LTP. In the *Fmr1* KO mice, LTP has been found altered in hippocampus only after theta burst stimulation (Lauterborn et al. 2007), a stimulation able to induce LTP in close resemblance to physiological hippocampal frequency of theta rhythm (5–10 Hz) (Capocchi et al. 1992). Only recently, a reduction of LTP in dentate gyrus has been observed in *Fmr1* KO mice possibly due to a reduction of NMDA excitatory postsynaptic currents (EPSCs), which is a consequence of a reduced ratio of NMDA/AMPA receptors (Eadie et al. 2010; Yun and Trommer 2011). On the other hand, high-frequency stimulation (100–400 Hz) does not affect LTP in hippocampus of *Fmr1* KO mice (Godfraind et al. 1996; Li et al. 2002; Paradee et al. 1999; Zhang et al. 2009). At the level of cortex, the LTP responses are different and indeed appear impaired in the *Fmr1* KO mice (Hayashi et al. 2007; Larson et al. 2005; Li et al. 2002; Meredith et al. 2007; Zhao et al. 2005). Additionally, induction of LTP has also been shown to be altered in lateral amygdala (Suvrathan et al. 2010; Zhao et al. 2005). FMRP is required for glycine-induced LTP (Gly-LTP) in the CA1 of hippocampus. This form of LTP requires activation of postsynaptic NMDA receptors and metabotropic glutamatergic receptors, as well as the subsequent activation of extracellular signal-regulated kinase (ERK) 1/2. Genetic deletion of FMRP interrupts the phosphorylation of ERK1/2, suggesting the possible role of FMRP in the regulation of the activity of ERK1/2 (Shang et al. 2009).

Effects on LTD. LTD is altered in hippocampus of *Fmr1* KO mice either applying low-frequency-paired-pulse stimulation (PP-LFS), which is rescued by FMRP replacement through viral strategy (Zeier et al. 2009), or with application of the group I mGluR agonist DHPG (Huber et al. 2000). mGluR5-LTD and FMRP are also connected: DHPG induces FMRP expression in synaptosomes and cultured neurons (Antar et al. 2004; Ferrari et al. 2007; Kao et al. 2010; Weiler et al. 1997), and DHPG-induced LTD is increased in *Fmr1* KO mice (Hou et al. 2006; Huber et al. 2002). Therefore, mGluR5-induced LTD in the context of Fragile X has received a large attention. CA1 LTD is reported to be translation dependent and transcription independent when explored in WT mice (Hou and Klann 2004; Huber et al. 2000, 2002), in line with the signaling pathways described above and activated by mGluR5 which involve protein synthesis (Dolen et al. 2010). mGluR LTD occurs in a protein-synthesis-dependent manner and correlates glutamatergic receptors (NMDA and AMPA) regulation to morphologic changes in spine number, shape, and size (Dolen et al. 2010).

mGluR-induced LTD (via DHPG) is increased in *Fmr1* KO mice (Huber et al. 2002) and is protein synthesis independent, possibly due to deregulated local protein synthesis (Lu et al. 2004; Muddashetty et al. 2007; Zalfa et al. 2007), which in turn enhances a constant AMPAR internalization (Huber et al. 2002; Nosyreva and Huber 2006; Ronesi and Huber 2008). This mechanism has been described as the base of the so-called mGluR theory (Bear et al. 2004) which hypothesize that an excessive group 1 mGluRs activation, upstream to the lack of FMRP, increases the protein synthesis and induces excessive AMPAR internalization, responsible for the consequent increase in LTD. The theory has been validated by a genetic rescue of the *Fmr1* KO mouse model crossing it with the heterozygous mouse for mGluR5. The crossed *Fmr1* KO/mGluR5 (+/−) mice demonstrated amelioration of several mouse phenotypes. Specifically, 50% reduction of mGluR5 level of expression in the *Fmr1* KO background restored the altered protein expression, which in turn reduced the abnormal response to mGluR5-induced LTD, rescued the abnormal spine morphology, and ameliorated some behavioral phenotypes including a reduced incidence of the audiogenic seizures (AGS) (Dolen et al. 2007).

Nevertheless, the excessive activity of mGluR5 seems not to be the only possible cause of seizures in the *Fmr1* KO mouse model. As previously mentioned, another possible cause to consider is the deficit of GABA neurotransmission. Indeed, several lines of research have proposed that absence of FMRP leads to a dysfunction in the GABAergic system (Olmos-Serrano et al. 2010). Recent studies demonstrate that GABAergic inhibition is impaired at cellular (Selby et al. 2007), physiological (Centonze et al. 2008; Curia et al. 2009), and molecular level (Curia et al. 2009; D'Hulst et al. 2006; El Idrissi et al. 2005). Therefore, decrease of interneuron number, altered GABAergic transmission, and/or altered $GABA_A$ subunit expression may be the cause for epileptic seizures and/or EEG abnormalities associated to FXS. Moreover, an involvement of G-protein-coupled $GABA_B$ receptors demonstrated to be effective in attenuating the AGS phenotype in *Fmr1* KO mice (Pacey et al. 2009). The mechanism, possibly involved in efficacy

for GABA$_B$ receptors, seems to be the coupling of GABA$_B$ receptors to regulator of RGS4 (Fowler et al. 2007) which in turn results as an inhibitor of Gp1 mGluRs (Saugstad et al. 1998). In agreement with this hypothesis, a double KO for *Fmr1* and RGS4 mouse model has reduced susceptibility to seizures (Pacey et al. 2009).

23.4.4   Spine Dysgenesis

Alterations of dendritic spines represent a common hallmark of mental retardation diseases and other synaptopathies (Purpura 1974). Although FXS is not characterized by gross brain defects, a consistent microanatomical phenotype is an increased spine density and an altered ratio of mature and immature spines (Comery et al. 1997; Irwin et al. 2001). During development, spines are stabilized and change their dynamic properties and morphology. Motility and turnover decrease, and thin, filopodia-like protrusions mature in stubby and mushroom-like shapes (Portera-Cailliau et al. 2003). A variety of intermediate shapes can exist, giving rise to a filopodia–spine continuum (Irwin et al. 2001).

The first evidence of spine dysgenesis in individuals with FXS was obtained by Golgi impregnation of autopsy material from a 62-year-old FXS patient (Rudelli et al. 1985). This analysis revealed long, tortuous, and thin dendritic spines on apical dendrites of pyramidal neurons from layers III and V of the parieto-occipital cortex (Rudelli et al. 1985). Further studies of three FXS adult individuals estimated higher spine density and length along the entire dendritic tree of cortical layer V pyramidal neurons (Irwin et al. 2001).

Mouse models of FXS (*Fmr1* KO) recapitulate the spine dysgenesis observed in patients. Mutant mice present increase density of long, immature spines in visual cortex, somatosensory cortex, and hippocampal dentate gyrus (Comery et al. 1997; Dolen et al. 2007; Galvez and Greenough 2005; Grossman et al. 2010; Irwin et al. 2002; Nimchinsky et al. 2001; Restivo et al. 2005). Spine defects in cortical neurons were detected during early postnatal development (1–3 weeks) and adulthood but not in 4-week-old mice (Galvez and Greenough 2005; Nimchinsky et al. 2001). However, in contrast with neocortex, spine abnormalities in the dentate gyrus remain constant during development (Grossman et al. 2010). Data obtained on *ex vivo* and *in vitro* systems do not consistently corroborate in vivo observations. Spine defects were not detected in neocortical and hippocampal organotypic cultures (Nimchinsky et al. 2001; Pfeiffer et al. 2010). Increased spine length and excessive filopodia protrusions were reported in primary *Fmr1* KO hippocampal neurons (Antar et al. 2006; Bilousova et al. 2009), although earlier reports produced divergent results (Braun and Segal 2000).

Excess of spines with immature morphology may be due to defects in spine dynamics and maturation, especially altered pruning (Galvez et al. 2003). Two recent studies examined spine plasticity in living *Fmr1* KO animals using transcranial two-photon imaging of somatosensory cortex. Both reports highlighted that the major abnormality is the augmented spine turnover, a process that includes formation of new spines and elimination of existing spines (Cruz-Martin et al. 2010; Pan et al. 2010).

In *Fmr1* KO mice, spine turnover fails to rapidly decrease during the first 2 postnatal weeks, leading to a delay in spine stabilization and transition from immature to mature spine types (Cruz-Martin et al. 2010). Increased spine turnover is maintained in late development (4 postnatal weeks) and in adulthood and may be due to a larger population of short-lived spines observed in KO mice (Pan et al. 2010). Since these transient spines display smaller head and longer neck, they could contribute to the immature spine morphology in KO animals (Pan et al. 2010).

Spine dynamics are known to change in response to experience-dependent modulation of specific circuits in the somatosensory and visual cortex (Holtmaat and Svoboda 2009). Although *Fmr1* KO animals display hypersensitivity to sensory stimuli (Chen and Toth 2001), this effect does not cause the enhanced spine dynamics, which in fact was not hampered by somatosensory deprivation (whisker trimming) (Pan et al. 2010). Furthermore, KO mice lack spine plasticity to somatosensory modulation (Pan et al. 2010). However, other circuits of experience-dependent neuronal plasticity seem to be preserved, since environmental enrichment rescues the spine abnormalities in visual cortex (Restivo et al. 2005).

Evidence of defects in spine maturation are compatible with the overall decrease of functional synapses, measured as dendritic protrusions juxtaposed with presynaptic markers in cultured hippocampal neurons (Antar et al. 2006; Braun and Segal 2000). Of interest, loss of synapses in *Fmr1* KO mice is also corroborated by electrophysiological data (Pfeiffer and Huber 2007; Pfeiffer et al. 2010).

The mechanisms leading to spine dysgenesis are not fully understood. Despite recent reports suggesting the involvement of the transcription regulator MEF2 (Pfeiffer et al. 2010) and neuronal microRNAs (Edbauer et al. 2010), the spine phenotype is likely multifactorial. As mentioned above, FMRP regulates the synthesis of a variety of proteins crucial for proper synaptic morphology and functionality (Bassell and Warren 2008). However, interfering with some of the signaling pathways altered in *Fmr1* KO mice – group I mGluRs, PI3K, and PAK1 RGS4 – is effective to partially rescue the spine anomalies.

As for the mGluR cascade, DHPG administration in cultured hippocampal neurons increases spine length in a protein-synthesis-dependent manner (Vanderklish and Edelman 2002). Hampering the excessive mGluR signaling by administration of the antagonist MPEP (de Vrij et al. 2008; Su et al. 2010) or genetic reduction of mGluR5 (Dolen et al. 2007) ameliorates the spine phenotype in *Fmr1* KO neurons. Furthermore, inhibition of PI3K, a signaling molecule excessively translated in the absence of FMRP, reduces spine density in cultured neurons (Gross et al. 2010). Similarly, inhibition of PAK1, a signaling cascade controlling actin cytoskeleton, partially rescues the defects in spine density and length (Hayashi et al. 2007). Finally, pharmacological treatments with lithium and minocycline, whose mechanisms are not fully elucidated, may also alleviate the spine phenotype in FXS mice (Bilousova et al. 2009; Liu et al. 2010).

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